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Genome-wide analysis of Notch function in early T cell
development

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Abstract

Acquired immunity to pathogens critically depends on functional T cells, which require the unique microenvironment of the thymus for their development. Exposure of thymus-seeding progenitors to Notch ligands expressed by thymic stroma initiates execution of the T cell differentiation program. Notch functions as the T lineage commitment factor at the early stages of differentiation and is required for survival, proliferation and further developmental progression later on. Although the importance of Notch signalling in T cell differentiation is long-established and some Notch target genes important in this process were identified, no systematic attempt to characterise the molecular program downstream of Notch signalling in developing T cells was made so far. In fact, due to the lack of appropriate tools until today, a systematic search for Notch targets in any system was largely restricted to studies in cell lines and conditions of artificially overactivated Notch signalling.

To bypass this limitation, I made use of biotin-tagged murine knock-in alleles of *Notch1* and its DNA-binding cofactor *Rbpj* generated in our research group. I combined ChIP-seq and RNA-seq approaches to generate a comprehensive map of the Notch-regulated transcriptome throughout T cell development—in uncommitted T cell progenitors as well as T lineage-committed pre- β -selection DN3a cells and post- β -selection DN4 thymocytes. To identify putative *cis*-regulatory elements, through which Notch controls expression of its target genes, I mapped DNase I hypersensitive sites, transcription start sites and histone modifications in DN3a thymocytes. These approaches led to the identification of a number of novel Notch targets, including an unexpected group of genes that are induced by Notch signalling in the earliest thymocytes but are rapidly downregulated by the time of commitment to the T cell lineage. This group is enriched for genes, which are commonly expressed in natural killer or myeloid cell lineages.

It was previously reported that Notch signalling is required for rearrangement at the *Tcrb* locus, but the molecular mechanism for this remained unknown. I identified prominent Notch1 and Rbpj occupancy in the TCR β enhancer and demonstrated that activation of Notch signalling in bone marrow progenitors enhanced germline transcription of the *Tcrb* locus. These results provide a likely molecular explanation for the role of Notch in TCR β recombination. Unexpectedly, in contrast to the textbook view on the Notch pathway, which suggests that Rbpj is constantly bound to DNA even in the absence of Notch signalling, my results revealed Notch-dependent Rbpj binding at the majority of sites.

Finally, although Notch signalling strength is known to decrease upon pre-TCR expression, Notch acquires a mitogenic function at this transition, suggesting that a spectrum of its target genes may change. The comparison of the Notch-regulated transcriptomes of DN3a and DN4 cells showed that Notch targets in pre-TCR-positive cells represent a subset of Notch targets prior to pre-TCR expression and that no new Notch targets emerge at this transition.

Zusammenfassung

T-Zellen sind ein zentraler Bestandteil der adaptiven Immunabwehr, die für ihre Entwicklung die besondere Mikroumgebung des Thymus benötigen. Nach dem Eintritt in den Thymus sind lymphoide Vorläuferzellen einer hohen Dichte an Notch Liganden ausgesetzt, was dazu führt, dass in den Vorläuferzellen ein T-Zellentwicklungs-spezifisches Genexpressionsprogramm aktiviert wird. In frühen T-Zellstadien dient Notch dem Determinierungsprozess der Zellen in Richtung T-Zelllinie. In späteren Entwicklungsstadien hingegen reguliert Notch das Überleben der Zellen, sowie die Zellteilung und die weitere Zellentwicklung. Die zentrale Rolle des Notch-Signalweges in der T-Zellentwicklung ist eingehend bekannt und es konnten einige Zielgene von Notch gefunden werden. Bisher wurde jedoch noch keine systematische Analyse durchgeführt, um das von Notch induzierte molekulare Programm in verschiedenen T-Zellentwicklungsstadien zu entschlüsseln. Angesichts fehlender molekularer Werkzeuge war bis heute das Auffinden direkter Notch-Zielgene auf Studien in Zelllinien beschränkt und wurde meist unter artifiziell erhöhten Notch-Signalen durchgeführt.

Um diese Einschränkungen zu umgehen, habe ich murine Knock-in Allele von *Notch1* und dem DNA-bindenden Co-Faktor *Rbpj* verwendet, die unter anderem für eine Biotinylierungssequenz kodieren. Ich habe eine Kombination aus ChIP-seq und RNA-seq Ansätzen angewendet, um eine umfassende Kartierung des Notch-regulierten Transkriptoms während der T-Zellentwicklung zu erstellen – in undeterminierten T-Vorläuferzellen sowie T-Zelllinie-determinierten pre-T-Zellrezeptor (pre-TCR) negativen DN3a und pre-TCR-positiven DN4 Thymozyten. Um potentielle *cis*-regulatorische Elemente zu beschreiben, die von Notch für die Regulation der Transkription der Zielgene genutzt werden, habe ich ebenfalls DNase I hypersensitive Stellen, Transkriptionsstartstellen und Histonmodifikationen in DN3a Thymozyten kartiert. Diese Ansätze haben zahlreiche neue Notch Zielgene identifiziert; unter anderem eine unerwartete Gruppe von Genen, die in den frühesten Thymozyten von Notch aktiviert werden und wiederum sogleich herabreguliert werden, bevor die Zellen den T-Zellliniendeterminationspunkt erreichen. Innerhalb dieser Gruppe sind Gene angereichert, die in der Regel in hohem Maße in natürlichen Killerzellen oder in myeloiden Zellen exprimiert werden.

Es wurde gezeigt, dass der Notch-Signalweg für die Rearrangierung des *Tcrb* Locus benötigt wird. Jedoch konnte dafür bisher keine Erklärung auf molekularer Ebene erbracht werden. Ich habe zum einen eine deutliche Bindung von Notch1 und Rbpj an dem TCRβ-Enhancer festgestellt. Zum anderen verstärkte die Aktivierung von Notch in Vorläuferzellen aus dem Knochenmark die Expression von „sterilen“ *Tcrb* Transkripten. Diese Ergebnisse könnten eine molekulare Erklärung für die Funktion von Notch in der Rearrangierung des *Tcrb* Locus darstellen.

Unerwarteterweise und konträr gegenüber der gängigen Lehransicht des Notch-Signalweges, die besagt, dass Rbpj an die DNS bindet, auch wenn der Notch-Signalweg inaktiv ist, haben meine Resultate eine Notch-abhängige Bindung von Rbpj an der Mehrzahl der Bindestellen gezeigt.

Es ist bekannt, dass die Signalstärke des Notch-Signalweges nach der Expression eines pre-TCR nachlässt. In pre-TCR-exprimierenden Zellen erfüllt der Notch-Signalweg jedoch immer noch eine mitogene Funktion. Dies könnte bedeuten, dass die Transition zur Änderung des Zielgenspektrums von Notch führt. Vergleiche der genomweiten Bindung von Rbpj in DN3a und DN4 Zellen sowie der Notch-regulierten Transkriptionsprofile in beiden Zellstadien zeigten, dass in pre-TCR-positiven Zellen keine neuen Notch-Zielgene hervorgehen, die nicht schon vor der Ausbildung des pre-TCR durch Notch reguliert werden.

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Abbreviations

2-ME	2-Mercaptoethanol
6-AA	aminocaproic acid
aa	amino acid
ALP	all-lymphoid progenitor
AP	active promoter
APC	antigen-presenting cell
ATP	adenosine triphosphate
Bio	biotin
BLP	B-cell-biased lymphoid progenitor
bp	base pair
BSA	bovine serum albumin
C	constant
C-terminal	carboxy-terminal
CAGE	cap analysis gene expression
CD	cluster of differentiation
cDNA	complementary DNA
ChIP	chromatin immunoprecipitation
CLP	common lymphoid progenitor
D	diversity
dATP	deoxyadenosine triphosphate
DC	dendritic cell
dCTP	deoxycytidine triphosphate
DE	distal element
dGTP	deoxyguanosine triphosphate
DHS	DNase I hypersensitive
DN	CD4 & CD8 double-negative
DNA	desoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DP	double-positive
ds	double-stranded
DSG	disuccinimidyl glutarate
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
dUTP	deoxyuridine triphosphate

ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetraacetic acid
EGTA	ethyleneglycol tetraacetic acid
EtOH	ethanol
ETP	early thymocyte precursor
FA	formaldehyde
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FSC	forward scatter
GLT	germline transcript
GFP	green fluorescent protein
hCd2t	truncated human <i>CD2</i>
HDAC	histone deacetylase
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HRP	horseradish peroxidase
HSC	haematopoietic stem cell
IAP	inactive promoter
ICN	intracellular domain of Notch
IMDM	Iscoe's modified Dulbecco's Medium
IP	immunoprecipitation
IRES	internal ribosomal entry site
J	joining
kb	kilobase pairs
ko	knockout
LMPP	lymphoid-primed multipotent progenitor
MACS	magnetic activated cell sorting
MAD	median absolute deviation
mRNA	messenger RNA
N-terminal	amino-terminal
NK	natural killer
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PC	principal component
PcG	Polycomb group
PCR	polymerase chain reaction
pH	potentia hydrogenii

PMSF	phenylmethanesulfonylfluoride
PRC2	polycomb repressive complex 2
PVDF	polyvinylidene difluoride
rIL-7	recombinant interleukin-7
RNA	ribonucleic acid
RPKM	reads per kilo base pairs per million mapped reads
RPM	reads per million mapped reads
RT	room temperature
SA	streptavidin
SD	standard deviation
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
seq	sequencing
SP	single positive
SPS	sequence-paired site
SSC	side scatter
T-ALL	T cell acute lymphoblastic leukaemia
TBS	tris-buffered saline
Tris	tris(hydroxymethyl)aminomethane
TSP	thymus-seeding progenitor
TSS	transcription start site
TTS	transcription termination site
Tween	polyoxyethylene sorbitan monolaurate
UTR	untranslated region
V	variable
wt	wild-type

Units, chemical elements, amino acids and nucleotides are written according to the standard IUPAC-nomenclature.

Abbreviations of gene and protein names are not listed and can be found in publicly accessible databases such as UCSC and UNIPROT.

1 Introduction

1.1 Hierarchical development of cellular components of the haematopoietic system

The haematopoietic system consists of organs, such as bone marrow, spleen, thymus and lymph nodes, which are involved in the production of the cellular components of the blood. Due to the limited life span of most mature blood cell types, their generation has to continue throughout the life of an organism. This process is ensured by pluripotent haematopoietic stem cells (HSCs), a rare cell population residing in the bone marrow of an adult organism. Like other somatic stem cells, HSCs have the capacity to self-renew, as well as the ability to differentiate and thereby generate all cell types of a given organ. Hence, HSCs can be defined operationally by their capability to reconstitute the entire blood system of a recipient (Orkin and Zon, 2008). HSCs reside within defined marrow niches, which ensure that the cells receive appropriate signals from the extracellular matrix such as stromal cells, osteoblasts and vascular cells (Calvi et al., 2003; Zhang et al., 2003; Kiel and Morrison, 2006). Like most haematopoietic lineages, HSCs can be defined by a combination of specific cell surface markers. Adult bone marrow HSCs can be subdivided into long-term HSCs ($\text{Lin}^- \text{c-Kit}^+ \text{Sca1}^+ \text{CD150}^+ \text{CD48}^- \text{CD34}^- \text{Flt3}^-$) and short-term HSCs ($\text{Lin}^- \text{c-Kit}^+ \text{Sca1}^+ \text{CD150}^+ \text{CD48}^- \text{CD34}^+ \text{Flt3}^-$), which have lost their self-renewing capacity and are referred to as multipotent progenitors (MPPs). MPPs in the bone marrow become progressively restricted to several or single lineages (Orkin, 2000). This differentiation process is largely mediated by a temporal and stage-specific expression of transcription factors. The requirements for several transcription factors at specific developmental stages could be shown by loss-of-function and gain-of-function studies (reviewed by Orkin and Zon, 2008) and are summarised in Figure 1.1.

The 'classical' textbook view of adult haematopoiesis proposes a bifurcation of myeloid- and lymphoid-lineages beyond the ST-HSC stage and is based on the identification of common myeloid and common lymphoid progenitors (CMPs and CLPs; Akashi et al., 2000; Kondo et al., 1997; Koch and Radtke, 2010). While CLPs give rise to lymphoid cells of the B and T cell lineages as well as natural killer (NK) cells, they do not develop into cells of the myeloid lineage (Kondo et al., 1997). CMPs, however, have the potential to subsequently differentiate into megakaryocyte/erythroid and granulocyte/macrophage progenitors (MEPs and GMPs; Akashi et al., 2000).

Continuous research on the haematopoietic hierarchy leads to constant modifications of the classical textbook model. For instance, the identification of lymphoid-primed MPPs (LMPPs), which are characterised by high levels of the fms-like tyrosine kinase (Flt3) among

the adult bone marrow $\text{Lin}^- \text{c-Kit}^+ \text{Sca1}^+ \text{CD150}^- \text{CD48}^+ \text{CD34}^+ \text{Flt3}^+$ subset, suggested a revised road map for adult haematopoiesis (Adolfsson et al., 2005; Lai and Kondo, 2006). LMPPs exhibit a vastly reduced megakaryocyte/erythroid potential but retain the capacity to differentiate into B and T cells or cells of the granulocyte/macrophage lineage. Furthermore, LMPPs express lymphoid-associated proteins such as Rag proteins and the terminal deoxynucleotidyl transferase (TdT, also known as DNA terminal transferase, or Dntt) as well as sterile immunoglobulin transcripts (Månsson et al., 2007; Ng et al., 2009; Yoshida et al., 2006).

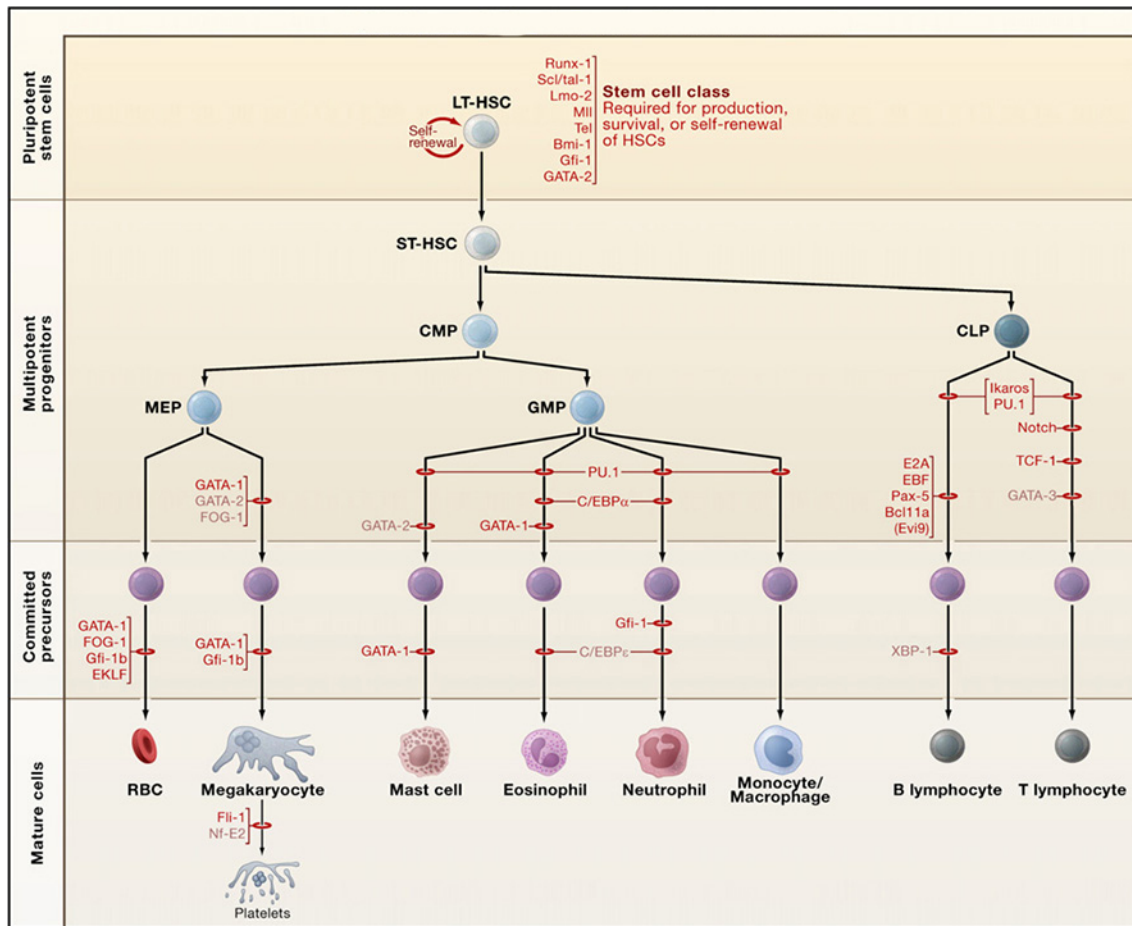


Figure 1.1 Hierarchical development of cellular components of the haematopoietic system (adapted from Orkin and Zon 2008). Schematic representation of the hierarchical relationships between blood cell types is shown. Critical transcription factors are shown in red, and the block of the lineage development upon loss of the transcription factor is indicated by a red bar. See main text for abbreviations.

The Weissman lab showed that the CLP subset can be subdivided into two distinct populations that mark the branch point between B and T cell lineages based on Ly6d surface levels (Inlay et al., 2009). Ly6d^- CLPs retain full *in vivo* lymphoid potential as well as low myeloid potential and produce more thymocytes at early time points than LMPPs. Their Ly6d^+ counterparts, however, behave essentially like B cell progenitors, as they upregulate B cell-specific

genes such as *Ebf1* and *Pax5*. The authors suggested that both cell types are located downstream of LMPPs and might represent the point at which the lymphoid lineage restriction takes place (Fig. 1.2).

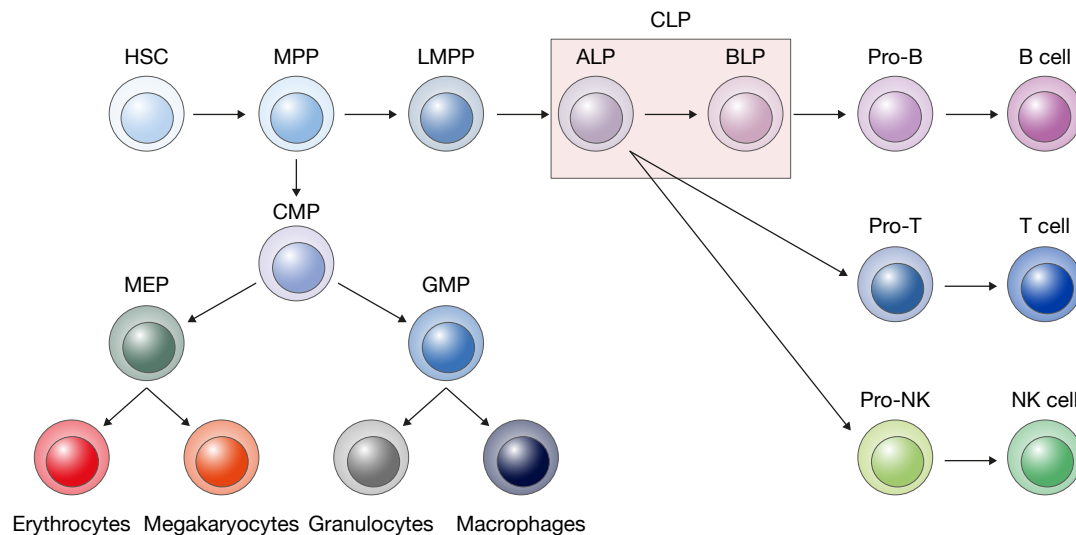


Figure 1.2 Alternative model of the haematopoietic system including LMPP, ALP and BLP populations (adapted from Choukrallah and Matthias 2014). Schematic representation of the hierarchical relationships between blood cell types is shown. Note the additional LMPP, ALP and BLP stages compared to Figure 1.1.

1.2 Development of the T cell lineage

1.2.1 Thymus-seeding progenitors

In contrast to most blood cell types, which mature in the bone marrow, cells of the T lineage complete their maturation in the thymus. Under physiological conditions, thymocytes do not self-renew. Hence, a continuous generation of thymocytes requires that haematopoietic progenitors developing in the bone marrow migrate to the thymus. The number of such thymus-seeding progenitors (TSPs), that colonise the thymus every day, is estimated to be very low (less than 20; Wallis et al., 1975; Ceredig et al., 2007; personal communication with Andreas Krueger, Hannover Medical School), which makes the analysis of TSPs in the adult thymus difficult. Functionally, such T cell progenitors have to fulfil three criteria: *i*) They must be able to leave the bone marrow niche and enter the blood stream; *ii*) they must be able to exit circulation and colonise the thymus; *iii*) they must be competent to receive Notch signals and differentiate into T lineage cells.

Depending on the experimental setup, the results of the studies that aimed to identify TSPs are contradictory and the identity of the TSP remains controversial (Richie Ehrlich et al., 2011). LMPPs and CLPs rapidly develop into T cells, suggesting that there are physiological thymic seeding progenitors among these populations (Schwarz et al., 2007). In contrast to this, the generation of T lymphocytes from upstream populations, such as HSCs, shows greatly delayed kinetics. This might indicate that these upstream progenitors need to home to the bone marrow before they or their progeny are able to seed the thymus.

Upon identification of bone marrow-resident CLPs, it was proposed that TSPs derive from a lymphoid lineage-committed population (Kondo et al., 1997; Gounari et al., 2002; Martin et al., 2003; Krueger and von Boehmer, 2007). However, Allman et al. (2003) reported that ETPs can develop via a CLP-independent pathway, as Ikaros-deficient mice generate early thymic progenitors (ETPs), even though haematopoiesis is blocked prior to the CLP stage in these mice. The hypothesis, that haematopoietic progenitor cells more undifferentiated than CLPs seed the thymus, was further strengthened by the finding that ETPs from adult mice have the potential to differentiate into myeloid cells and B cells (Bell and Bhandoola, 2008; Wada et al., 2008).

In a lineage tracing study, Schlenner et al. (2010) visualised the history of interleukin-7 receptor α (IL7R) expression in haematopoietic cells. IL7/IL7R signalling is essential for lymphopoiesis *in vivo* (Peschon et al., 1994; Fischer et al., 1997; Puel and Leonard, 2000). While *Il7r* expression is absent in the earliest thymocytes (Allman et al., 2003; Rothenberg et al., 2008), IL7R surface expression marks both bone marrow-residing lymphoid progenitors (Kondo et al., 1997; Igarashi et al., 2002; Martin et al., 2003) as well as thymocyte progenitors in the blood (Rodewald et al., 1994; Krueger and von Boehmer, 2007). IL7R fate mapping labelled nearly all T cells but hardly any myeloid cells. This suggested that: *i*) distinct origins of both lineages exist; *ii*) lymphoid-restricted progenitors give rise to nearly all T cells; *iii*) the myeloid potential of ETPs is hardly realised *in vivo* (Schlenner et al., 2010). The discrepancies between *in vitro* and *in vivo* obtained results might be caused by artificial induction of non-physiological differentiation pathways *in vitro*, as even CLPs can give rise to myeloid cells when cultured *in vitro* in the presence of the Notch-ligand DLL4 (Bell and Bhandoola, 2008; Schlenner et al., 2010; Ramond et al., 2014). TSPs were also described in unborn mice, at developmental stages prior to proper structural development of the thymus (Harman et al., 2005; Masuda et al., 2005). These cells are also found in the fetal liver as well as blood stream and were committed to the T/NK cell lineages, which suggested that commitment occurs prior to contact with the thymic microenvironment (Rodewald et al., 1994; Douagi et al., 2002). However ETPs of newborn mice functionally resemble LMPPs as they lack megakaryocyte/erythroid potential but exhibit robust T, myeloid and B lineage potential, of which the

latter ceases after birth and in response to Notch signalling (Ceredig et al., 2007; Luc et al., 2012).

Recently, Ramond et al. (2014) suggested that the fetal thymus is colonised by two waves of distinct haematopoietic progenitor cells. Their model reconciles the divergent views of the previous studies on the nature of TSPs. The first wave of TSPs, between E12 and E15, has a phenotype similar to that of CLPs, as myeloid and B cell potential is lost prior to thymus entry. These cells possess limited capacity for population expansion but confer partial reconstitution after transfer by rapidly differentiating into $\alpha\beta$ -T cells as well as invariant $V\gamma 3^+$ dendritic epithelial cells, generation of which is a hallmark of embryonic T cell development (Havran and Allison, 1990; Ikuta et al., 1990). In contrast, TSPs of the subsequent wave, starting at E16, phenotypically resemble LMPPs, as myeloid and B cell potential is lost during intrathymic development. Furthermore, those cells also lost embryonic $\gamma\delta$ -T cell potential, and thus, resemble postnatal ETPs. These ETPs generate large numbers of CD4 and CD8 double-positive (DP) thymocytes by extensive expansion of their population before maturation. Compared to TSPs from the first wave, differentiation capacity is delayed in contrast to a higher proliferative capacity, which might be caused by high expression of cyclin D1 in combination with low expression of cyclin-dependent kinase inhibitors.

It has to be noted that thymopoiesis differs between the fetal and adult mice. Compared to adult mice, fetal mice exhibit higher levels of haematopoietic progenitors in the blood (Rodewald et al., 1994). The ETP to total thymocyte ratio in fetal mice is also higher compared to adult mice (Hozumi et al., 2008; Belyaev et al., 2012). This suggests that in fetal mice more progenitors colonise the thymus and differentiate into ETPs. However, adult progenitors are likely to compensate for their reduced thymic seeding through an increased proliferative ability, as they are 100-fold more efficient in generating T cells after intrathymic transfer than fetal progenitors (Lu et al., 2005). In line with this result, the gene expression profile of fetal ETPs resembles more the profile of adult DN2 cells than adult ETPs (Belyaev et al., 2012). Taken together, this shows that adult and fetal TSPs are functionally different.

1.2.2 Mobilisation of thymus-seeding progenitors into the circulation

Before seeding the adult thymus, progenitors with T lineage potential must leave the bone marrow niche into the blood. MPPs, LMPPs and CLPs can all be detected in the blood, and HSCs are known to be present in the blood, since blood transfers provide long-term multilineage reconstitution (Schwarz and Bhandoola, 2004; Perry et al., 2006; Lai and Kondo, 2007). In the bone marrow niche, progenitors are localised along perivascular sites at the endosteum of the bone (Kunisaki and Frenette, 2012). In this niche, the progenitors reside in the vicin-

ity of osteoblasts, perivascular mesenchymal stem cells as well as sinusoidal endothelial cells (Lymperi et al., 2010).

The factors, required to tether the progenitors in the bone marrow, are presented by these niche cells and comprise beside others, adhesion molecules as well as chemokines. These include vascular cell adhesion molecule 1 (VCAM1; Simmons et al., 1992) or the chemokine receptor CXCR4 with its ligand CXCL12 (also known as stromal cell derived factor-1 or SDF-1 Pitchford et al., 2009). Granulocyte colony-stimulating factor (G-CSF) is known to promote mobilisation of bone marrow progenitors. On one hand, G-CSF is known to disrupt the CXCR4/CXCL12 chemotactic interaction (Levesque et al., 2003). On the other hand, G-CSF-mediated stimulation of macrophages and neutrophils leads to the release of cytokines and metalloproteases that degrade the extracellular matrix (Liu et al., 2000). Several cytokines were reported to promote progenitor mobilisation such as CCL3 (MIP1a), CXCL8 (IL8) and CXCL2 (GRO β ; reviewed by Zhang and Bhandoola, 2014).

Another effect of G-CSF is the upregulation of sphingosine-1 phosphate (S1P) levels in the blood plasma, which facilitates progenitor egress via signalling through S1P receptors on both haematopoietic progenitors and bone marrow stromal cells (Golan et al., 2012). This model suggests a regulated rate of progenitor egress from the bone marrow, in which, under homeostatic conditions, most cells receive a high level of retention signal and remain in the bone marrow niche, while specific progenitor subsets are able to egress. An alternative model suggests that progenitor mobilisation is a non-regulated process caused by passive leakage from the bone marrow in such a way that small numbers of progenitors randomly escape from the bone marrow into the blood (Zhang and Bhandoola, 2014). On one hand, this model is supported by the finding that several progenitor populations are present at equivalent ratios in the blood and bone marrow (Schwarz and Bhandoola, 2004). On the other hand, mobilisation as well as thymus seeding are cyclic, with a periodicity of approximately 3-5 weeks in adult mice, suggesting that there is regulation and communication (Foss et al., 2001; Donskoy et al., 2003).

1.2.3 Molecular basis of homing to the thymus

The homing process to the thymus was shown to depend on several signalling modules such as selectin ligands, chemokine receptors and integrins. Like general leukocyte homing, the process is executed in a multistep cascade, which includes rolling adhesion, chemoattraction, tight adhesion and transendothelial migration (Scimone et al., 2006). Haematopoietic progenitors expressing P-selectin glycoprotein ligand 1 (PSGL1) and CCR7 or CCR9 on their cell surface

interact though these molecules with targets on the endothelium for migration into the tissue (Rossi et al., 2005; Scimone et al., 2006; Krueger et al., 2010; Zlotoff et al., 2011).

PSGL1 binds to the P-selectin receptor that is presented by the endothelial cells lining the thymic vasculature (Rossi et al., 2005; Sultana et al., 2012). Periodical fluctuation of endothelial P-selectin levels might explain the cyclic colonisation of the thymus as well as the linked mobilisation of progenitors (Foss et al., 2001; Donskoy et al., 2003; Gossens et al., 2009). Functional PSGL1 is critical for an efficient generation of thymocytes and requires post-translational modification of the ligand (Li et al., 1996; Rossi et al., 2005).

In addition to the adhesion mediated by selectins, the interaction between $\alpha\text{L}\beta 2$ or $\alpha 4\beta 1$ integrins and ICAM1 or VCAM1 surface receptors is thought to slow down haematopoietic progenitors in the blood stream. This process leads to firm adhesion of rolling progenitors in the thymic vasculature, which facilitates transendothelial migration into the thymus (Ruiz et al., 1995). Consequently, the integrin inhibition of bone marrow progenitors prior to intravenous transfer results in reduced homing to the thymus (Scimone et al., 2006).

Engagement of the chemokine receptors CCR7 and CCR9 on haematopoietic progenitors with their cognate ligands, produced by stromal epithelial cells in the thymus, is associated with thymus homing in fetal and adult mice (Uehara et al., 2002; Liu et al., 2006; Krueger et al., 2010; Zlotoff et al., 2011). In competitive bone marrow transplants, single-deficiency of any of these receptors as well as double-deficiency have no effect on the bone marrow compartment, while the generation of thymocytes (in particular ETPs) is reduced (Schwarz et al., 2007; Krueger et al., 2010; Zlotoff et al., 2011). This developmental defect can be overcome by injection of the progenitors into the thymus. CCL19 and CCL21—the ligands for CCR7—are produced by medullary epithelial cells, while CCL25, the ligand for CCR9, is ubiquitously expressed by all thymic epithelial cells (TECs; Wurbel et al., 2000; Misslitz et al., 2004; Ueno et al., 2004; Ciofani and Zúñiga-Pflücker, 2007). CCRs are G protein coupled receptors. Consequently, signalling through CCRs is abrogated by Pertussis toxin treatment of bone marrow progenitors prior to intravenous transfer. This treatment results in diminished thymic ETP numbers (Jin and Wu, 2008). A similar effect is seen in fetal mice deficient in the guanine nucleotide exchange factors (GEFs) DOCK2 and DOCK180 (Lei et al., 2009). Taken together, this indicates that $G\alpha_i$ signalling in bone marrow progenitors is required for thymic homing. Besides these chemokine receptor/ligand interactions, CXCR4 and CCR5 are also implicated in thymus homing (Robertson et al., 2006).

Based on the requirements of the aforementioned molecules, the potential TSP population can be narrowed down. HSC and MPP do not express either CCR9 or CCR7. Furthermore, they exhibit a low level of functional PSGL1, as they weakly express the receptor and the enzymes, which catalyse these PSGL1 modifications. In contrast, CCR9 and CCR7 are expressed

on downstream subsets of LMPPs, and nearly all CLPs express CCR7 and some also express CCR9. In addition, CCR9⁺ LMPPs as well as Ly6d⁻ CLPs (ALPs) have elevated levels of PSGL1 and its modifiers, the glycosyltransferases Fut4 and Fut7 (Sultana et al., 2012). In line with these findings, CLPs and LMPPs closely resemble the gene expression profile as well as lineage potential of ETPs (Luc et al., 2012), supporting the idea that there are physiological thymic seeding progenitors among these populations. Therefore, it is also likely that the TSP is a heterogeneous population, which consists of both lineage-restricted as well as multipotent progenitors (Rodewald et al., 1994; Bhandoola et al., 2007).

1.2.4 The thymus-seeding progenitor niche

TSPs are thought to occupy a niche in the thymus that only becomes vacant, when the progenitors residing in this niche further differentiate, and thus, leave the niche. This process, which happens in periodic waves, is referred to as 'gating' (Donskoy et al., 2003). The TSPs niche in the thymus might be close to the corticomedullary junction (CMJ), where there are large post-capillary venules and where engraftment of the thymus is seen first (Lind et al., 2001; Porritt et al., 2003). Based on intrathymic injections of HSCs, the size of the niche was estimated to fit 200 cells (Spangrude and Scollay, 1990). However, this might be an underestimation, because HSCs generate thymocytes more efficiently than TSPs, since intrathymic administration of HSCs sustains T cell differentiation for longer periods than transfer of physiological TSPs (Adjali et al., 2005).

Secondary intravenous transplantation of cells that engrafted the thymus after primary intrathymic injection, fails to provide long-term multi-lineage reconstitution (Vicente et al., 2010). This suggests that self-renewal of HSCs is not supported by the thymic progenitor niche. In addition, early thymic transplantation experiments showed that thymus-resident cells are replaced by host-derived cells approximately four weeks after transplantation of wild-type thymus into mice with competent progenitor populations in the bone marrow (Berzins et al., 1998). Similarly, thymus grafting in immunodeficient mice lacking functional T cell receptors results in a single wave of thymopoiesis prior to re-colonization of the thymus by host-derived progenitors and concomitant arrest of T cell development (Frey et al., 1992; Takeda et al., 1996). These findings suggest that thymocytes are seemingly short-lived, as well as that progenitors in the thymus lack self-renewal capacity, and consequently, that thymopoiesis depends on constant influx of haematopoietic progenitors from the blood.

However, two recent studies revealed that thymus-autonomous T cell development lasts several months if the intrathymic niche space is not contested through immigrating host-derived haematopoietic progenitors. This suggests that: *i*) Many thymocytes can self-renew

under these conditions; *ii*) under normal conditions, in addition to the inherent short life span of the thymocytes, the turnover of each generation is also driven by fresh cells entering the progenitor niche and forcing out resident cells; *iii*) the factors, which determine this niche competition between thymus-resident and bone marrow-derived progenitors are IL7 and possibly other growth or differentiation factors such as Flt3 and Notch ligands (Boehm, 2012; Martins et al., 2012; Peaudecerf et al., 2012).

1.2.5 Functional classification of T cells

Peripheral T cells are key mediators in the adaptive immune response against pathogens, and, at the same time, prevent an inappropriate immune response to self (Stritesky et al., 2012). Through their T cell receptor (TCR), they are able to recognise antigens in the context of major histocompatibility complex (MHC) molecules on antigen presenting cells (APCs). Based on the composition of the mostly heterodimeric TCR, T cells are subdivided into either $\alpha\beta$ - or $\gamma\delta$ -T cells. Only few T cells express $\gamma\delta$ -TCRs, while the bulk intra- and extrathymic T cell pools are constituted by T cells of the $\alpha\beta$ -lineage. Peripheral $\alpha\beta$ -T cells express either CD4 or CD8 co-receptors on their surface. Depending on the co-receptor, these T cells have different capacities of engaging antigens on APCs, and subsequently, mounting an immune response through their TCR.

Naïve CD8⁺ cytotoxic T cells recognise antigens in context of MHC class I and become effector T cells, which release cytotoxic proteins, such as perforin or granzyme B, to destroy their target cells (reviewed by Kaech and Cui, 2012; Gravano and Hoyer, 2013). After a response, some cells differentiate into memory T cells, which quickly expand upon re-encounter of the cognate antigen.

CD4⁺ T helper (T_H) cells recognise antigens in context of MHC class II on DCs. The group of T_H cells is heterogeneous and consists of several subsets, which have specific functions within the immune system, and which upon stimulation, secrete specific cytokines to modulate other components of the immune system: T_H1 cells release interferon γ (IFN γ) to activate macrophages; T_H2 cells secrete IL4 to activate B cells; T_H17 cells are pro-inflammatory and release IL17 to recruit neutrophils; T_H9 cells secrete pro-inflammatory IL9 to activate mast cells and promote basophil and eosinophil function; IL22 is produced by T_H22 and T_H17 cells to activate epithelial cells to produce anti-microbial peptides; TGF β and IL10 secreted by regulatory T cells (T_{Reg}) are required for the suppression of effector T cells and to maintain tolerance to self antigens. Within secondary lymphoid organs, follicular T_H cells (T_{FH}) secrete IL21 and interact with clonally-selected B cells to produce humoral immunity (reviewed by Zhou et al., 2009; Zhu et al., 2010; Lee, 2014; Kara et al., 2014).

1.2.6 The thymus and intrathymic T cell development

T cell development requires signals provided by the specific microenvironment of the thymus, which is an evolutionarily conserved primary lymphoid organ (Boehm, 2012). The importance of the thymus remained unknown for a long time, which for instance is reflected by a statement of Sir Peter Medawar in 1963: “*We shall come to regard the presence of lymphocytes in the thymus as an evolutionary accident of no very great significance*”(Medawar, 1963). The immunological function of the thymus was discovered in the 1960s by Miller and colleagues using neonatally thymectomized mice (Miller, 1961, 1963, 2011).

Thymus development starts in the fetus from mesenchymal cells as well as epithelial cells of the third and fourth pharyngeal pouches. The thymus is composed of two lobes, each of which can be divided into a central medullary region and a peripheral cortical region that is surrounded by a capsule. Cells within the thymus are either thymic stromal cells or cells of haematopoietic origin. There are four major compartments within the organ, which are structurally and functionally distinct, as they provide specific microenvironments to guide thymocyte development (reviewed by Petrie and Zúñiga-Pflücker, 2007).

T cell progenitors enter the thymus at the corticomedullary junction (CMJ), which contains a dense network of endothelial cells that facilitate progenitor entry as well as exit of mature thymocytes from/into the bloodstream. Thymocytes first migrate towards the subcapsular zone (SCZ), thereby passing through the cortex, which contains mainly cortical thymic epithelial cells (cTECs) as well as fibroblasts and macrophages. After arrival in the SCZ that contains a dense cTEC network, thymocytes migrate back through the cortex and CMJ into the medulla. Compared to the cortical region, the medulla consists of a more loosely organised stromal network of medullary thymic epithelial cells (mTECs) as well as DCs (Fig. 1.3).

The distinct stages of thymocyte development can be identified through tracing of gradual alterations in cell surface marker expression profile of various proteins, which include the rearranged TCR chains; the TCR co-receptors CD4 and CD8; the T cell-activation marker CD25 (IL2 receptor α chain); the activation and memory marker CD44 (hyaluronic acid receptor); and the stem cell marker CD117 [c-Kit; stem cell factor (SCF) receptor]. Based on the expression of CD4 and CD8, thymocytes are separated into three main groups: CD4 and CD8 double-negative (DN), CD4 and CD8 DP or CD4 and CD8 single-positive (SP) cells.

DN cells (3 - 5% of all thymocytes) represent the most immature thymocytes and consist of four main groups (DN1 - DN4), which all have unique combinations of CD25, CD44 and c-Kit surface marker profiles (Godfrey et al., 1994; Ceredig and Rolink, 2002). Among the heterogeneous DN1 population are early T cell progenitors (ETPs), which are CD25⁻ CD44⁺ c-Kit^{hi} (Allman et al., 2003). This population contributes to only approximately 0.01% of all

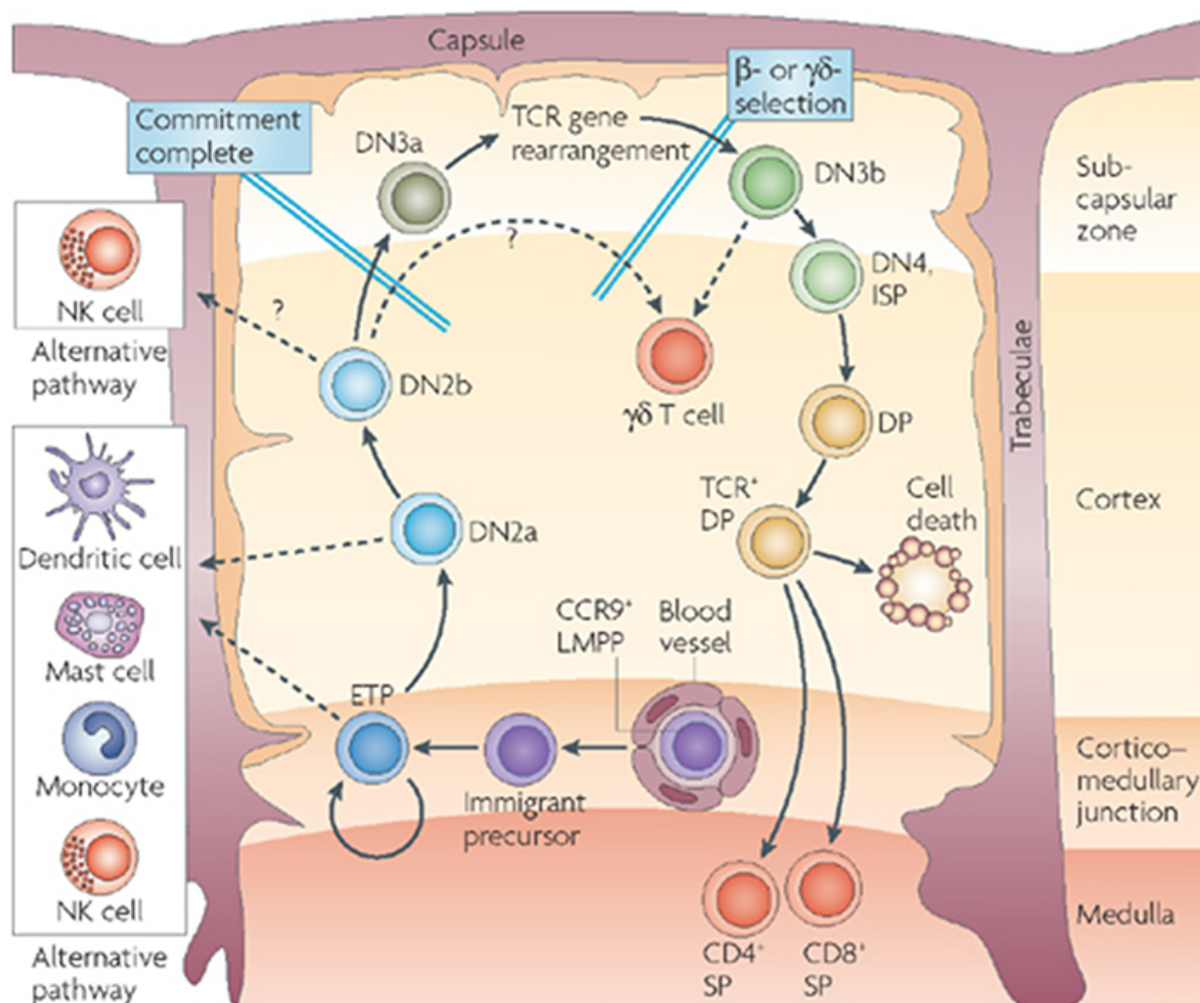


Figure 1.3 Stages of intrathymic T cell development (Rothenberg et al., 2008). Schematic cross-section of the thymus presenting the different compartments of the organ as well as the thymocytes, which migrate through these regions. Each compartment provides a specific microenvironment and thereby promotes differentiation of the haematopoietic progenitors into the T cell lineage. Haematopoietic progenitors with multi-lineage potential enter the thymus and become restricted to the T cell lineage fate in a sequential manner through the early ETP, DN2 and DN3a stages. After the determination of the $\alpha\beta$ - or $\gamma\delta$ -lineage fate, the cells expand massively and migrate from the cortical region towards the medulla. Before a mature T cell can exit into the periphery, it has to undergo positive and negative selection. Dashed arrows indicate potential alternative lineage fates of the progenitors. See main text for abbreviations.

thymocytes and represents the earliest thymic T lymphocyte progenitors. ETPs can be further subdivided by surface levels of the B cell-differentiation marker CD24 (Heat-stable antigen or HSA), as a function of c-Kit (Porritt et al., 2004; Tan et al., 2011). Compared to other haematopoietic progenitors, ETPs constitute the most effective population in generating T cells (Allman et al., 2003; Porritt et al., 2004). Within the thymus, ETPs remain for approximately ten days in a confined region at the CMJ, where they receive the appropriate signals to undergo a proliferative burst (Porritt et al., 2003).

After this phase, ETPs asynchronously start to migrate towards the SCZ, where they interact with cTECs as well as fibroblasts and subsequently differentiate into CD25⁺ CD44⁺ c-Kit^{hi} DN2 cells (Porritt et al., 2003; Takahama, 2006; Petrie and Zúñiga-Pflücker, 2007). c-Kit surface levels allow a further subdivision of the DN2 compartment into c-Kit^{hi} DN2a cells and c-Kit^{int} DN2b cells (Ceredig and Rolink, 2002). The average lifespan for DN2 cells is approximately two days (Porritt et al., 2003). During this time of polarised migration, DN2 cells pass through the inner region of the cortex and receive mitogenic signals, resulting in on-going proliferation (Petrie and Zúñiga-Pflücker, 2007). The extensive (100-1000-fold) proliferation of ETPs and DN2 cells leads to a massive expansion until the cells reach the DN3 stage (Kawamoto et al., 2003).

DN3 cells downregulate CD44 and c-Kit and have a CD25⁺ CD44⁻ c-Kit^{lo} phenotype. Early DN3 cells migrate approximately for two days through the outer half of the cortex towards the SCZ. During this time, the cells rearrange the DNA at the variable (V), diversity (D) and joining (J) segments of the *Tcrb* (β chain, Fig. 1.4), *Tcrγ* (γ chain) or *Tcrδ* (δ chain) antigen receptor loci, through a process termed V(D)J recombination that will be discussed later in detail (Burtrum et al., 1996). In order for these rearrangements to take place, the cells arrest the cell cycle. Only productive in-frame rearrangements of these loci allow the expression of a functional (pre-)TCR and subsequent progression to the next developmental stage. If the cells do not receive the (pre-)TCR-mediated survival signal at this stage, they undergo programmed cell death (apoptosis; Michie and Zúñiga-Pflücker, 2002). Accordingly, deficiency of components of the recombination machinery or of the TCR signalling complex results in the developmental arrest at the DN3 stage (Mombaerts et al., 1992b,a; Shinkai et al., 1992; Fehling et al., 1995; Tanaka et al., 1995; von Boehmer, 2005). In the case of successful in-frame rearrangements of the *Tcrγ* and *Tcrδ* loci, cells signal through the γδ-TCR and enter the γδ-T cell lineage (Livak et al., 1995). Progenitors that are about to enter the αβ-T cell lineage, have successfully rearranged the *Tcrb* locus and express functional pre-TCRs that signal ligand-independently (Irving et al., 1998; Yamasaki et al., 2006), and consist of components of CD3 chains as well as a β chain paired to the invariant pre-TCRα chain (von Boehmer, 2005). This selection process is termed β-selection and represents the first out of three critical checkpoints during αβ-T cell development. DN3 cells prior to this checkpoint are termed DN3a cells.

Upon successful β-selection, cells receive a strong mitogenic stimulus, resulting in extensive proliferation (Michie and Zúñiga-Pflücker, 2002; Kawamoto et al., 2003). The cells in the first of these post-β-selection stages are referred to as DN3b cells. They can be distinguished from resting DN3a cells due to their larger cells size and presence of intracellular (ic) TCRβ (Ceredig and Rolink, 2002). As proliferation is inherently linked to an increased metabolic rate, DN3b cells exhibit elevated surface levels of CD71 (Transferrin receptor) and

CD98 (subunit of L-amino acid transporters), compared to DN3a cells (Kelly et al., 2007). Furthermore, DN3b cells upregulate CD27, which is a lymphocyte-specific member of the TNF receptor superfamily, and CD28. The latter is a member of a heterophilic cell adhesion complex as well as the receptor required for T cell activation, since it recognises the B7 antigen on APCs (June et al., 1990; Taghon et al., 2006; Teague et al., 2010; Zúñiga-Pflücker, 2012). T cell differentiation shortly after β -selection proceeds rapidly. Thus, the DN3b stage is only a transient stage, as the cells have an average lifespan of approximately one day or less, during which they reside mainly in the SCZ (Penit, 1988; Lind et al., 2001; Petrie and Zúñiga-Pflücker, 2007). Besides the proliferative burst, β -selection triggers the inhibition of further V(D)J recombination (Aifantis et al., 1997), and coincides with the down-regulation of CD25 expression (Petrie et al., 1990; Koch and Radtke, 2010).

The subsequent $\text{TCR}\beta^+ \text{CD}25^- \text{CD}44^- \text{c-Kit}^- \text{DN}4$ cells migrate out of the SCZ back into the cortex (Petrie and Zúñiga-Pflücker, 2007). Similar to the DN3b cells, the DN4 cells resemble a transient proliferating population with a lifespan of one day or less. These cells are the direct precursors of yet another transient population, the immature single-positive (ISP) cells. The rapidly proliferating ISPs are characterised by the presence of CD24 and low levels of CD3 in combination with either CD8 (mouse) or CD4 (human) expression (Paterson and Williams, 1987; MacDonald et al., 1988; Galy et al., 1993; Miyazaki, 1997; Spits, 2002).

Co-expression of CD4 and CD8 is upregulated during the ISP stage and approximately 19 days after thymic entry, the first CD4 and CD8 DP T cells are generated (Porritt et al., 2003). DPs are distributed across the whole region of the outer cortex and some early DPs can be found within the SCZ (Petrie and Zúñiga-Pflücker, 2007). They contribute the bulk of thymus cellularity (Penit et al., 1995) and can be separated into the early DP thymocytes which are large and blasting (approximately 20% of all DPs) and smaller resting DPs (approximately 80% of all DPs). The expression of *Rag* genes, which encode an endonuclease complex that is required for V(D)J recombination, is reinitiated in these small DP T cells. This allows the cells to rearrange the *Tcra* locus and to generate a functional $\text{TCR}\alpha$ chain. If the α and β chains assemble correctly, the cells present a TCR on their cell surface.

Further survival and differentiation then depend on the specificity and affinity of this $\alpha\beta$ -TCR for ligands, which are presented by MHCs on thymic stroma cells (reviewed by Klein et al., 2009, 2014). This differentiation step consists of a dual screening process termed positive and negative selection, which only approximately 2% of all thymocytes survive. Both are essential to produce a repertoire of T cells with TCRs, which on one hand, recognise self-peptides in the context of MHC molecules, and on the other hand, do not bind to self-antigens with high affinity—a mechanism that ultimately contributes to central tolerance, which is critical for a cellular adaptive immune response.

Positive selection takes place in the cortex and is the second critical checkpoint in T cell development (besides β -selection). In order for a DP T cell to be positively selected, its initially lowly expressed TCR needs to engage at least with a low affinity self-MHC complexes presented by cTECs as well as partially by DCs and fibroblasts in the cortex (Kisielow et al., 1988; Alam et al., 1996; Klein et al., 2009). Thymocytes with no affinity or very low affinity die by neglect (Egerton et al., 1990; Huesmann et al., 1991). The surviving thymocytes that resided for approximately two days in the cortex (Petrie and Zúñiga-Pflücker, 2007), upregulate the expression of the self-MHC-restricted TCRs with low to intermediate affinity. This is accompanied by a transient downregulation of CD8 before they ultimately terminate expression of CD4 or CD8 to become CD4⁺ CD8⁻ or CD4⁻ CD8⁺ SP cells, respectively (Brugnera et al., 2000).

SP thymocytes then migrate into the medulla where they are exposed to mTECs and DCs, which present a plethora of tissue-restricted antigens in the context of their MHCs. Here, the cells have to survive negative selection, which eliminates thymocytes with high-affinity TCRs for self-MHCs through apoptosis. Thus, this final selection process for thymocytes reduces the chance to generate autoreactive T cells (Sha et al., 1988; Baldwin et al., 2004; Hogquist et al., 2005; Klein et al., 2009; Stritesky et al., 2012). After approximately 7-10 days in the medullary region of the thymus (Petrie and Zúñiga-Pflücker, 2007), only mature CD4SP and CD8SP T cells with functional TCRs of low-intermediate affinity can exit the thymus. When these naïve CD4SP T_H cells or CD8SP cytotoxic T cells in the periphery get activated, they differentiate into effector T cells and eventually become memory T cells.

1.2.7 V(D)J recombination during thymocyte development

The ability of the immune system to respond to virtually any antigen is conferred through a diverse antigen receptor repertoire of T and B lymphocytes. B cells recognise antigens with immunoglobulin (Ig) receptors, which are composed of an Ig heavy (IgH) chain and an Ig light (Ig λ) chain or Ig kappa (Ig κ) chain. T cells recognise antigens in the context of MHCs through their TCR, which is composed of an α and a β chain or a γ and a δ chain. All these proteins are encoded in specific loci in the genome such as *Igh* (IgH), *Igk* (Ig κ), *Igl* (Ig λ), *Tcrb* (TCR β), *Tcr γ* (TCR γ), *Tcra* (TCR α) and *Tcrd* (TCR δ). These genes are highly unusual, as they exist in a non-functional state in the germline, with the 5' portion of each gene (which encodes the antigen binding domain) arranged as arrays of variable (V), diversity (D; only present in some loci) and joining (J) gene segments as well as 3' located constant (C) exons (Fig. 1.4; Schatz and Ji, 2011).

During lymphocyte development, the V, D and J segments become nearly randomly re-arranged prior to transcription of a mature mRNA. This stepwise somatic recombination of B and T cell receptor genes is termed V(D)J recombination (reviewed by Schatz and Ji, 2011). V(D)J recombination requires the introduction of chromosomal DNA double-strand breaks. This reaction is catalysed by the Rag recombinase (referred to hereafter as Rag)—a complex consisting primarily of the proteins encoded by recombination activating gene 1 (*Rag1*) and *Rag2*. Each V, D and J gene segment is flanked by recombination signal sequences (RSSs), which are bound and cleaved by Rag. The ends of the DNA at the generated lesions are first processed and subsequently repaired through a multiprotein complex composed of many components of the non-homologous end joining (NHEJ) repair pathway. Every recombination step results in deletion or inversion of the interspersed sequence between the two joined segments, which can be a few hundred base pairs or several million base pairs apart from each other.

Rag-mediated lesions introduce genome instability that can result in chromosomal translocations. Hence, V(D)J recombination is regulated at several levels: *i*) The expression of *Rag* genes as well the activity and turnover of Rag is spatially and temporally restricted to the early stages of lymphocyte development; *ii*) the chromatin status (open/accessible or condensed/inaccessible) at the RSSs is determined by several factors, which act in *cis* and in *trans* and ultimately control the 'accessibility' of the RSSs for the recombination machinery; *iii*) such *cis*-regulatory regions as well as widely spaced coding gene segments of antigen receptor loci are often juxtaposed through chromosome looping, which is regulated by the position and three-dimensional conformation of antigen receptor loci in the nucleus.

V(D)J recombination is an ordered process, as at loci containing J segments, D-to-J recombination invariably precedes V-to-DJ recombination. This control is partially exerted by RSSs themselves. They contain either a 12 bp or 23 bp spacer region flanked by a heptamer and a nonamer element (forming 12-RSSs and 23-RSSs). There is a strong preference for recombination between a 12-RSS and a 23-RSS (12-23 rule). A 23-RSS 3' of a D segment and a 12-RSS 5' of a J segment promote D-to-J recombination; while a 23-RSS 3' of a V segment and a 12-RSS 5' of a D segment promote V-to-DJ recombination. Illegitimate V-to-J rearrangements of *Tcrb*, *Tcrg* and *Tcrd* are prevented by an additional mechanism (termed beyond 12-23 rule or B12-23; Bassing et al., 2000; Banerjee and Schatz, 2014). Finally, the ordered fashion might be achieved through binding sites for the transcription factor AP1/c-Fos within the 23-RSSs 3' of D segments, as it was shown for the *Tcrb* locus (Wang et al., 2008a). c-Fos recruitment to these sites is thought to sterically interfere with Rag activity at the nearby 12-RSSs 5' of D segments. As the inhibitory 23-RSSs 3' of D is removed upon D-to-J recombination, the 12-RSSs 5' of D segments can be processed, which promotes V-to-DJ rearrangement.

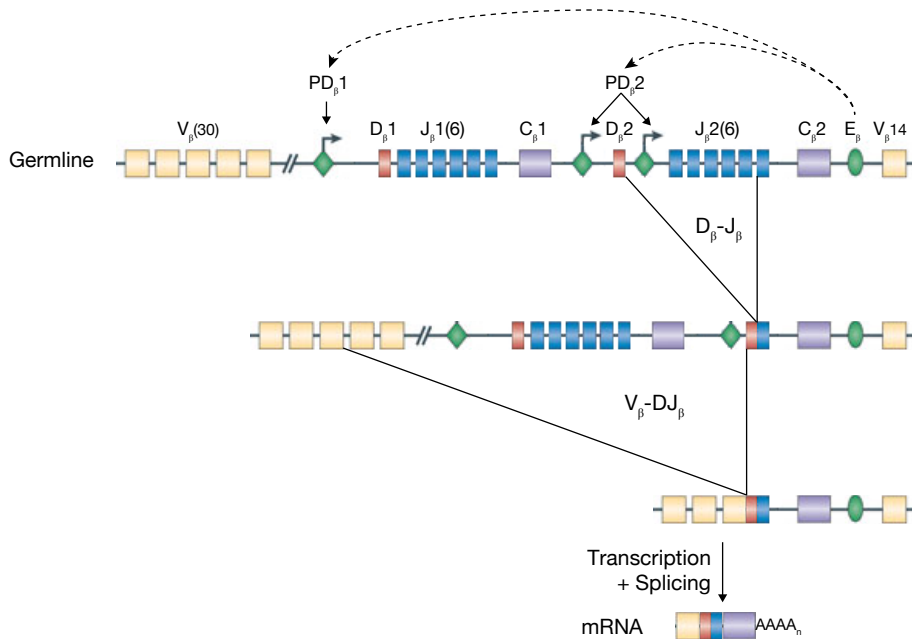


Figure 1.4 *Cis*-regulatory elements controlling the rearrangement of the *Tcrb* locus (adapted from Schatz and Ji 2011). Schematic representation of the *Tcrb* locus with the variable (V), diversity (D), joining (J) and constant (C) gene segments depicted as rectangles. The upper panel represents the locus in germline configuration with the *cis*-regulatory elements shown in green. Dashed arrows depict the interaction of the E_{β} enhancer (oval) with the germline promoters $PD_{\beta}1$ and $PD_{\beta}2$ (diamonds). Rag-mediated cleavage results first in D_{β} -to- J_{β} rearrangement followed by V_{β} -to- DJ_{β} rearrangement. The transcript of the fully rearranged *Tcrb* gene is spliced prior to its translation into a TCR β chain protein.

While D-to-J rearrangements occur on both *Tcrb* alleles, only one allele undergoes V-to-DJ rearrangement—a process termed ‘allelic exclusion’. Allelic exclusion initially depends on asynchronous recombination, as one allele at a time must attempt to undergo V-to-DJ rearrangement. This is promoted by a stochastic mechanism, which depends on the interaction of *Tcrb* alleles with inhibitory (for recombination) subnuclear compartments such as the nuclear lamina and pericentromeric heterochromatin. Ultimately, these interactions reduce the likelihood that two *Tcrb* alleles attempt V-to-DJ recombination simultaneously (Schlimgen et al., 2008; Krangel, 2009; Chan et al., 2013).

The maintenance of the unrearranged state of one *Tcrb* allele is established by feedback inhibition through signalling of the pre-TCR, which promotes downregulation of *Rag* gene expression (Michie and Zúñiga-Pflücker, 2002; Krangel, 2009). Compared to DN T cells, DP thymocytes exhibit a decontracted *Tcrb* conformation, since both ends of the locus are far apart (Skok et al., 2007). This might prevent synapsis formation during the *Rag* re-expressing DP stage between V_{β} and DJ_{β} segments, which are separated by hundreds of kb of DNA. Furthermore, the DN to DP transition coincides with a less-accessible chromatin conformation

at the V_β segments, which might be introduced through the inhibition of the transcription factor E2a upon pre-TCR signalling (Tripathi et al., 2002; Agata et al., 2007; Krangel, 2009).

During T cell development, V(D)J recombination takes place first at the *Tcrb* and/or the *Tcrg* locus during the DN2 and DN3a stages, where Rag protein levels peak for the first time. The cells commit either to the $\gamma\delta$ -lineage or the $\alpha\beta$ -lineage, concurrent with, and probably as an outcome of these TCR recombination events (Hayday and Pennington, 2007; Krangel, 2009; Ciofani and Zúñiga-Pflücker, 2010). Successful *Tcrg* and *Tcrd* rearrangements facilitate the expression of a functional $\gamma\delta$ -TCR and subsequent signalling through this receptor promotes entry into the $\gamma\delta$ -lineage. In contrast, successful *Tcrb* rearrangement, followed by expression of a functional β chain and assembly of a pre-TCR, promotes entry into the $\alpha\beta$ -lineage. The *Tcra* locus is then rearranged in resting DP T cells, in which *Rag* genes are re-expressed.

The *Tcrd* locus is located within the *Tcra* locus. While V_δ -to- D_δ -to- J_δ recombination occurs in DN cells, V_α -to- J_α recombination, which deletes partially or fully rearranged *Tcrd* genes, occurs in DP cells (Livak et al., 1995; Nakajima et al., 1995; Wilson et al., 1996). This developmental stage-specific control is achieved through spatiotemporal enhancer activity of E_δ and E_α (Sleckman et al., 1997; Hernández-Munain et al., 1999; Monroe et al., 1999). The V gene usage of *Tcra* and *Tcrd* is highly regulated. Only few V genes contribute substantially to the *Tcrd* repertoire and their usage correlates with their promoter activity during DN stages (Hawwari and Krangel, 2005). Long distance E_α enhancer activity in DP thymocytes results at first in proximal (<500 kb to J_α) V gene usage for primary rearrangements. E_α also regulates two promoters in the locus—T-early α (TEA) and $J_\alpha 49$. Transcription from TEA is required to allow chromatin remodelling as well as accessibility of the recombinase complex to J_α segments through introduction of H3K4me3—a modification that promotes binding of Rag proteins (Abarrategui and Krangel, 2006, 2007; Liu et al., 2007; Matthews et al., 2007). As there are multiple rounds of V_α -to- J_α rearrangements in DP thymocytes (on average five rounds on each allele), secondary V_α -to- J_α rearrangements assure efficient usage of distal 5' V_α as well as 3' J_α segments (Hawwari and Krangel, 2007).

The *Tcrg* locus exhibits certain developmentally programmed V_γ -to- D_γ recombination, where V_γ gene usage instructs the migration of $\gamma\delta$ -T cell subsets to distinct anatomical locations (Hayday and Pennington, 2007; Krangel, 2009). This process is not instructed via enhancer elements in the locus, but rather depends on the order of V_γ genes. There is a bias towards a J_γ segment-proximal V_γ usage during fetal life. Suppressive mechanisms, mediated by the transcription factor E2a, overcome this bias in adults through enforced repression of proximal V_γ gene promoter activity (Bain et al., 1999; Xiong et al., 2008). V_γ -to- J_γ rearrangements are directed through cytokines such as IL7 and IL15 (Krangel, 2009). IL7/IL7R signalling during DN stages is essential for *Tcrg* recombination. Stat5, the transcriptional me-

diator downstream of IL7R, occupies several sites in the *Tcrq* locus and is thought to promote active J $_{\gamma}$ chromatin as well as V $_{\gamma}$ -to-J $_{\gamma}$ recombination (Yao et al., 2006). The accessibility control of the *Tcrq* locus through the IL7 signalling cascade is mediated in part by Stat5-dependent induction of germline transcription (Ye et al., 1999). Furthermore, the specific V $_{\gamma}$ 5-to-J $_{\gamma}$ 1 rearrangement, which is mediated by IL15, also depends on Stat5 (Zhao et al., 2005b).

1.2.8 Elements controlling *Tcrb* recombination

The *Tcrb* locus spans approximately 700 kb in the mouse germline. Within its 5' region there are 22 functional V $_{\beta}$ genes and 13 non-functional pseudogenes, while the 3' region consists of a duplicated cluster of D $_{\beta}$ -J $_{\beta}$ -C $_{\beta}$ gene segments and one downstream V $_{\beta}$ gene (Fig. 1.4). During development, the D $_{\beta}$ -to-J $_{\beta}$ recombination occurs mainly in DN2 thymocytes (some rearrangements occur in DN1 thymocytes) while V $_{\beta}$ -to-DJ $_{\beta}$ recombination takes place in DN3a thymocytes (Godfrey et al., 1994).

Promoters that initiate sterile transcription in a developmentally regulated fashion flank V $_{\beta}$ and D $_{\beta}$ segments. The *Tcrb* locus contains a single enhancer element termed E $_{\beta}$, which is located between the C $_{\beta}$ 2 and the 3' terminal V $_{\beta}$ 14 element (Krimpenfort et al., 1988; McDougall et al., 1988). The enhancer interacts with and regulates the PD $_{\beta}$ 1 and PD $_{\beta}$ 2 promoters upstream of D $_{\beta}$ -J $_{\beta}$ -C $_{\beta}$ segments but does not control V $_{\beta}$ expression in a non-rearranged locus (Bories et al., 1996; Bouvier et al., 1996; Leduc et al., 2000). However, the assembly of a complete VDJ $_{\beta}$ variable region places the promoter of the rearranged V $_{\beta}$ segment in the vicinity and under the control of E $_{\beta}$ (Busse et al., 2005). This results in enhanced transcription of an immature mRNA consisting of the variable VDJ $_{\beta}$ element and the constant C $_{\beta}$ exons, which is then spliced to produce a full-length TCR β chain.

Analysis of mice, in which E $_{\beta}$ is deleted (E $_{\beta}$ ^{-/-}), showed that E $_{\beta}$ is required for *Tcrb* gene rearrangements in addition to sustaining the high level expression of the rearranged *Tcrb* allele and initiating of germline expression. Consequently, T cell development is partially blocked at the DN cell stage and peripheral $\alpha\beta$ -T cells are absent in E $_{\beta}$ ^{-/-} mice (Bories et al., 1996; Bouvier et al., 1996; Leduc et al., 2000). However, replacement of E $_{\beta}$ with other enhancers indicates that other *cis*-regulatory elements are also involved in the lineage- and developmental stage-specific control of *Tcrb* rearrangement (Sikes et al., 2002). The germline promoters PD $_{\beta}$ 1 and PD $_{\beta}$ 2 that neighbour the D $_{\beta}$ gene segments are likely to be such elements.

Activation of these promoters results in the generation of sterile D $_{\beta}$ -J $_{\beta}$ -C $_{\beta}$ transcripts, which are in germline configuration (Sikes et al., 1998, 1999; McMillan and Sikes, 2008; Abarrategui and Krangel, 2009). Expression of these transcripts is thought to occur during ETP/DN1 stages, while some studies report initiation of germline expression already in haem-

atopoietic precursors in the bone marrow (Soloff et al., 1995; García-Ojeda et al., 2005). Initiation of germline transcription as well transcriptional elongation facilitates the accessibility of transcribed segments to Rag by initiating chromatin remodelling as well as nucleosome repositioning (Osipovich et al., 2007; Kondilis-Mangum et al., 2010). On one hand, transcription results in a phasing of nucleosomes proximal to the transcription start site (TSS), which depletes nucleosomes at RSSs and makes it accessible for Rag (Kondilis-Mangum et al., 2010). On the other hand, transcription introduces H3K4me3, and thereby enhances the recruitment of Rag proteins (Abarrategui and Krangel, 2006, 2007; Liu et al., 2007; Matthews et al., 2007).

Replacement of PD β 1 with other promoters showed that promoter activation rather than activity of a specific promoter is required for D β -to-J β recombination (Sikes et al., 1999). In this context, the recruitment of the SWI/SNF (switch/sucrose non-fermentable) chromatin remodelling complex is sufficient to induce D β transcription and recombination in the absence of a promoter, as it is thought to facilitate recombination by catalysing the displacement of histones from RSSs (Osipovich et al., 2007). Both D β elements are flanked by 5' promoters that presumably enforce DJ β accessibility during V-to-DJ recombination. The 5' promoter of the D β 2-J β 2-C β 2 cassette is suppressed through upstream stimulatory factor (USF)-1 recruitment to the D β 2 12-RSS in the germline (Stone et al., 2012). Transcription of the D β 2-J β 2-C β 2 element initially depends on a second promoter located between D β 2 and J β 2. Promoter activity is then redirected upstream of D β 2 upon D β -to-J β joining (McMillan and Sikes, 2009; Sikes et al., 2010). These different activities of the promoters as well as their positions relative to the D β and/or J β segments and their RSSs result in preferential accumulation DJ β 1 joints compared to DJ β 2 joints (Born et al., 1985; Haars et al., 1986; Lindsten et al., 1987; Uematsu et al., 1988). In the case of frame-shifted V(D β 1)J joints, DJ β 2 joints can act as a reserve substrate for secondary V β rearrangements to maximise β -selection survival and TCR β repertoire diversity (Sikes et al., 2002; McMillan and Sikes, 2009; Sikes et al., 2010).

Activation of PD β 1 and to a lesser extent PD β 2 depends on E β , which was shown to physically interact with both germline promoters (Oestreich et al., 2006). DN cells of E β ^{-/-} mice fail to undergo D β 1-to-J β 1 rearrangements and D β 2-to-J β 2 joining is approximately 25-fold reduced compared to wild-type cells (Ryu et al., 2003). In addition to this, E β enhancer deficiency often results in aberrant D β -to-J β recombination events such as D β 1-D β 2-J β 2 (Ryu et al., 2003). The E β -mediated regulation is directional and confined to an ~25 kb large region 5' of the enhancer, encompassing both D β -J β -C β clusters (Mathieu et al., 2000). Multiple transcriptional regulators, such as Ets proteins, Runx proteins, bHLH proteins and Gata3, bind to E β (Leiden, 1993; Carvajal and Sen, 2000; Tripathi et al., 2000; Anderson, 2006; Bonnet et al., 2009b,a), which results in T cells lineage-specific activation of the enhancer early in development (Capone et al., 1993). A conserved core region of E β is sufficient to sustain

Tcrb gene expression and promote $\alpha\beta$ -T cell development (Bonnet et al., 2009b). However, early thymocytes carrying only the core enhancer fragment barely achieve D_β - J_β - C_β germline transcription and recombination.

In contrast to the enhancer, loss of the $PD_\beta 1$ promoter results in a specific defect in $D_\beta 1$ -to- $J_\beta 1$ recombination, while $D_\beta 2$ -to- $J_\beta 2$ is not affected (Whitehurst et al., 1999). Disruption of $PD_\beta 1$ causes DNA hypermethylation at RSSs and their surrounding regions as well as reduced histone acetylation, thereby reducing accessibility and recombination (Whitehurst et al., 2000). This defect can be rescued by reintroduction of another promoter, which illustrates how the promoters rely on tissue-specific enhancer activity to control V(D)J recombination and chromatin accessibility (Sikes et al., 1999). Mechanistically, the interaction of E_β with PD_β promoters is thought to establish a functional nucleoprotein complex at the promoters, including several transcription factors as well as components of the basal transcription machinery such as RNA polymerase II and TBP (Spicuglia et al., 2002; Oestreich et al., 2006). This E_β / PD_β -based holocomplex is thought to allow stable recruitment of the SWI/SNF chromatin-remodelling complex to promote germline transcription as well as subsequent recombination (Bonnet et al., 2009a). p300/CBP and PCAF, two histone acetylases that are known to be recruited upon Notch activation, are recruited to $PD_\beta 1$ in an E_β -dependent manner (Spicuglia et al., 2002). Furthermore, conditional inactivation of *Notch1* in DN2/3 thymocytes leads to a defect in *Tcrb* rearrangement (Wolfer et al., 2002). However, a molecular explanation for this phenotype is missing.

1.2.9 Signalling pathways during early T cell development

Several signalling pathways regulate intrathymic T cell development. The chemokines CCL25 and CXCL12, and their receptors, CCR9 and CXCR4, respectively, are required for thymus homing and seeding of TSPs. Furthermore, both pathways are involved in thymocyte migration from the CMJ to the cortex (Wurbel et al., 2001; Ara et al., 2003; Plotkin et al., 2003; Benz et al., 2004; Liu et al., 2006). CXCR4 also mediates cell survival and proliferation during β -selection together with pre-TCR signalling (Tramont et al., 2010). CXCR4 downregulation in DP T cells is paralleled by CCR7 upregulation, which, together with its activating chemokines CC19 and CCL21, regulates migration of SP T cells from the cortex into the medulla (Kwan and Killeen, 2004; Ueno et al., 2004; Kurobe et al., 2006; Davalos-Misslitz et al., 2007). In addition to CCR9 and CXCR4, CCR7 is required for efficient thymus seeding of haematopoietic progenitors (Krueger et al., 2010; Luche et al., 2010).

Thymic stromal cells also express stem cell factor (SCF or kit-ligand), which signals via the c-Kit receptor protein-tyrosine kinase (CD117). c-Kit levels are high on HSCs, lymphoid

progenitors in the bone marrow as well thymic ETP/DN1 and DN2 cells (Ogawa et al., 1991; Wu et al., 1991; Godfrey et al., 1992). c-Kit pathway inhibition results in a reduction of the ETP/DN1 compartments (Rodewald et al., 1995; Di Santo and Rodewald, 1998) and also affects the DN2 but not DN3 cells *in vitro* (Massa et al., 2006). Wang et al. (2006b) suggested that SCF can maintain cells in an undifferentiated state by its mitogenic function and that loss of signalling results in accelerated T cell lineage maturation of lymphoid progenitors.

TECs produce IL7, which is another cytokine required for early T cell development (reviewed by Hong et al., 2012). IL7/IL7R signalling is mediated via the signal transducer and activator of transcription 5 (Stat5) and the phosphatidylinositol-3-kinase (PI3K) pathway. IL7 counteracts the inhibitory effect of PTEN on the PI3K pathway, which promotes the turnover of the proapoptotic factors Bax or Bim and thereby prevents apoptosis (reviewed by Fayard et al., 2010). Hence, IL7 signals are required for proliferation, survival and differentiation of DN2 and DN3 thymocytes (von Freeden-Jeffry et al., 1997), and IL7 deficiency results in a block of T cell development at the DN2 stage (reviewed by Ciofani and Zúñiga-Pflücker, 2006, 2007). Expression of the IL7 receptor is downregulated during β -selection, and DP cells remain refractory to IL7 signals until cells undergo positive selection (Sudo et al., 1993; Van De Wiele et al., 2004; Yu et al., 2006). Moreover, IL7/IL7R signalling is essential for *Tcr* locus accessibility as well as rearrangement, and thus, for $\gamma\delta$ -T cell development (Maki et al., 1996; Durum et al., 1998).

The binding of secreted Hedgehog (Hh) proteins, such as Sonic Hh (SHh) or Indian Hh (IHh), to the bipartite Patched (Ptch) and Smoothened (Smo) transmembrane receptor, activates the Gli family of transcription factors, which are the downstream effectors of the Hh pathway (reviewed by Crompton et al., 2007). The requirement for Hh signalling in T cell development is controversial (reviewed by Shah and Zúñiga-Pflücker, 2014). Previous results suggested a requirement of the pathway at multiple thymocyte stages (El Andaloussi et al., 2006; Rowbotham et al., 2009; Drakopoulou et al., 2010). However, Uhmman et al. (2011) showed that intrathymic T cell development is independent of T cell-intrinsic Ptch expression and proposed that Hh is an exclusive T cell-extrinsic factor necessary for proper development of T cells at their pre-thymic stage.

The Wnt signalling pathway is activated by engagement of Wnt proteins with their receptor complex consisting of the frizzled family receptor and low-density lipoprotein receptor-related-protein (LRP5 or LRP6) co-receptor (reviewed by Staal and Clevers, 2005). This protects β -catenin from proteasomal degradation and allows its translocation into the nucleus. Nuclear β -catenin acts as a transcriptional switch, as it binds to the transcription factors TCF-1 (T cell factor) or LEF (lymphocyte enhancer binding factor), turning them from transcriptional repressors into activators (Ciofani and Zúñiga-Pflücker, 2007). Many studies suggested piv-

otal roles of Wnt signalling, such as proliferation and survival, during DN and DP stages of T cell development (Weerkamp et al., 2006a reviewed by Staal and Clevers, 2005; Ciofani and Zúñiga-Pflücker, 2007; Shah and Zúñiga-Pflücker, 2014). Ioannidis et al. (2001) showed that T cell maturation depends on the presence of the β -catenin-binding domain in TCF-1. However, deficiency of β -catenin alone (Cobas et al., 2004) or in combination with γ -catenin (Koch et al., 2008b) does not perturb haematopoiesis or lymphopoiesis. Therefore, the dependency of the earliest thymocytes on TCF-1 might point towards a Wnt-independent role (as an repressor) of this transcription factor (Schilham et al., 1998; Weber et al., 2011; Germar et al., 2011; Yu et al., 2012a).

Bone morphogenic proteins (BMP) belong to the transforming growth factor- β (TGF- β) superfamily of growth factors. While perturbation of TGF- β signalling does not affect the DN compartment, BMPs appear to negatively affect T cell development by limiting proliferation and differentiation (Graf et al., 2002; Hager-Theodorides et al., 2002; Cejalvo et al., 2007; Ciofani and Zúñiga-Pflücker, 2007). Furthermore, like Wnt proteins (Balciunaite et al., 2002), BMPs are required for the development of thymic stroma, as both affect the expression of FoxN1 (Bleul and Boehm, 2005), which is an essential transcription factor for the development of TECs (Zhang et al., 2012c).

The central pathway for intrathymic T cell development is the Notch pathway. It acts in concert with the pathways mentioned above to promote thymopoiesis. Physiological T cell development is not instructed through a single 'master regulator' that forces haematopoietic progenitors to adopt the T cell fate. It is rather a process, in which multipotent-progenitors that enter the thymus progressively lose their potential to give rise to cells of non-T lineages (commitment), while at the same time, these cells acquire their T cell potential through expression of T lineage-specific genes (specification; reviewed by Rothenberg, 2012). T cell specification and commitment take place during the pre- β -selection DN stages. During this phase, the cells are independent of TCR signals while they are highly dependent on the activation of their Notch1 receptors by DLL4 ligands presented by TECs (Hozumi et al., 2008; Koch et al., 2008a). Notch1 signals are required for T cell lineage specification (Radtke et al., 1999; Han et al., 2002; Schmitt and Zúñiga-Pflücker, 2002; Sambandam et al., 2005; Tan et al., 2005) as well as for commitment (Bell and Bhandoola, 2008; Wada et al., 2008; Feyerabend et al., 2009). Thus, ICN1 closely resembles the function of a 'master regulator' for T cell development. However, Notch signalling requires additional transcriptional regulators to promote thymocyte development.

1.2.10 Essential transcription factors during early T cell development

T cells upregulate the expression of the zinc-finger transcription factor *Gata3*, which is required for the execution of several developmental steps (Hosoya et al., 2010). While loss of *Gata3* does not affect haematopoietic stem cells or multipotent haematopoietic progenitors, the generation of functional ETPs depends on *Gata3* (Hosoya et al., 2009). *Gata3* is also required for DN1/ETP to DN2 transition by promoting a Notch-induced T cell program and for the suppression of a latent B cell potential in pro-T cells (García-Ojeda et al., 2013). Subsequently, *Gata3* levels are reduced through E2a to allow T lineage commitment and further differentiation (Xu et al., 2013). *Gata3* facilitates pre-TCR complex formation and is consequently required for β -selection by (Pai et al., 2003). Furthermore, *Gata3* is involved in the CD4 vs CD8 lineage choice (Singer et al., 2008) and it is the master regulator for Th2 lineage development of peripheral CD4⁺ T cells (Pai et al., 2004; Zhu et al., 2004; Hosoya et al., 2010). It was shown that Notch directly activates *Gata3* expression for this inductive Th2 differentiation process and that both transcription factors synergise to induce IL4 expression (Amsen et al., 2007; Fang et al., 2007). While *Gata3* expression is upregulated from the ETP to the DN3 stage, it needs to be restricted in DN2 cells to facilitate T lymphocyte lineage commitment. This repression of *Gata3* is achieved by E2a (Xu et al., 2013).

The E-proteins and bHLH transcription factors E2a (E47 and E12), HEB (HEBAIt and HEBCan) and to a lesser extent E2-2 (E2-2Alt and E2-2Can) play important roles throughout different stages of T cell development, as they regulate T cell specification, commitment and developmental plasticity (Wikström et al., 2008; Braunstein and Anderson, 2012; Miyazaki et al., 2014). E2a is required during T cell specification to restrict the myeloid potential, and consequently, E2a deficiency results in increased differentiation of lymphoid progenitors into the myeloid lineage. During this phase, E2a cooperates with Notch signalling at several genes, such as at the *Ptcra* locus (pre-TCR α), to promote T cell development (Reizis and Leder, 2001, 2002; Ikawa et al., 2006). Yashiro-Ohtani et al. (2009) demonstrated that E2a is an important activator of *Notch1* expression and that the upregulation of the E2a antagonist Id3 upon β -selection leads to a rapid downregulation of *Notch1* expression. Furthermore, E2a is required for effective V(D)J recombination at the *Tcrb* locus as it regulates chromatin accessibility in a dosage-dependent manner (Agata et al., 2007).

The HEB proteins, consisting of HEBAIt and HEBCan, function at several stages of T cell development including β -selection, *Tcrb* as well as *Tcra* gene rearrangement or regulation of pre-TCR α (D'Cruz et al., 2010; Braunstein and Anderson, 2012). Accordingly, HEB deficiency results in a developmental block at the β -selection checkpoint (Braunstein and An-

derson, 2010). Furthermore, HEBAIt promotes T cell specification in a Notch1-dependent manner (Wang et al., 2006a; Braunstein and Anderson, 2011; Braunstein et al., 2010).

TCF-1 is encoded by *Tcf7*, which is strongly induced during T cell development as well as upon *in vitro* induction of Notch signalling in pluripotent haematolymphoid progenitors (Taghon et al., 2005; Weber et al., 2011). *Tcf7* is thought to be a Notch target gene, since ICN1 and Rbpj occupy an enhancer element upstream of *Tcf7* in a T cell leukaemia cell line (Germar et al., 2011). Deficiency in TCF-1 results in a partial block of T cell development at the ETP stage, and *Tcf7*^{-/-} ETPs exhibit reduced DNA metabolism and die by apoptosis (Germar et al., 2011). This block can be rescued by overexpression of human TCF-1, which leads to the upregulation of many T cell lineage-specific genes, such as transcription factors Gata3 and Bcl11b, as well as components of the TCR, even in the absence of Notch signalling (Weber et al., 2011). Hence, TCF-1 is a critical regulator of T cell lineage specification.

Hes1 (hairy and enhancer of split 1) is a canonical Notch target gene, as its expression is induced in several tissues upon Notch activation (Jarriault et al., 1995; Nishimura et al., 1998). Its encoded basic helix-loop-helix (bHLH) transcriptional repressor Hes1 is important during T cell specification, where the Notch-Hes1 axis was shown to constrain myeloid gene expression programs in T cell progenitors. Hes1 occupies a binding site within the promoter of the *Cepba* gene, which encodes C/EBP- α , a critical regulator of the development of myeloid cells and dendritic cells (DCs; Zhang et al., 1997; De Obaldia et al., 2013b; Welner et al., 2013). The Hes1-mediated repression of *Cepba* is essential for efficient T cell development, as *in vitro* Notch-induced *Hes1*^{-/-} cells gave rise to myeloid cells, while Hes1 and C/EBP- α double-deficiency restores T cell development under the same culture conditions (De Obaldia et al., 2013b). Wendorff et al. (2010) demonstrated that the conditional deletion of *Hes1* results in impaired T cell lineage commitment in competitive bone marrow chimeras, while it does not affect thymocyte maturation. Furthermore, Hes1 is required during β -selection to facilitate activation of the PI3K/Akt pathway, which is required for pre-TCR-induced survival, differentiation and proliferation of developing $\alpha\beta$ -lineage T cells. During this stage, Hes1 is thought to repress the *Pten* gene, which encodes the PI3K/Akt pathway inhibitor Pten (Wong et al., 2012a; Shah and Zúñiga-Pflücker, 2014).

The zinc-finger transcription factor Bcl11b is sharply upregulated upon the DN2a to DN2b transition (Tydell et al., 2007). Its T cell-specific expression profile was shown to depend on an enhancer element that is 850 kb downstream of the *Bcl11b* locus (Li et al., 2013). This *cis*-regulatory element loops to the *Bcl11b* promoter and contains consensus motifs for several other transcription factors. *Bcl11b* is thought to be downstream of TCF-1 and Notch signalling as a TCF-1-dependent regulation of the enhancer was shown, while Notch is thought to regulate *Bcl11b* via its promoter or intragenic *cis*-regulatory elements (Li et al., 2013). Bcl11b

is required for T cell lineage commitment, since it represses several genes that are associated with the NK-cell lineage such as *Id2*, *Il2rb* and *Zfp15* (Ikawa et al., 2010; Li et al., 2010a). Accordingly, *Bcl11b* deletion in T cells results in the acquisition of NK cell properties and concomitant decrease in T cell-associated gene expression in these T cells (Li et al., 2010b).

The *Myc* gene encodes the basic region/helix-loop-helix/leucine zipper (bHLHZip) transcription factor c-Myc, which binds to E-box DNA motifs as a heterodimer with Max, resulting in transcriptional activation or suppression of genes (reviewed by Eilers and Eisenman, 2008). *Myc* is a critical oncogene, as it promotes cell cycle entry (Walker et al., 2005). This mitogenic effect of c-Myc is critical during malignant T cell development, where its expression is under the control of Notch signalling (Palomero et al., 2006; Sharma et al., 2006; Weng et al., 2006). During normal T cell development, c-Myc levels increase upon β -selection where c-Myc-mediated proliferation is required (Kreslavsky et al., 2012). However, this proliferation is dispensable for the developmental progression from the DN to the DP stage (Dose et al., 2006; Kreslavsky et al., 2012). While *Myc* expression in DN3 cells is Notch-dependent (Weng et al., 2006), c-Myc-induced proliferation is sufficient to overcome the Notch signalling requirement for proliferation during β -selection (Kreslavsky et al., 2012; Wong et al., 2012a).

Besides these transcription factors, numerous others were reported to regulate early T cell development such as Ikaros family members, Ets family members (PU.1, Ets1, Ets2, Erg), Gli, c-Myb and Runx1/CBF (reviewed by Rothenberg, 2012; Rothenberg et al., 2013; Rothenberg, 2014). In summary, thymopoiesis is a complex process, which is regulated through multiple transcription factors. These regulators interact or control each other to induce, repress, or limit a gene expression network that inhibits alternative fates and allows a stepwise progression into the T cell lineage (Koch and Radtke, 2010).

1.3 Mechanistic insights into the Notch pathway

About a century ago – in 1914, John S. Dexter reported a mutant *Drosophila* phenotype with notched wings (Dexter, 1914). Shortly thereafter Thomas Morgan Hunt characterised the mutant alleles of these flies (Morgan, 1917). In the 1980s, Spyros Artavanis-Tsakonas and Michael Young cloned the gene encoding the Notch receptor and attributed the wing-notching phenotype to haploinsufficiency of the gene (Wharton et al., 1985; Kidd et al., 1986). These studies are the cornerstones that led to the discovery of the Notch pathway. Ever since, a myriad of studies characterised the structural and mechanistic functions of the pathway.

The Notch pathway is a conserved signalling cascade in metazoan organisms that enables short-range communication between cells. The pathway is remarkably simple as it consists of three core components: *i*) The Notch receptor presented on the signal-receiving cell; *ii*) a ligand

present on the cell surface of the signal-sending cell; *iii*) the transcriptional mediator of Notch signalling—CSL (C-repeat binding factor 1 [CBF1] in humans; Suppressor of hairless [Su(H)] in flies; lin-12 -and glp-1 [LAG] in worms; Rbpj in mice). The encounter of a ligand by a Notch receptor results in the proteolytic release of an active Notch fragment, which translocates to the nucleoplasm where it binds Rbpj to mediate transcriptional activation of Notch target genes.

1.3.1 Domain architecture of Notch receptors

Mammals express four Notch receptor paralogues (Notch1-4) that display both redundant and unique functions. While there is only one Notch receptor in *Drosophila melanogaster*; the worm *Caenorhabditis elegans* possesses two redundant receptors (LIN12, GLP1). Notch receptors are large type I single-pass transmembrane glycoproteins that have an overall highly conserved domain organisation composed of a series of characteristic structural motifs (Fig. 1.5; reviewed by Gordon et al., 2008; Kopan and Ilagan, 2009; Chillakuri et al., 2012).

Membrane-bound receptors are heterodimers resulting from the cleavage of full-length Notch polypeptides in the secretory trans-Golgi pathway by furin-like convertases at site 1 (S1). The cleavage products NECD-NTMIC (Notch extracellular domain-Notch transmembrane and intracellular domain) are held together by non-covalent interactions between the hydrophobic heterodimerisation domain (HD) regions of the extra- and intracellular domain of Notch. The extracellular domain is required for ligand-mediated receptor activation and, depending on the type of Notch receptor, contains 29-36 tandem epidermal growth factor (EGF)-like repeats. It was shown in *D. melanogaster* that the N-terminal repeats 11-12 are required for *trans*-activation by ligands, while repeats 24-29 mediate *cis*-inhibition by preventing receptor activation through ligands presented on the same cell (Cordle et al., 2008). Proximal to the EGF repeats, Notch proteins contain a negative regulatory region (NRR) made up by three cysteine-rich Lin12-Notch repeats (LNRs). This region conceals and protects the cleavage site 2 (S2) from metalloproteases, thereby preventing ligand-independent Notch signalling (Sanchez-Irizarry et al., 2004).

Adjacent to the HD of NTMIC is the transmembrane domain (TMD), which anchors the receptor in the cell's plasma membrane. This domain also contains the cleavage site 3 (S3), which is targeted by the γ -secretase complex to liberate the C-terminal intracellular domain of Notch (ICN). The ICN contains the Rbpj association module (RAM) domain that is required for high-affinity binding to the transcriptional mediator Rbpj (Tamura et al., 1995). An unstructured linker contains one of the two nuclear localisation signal (NLS) domains and separates the RAM domain from a seven ankyrin repeat-containing (ANK) domain. While the RAM domain binds CSL with relatively high affinities, it is likely to mediate the initial association of

ICN and Rbpj (Lubman et al., 2007; Del Bianco et al., 2008; Johnson et al., 2010). This process enables formation of a CSL/ANK composite surface that recruits Mastermind-like (MAML) co-activator proteins. Formation of this heterotrimeric complex composed of ICN, MAML and Rbpj is essential for transcriptional activation of Notch target genes (Fryer et al., 2002; Wallberg et al., 2002; Maillard et al., 2004; Nam et al., 2006). Mammalian Notch1 and Notch2 but not Notch3 or Notch4 encode a transactivation domain (TAD) region. The TAD of Notch1 was shown to be required for the proper assembly of the ICN1/Rbpj/MAML trimolecular transcription complex in fetal haematopoietic stem cells (Gerhardt et al., 2014). The most C-terminal domain of the receptor contains conserved proline/glutamic acid/serine/threonine-rich motifs (PEST). The PEST domain encodes degradation motifs, which are phosphorylated by kinases and subsequently targeted by E3 ubiquitin-ligases. This process regulates the turnover of activated ICN, as it targets the ICN for proteasomal degradation (Fryer et al., 2004; Tsunematsu et al., 2004; O'Neil et al., 2007; Thompson et al., 2007).

1.3.2 Notch ligands

Notch ligands are type I transmembrane proteins and can be subdivided into Delta-like ligand (DLL) or Serrate/Jagged ligand families (Fig. 1.5; reviewed by D'Souza et al., 2008). While there is one ligand of each family (Delta and Serrate) in *D. melanogaster*, the mammalian genome encodes three Delta-like family members (DLL1, DLL3 and DLL4) as well as two Jagged family ligands (Jagged1 and Jagged2). All ligands contain a Notch-binding site within a Delta/Serrate/LAG2 (DSL) region. Jagged/Serrate ligands can be distinguished from Delta/Delta-like ligands by the presence of a cysteine-rich (CR) domain. All ligands encode EGF-like domains, which vary in number from 16 (Jagged family) and 5-9 (DLL family). Mammalian Jagged1 and Jagged2, as well as DLL1 are characterised by the presence of a Delta/OSM11 (DOS) domain—two N-terminal EGF-like repeats with unusual structure, which is absent in DLL3 and DLL4. Besides these canonical ligands, there were also non-canonical Notch ligands reported, such as soluble ligands, that function in concert with transmembrane adaptors (Aho, 2004; Chen and Greenwald, 2004; Komatsu et al., 2008).

The signalling activity of DSL ligands is regulated by endocytic trafficking of the ligand in the signal-sending cell, which requires: *i*) ubiquitination of the ligands by the E3 ubiquitin ligases Mindbomb (Mib) or Neuralized (Neur); *ii*) endocytic adaptors of the eosin family; *iii*) dynamin-dependent endocytosis (Fig. 1.6; reviewed by Le Borgne, 2006; Nichols et al., 2007; Musse et al., 2012). Two general models exist, explaining the importance of ligand endocytosis in Notch signalling. The 'recycling' model proposes that ubiquitinated ligands become endocytosed and are subsequently trafficked into cell surface microdomains. The 'pulling-force'

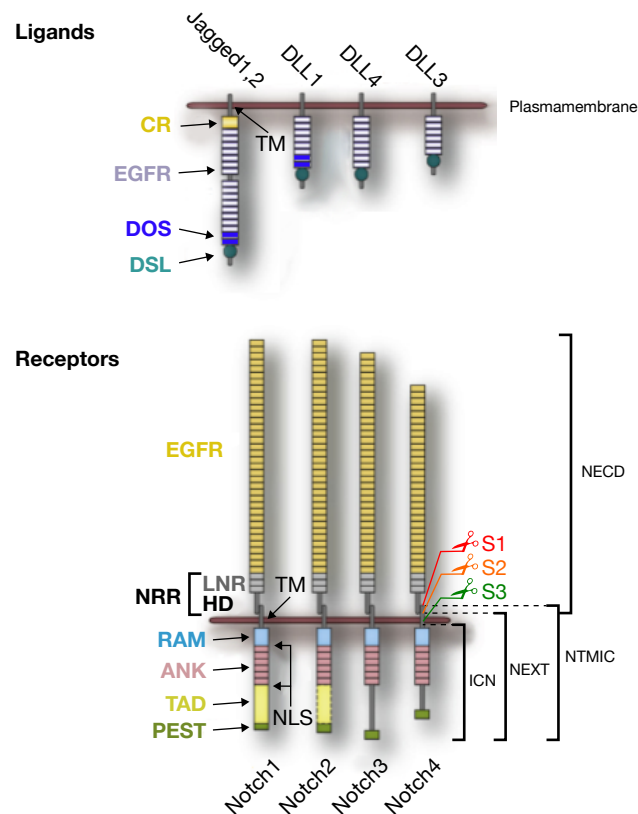


Figure 1.5 Domain architecture of Notch receptors and ligands (adapted from Radtke et al. 2010). Schematic depiction of the murine Notch ligands (upper panel) and the Notch receptors (lower panel). Abbreviations for ligand domains: CR - cysteine-rich; DSL - Delta/Serrate/LAG2; EGFR - epidermal growth factor like repeats; DOS - Delta/OSM1. Additional abbreviations for receptor domains: LNR - Lin12-Notch repeat; HD - heterodimerisation domain; NRR - negative regulatory region; NLS - nuclear localisation signal; RAM - Rbpj association module; ANK - ankyrin repeat-containing; TAD - transactivation domain; PEST - proline/glutamic acid/serine/threonine-rich. The products of the S1 cleavage site (red scissor symbol) are indicated: NECD - Notch extracellular domain, NTMIC - Notch transmembrane and intracellular domain; as well as the Notch extracellular truncation - NEXT product of the S2 cleavage site (orange scissor symbol) and the ICN - intracellular Notch product of the S3 (S4) cleavage (green scissor symbol).

or 'lift and cut' models suggest that ligand ubiquitination occurs upon receptor encounter and leads to the formation of epsin-dependent endocytic structures (Meloty-Kapella et al., 2012). The generated mechanical force on the receptor introduces a structural conformation change that unfolds the NRR domain of the Notch heterodimer, which allows proteolysis by metalloproteases (Fig. 1.6).

Within the thymic epithelial microenvironment, all Notch-ligands except DLL3 are expressed (Felli et al., 1999; Schmitt and Zúñiga-Pflücker, 2002; Harman et al., 2003; Schmitt et al., 2004a; Mohtashami et al., 2010). However the Notch1-DLL4 axis was shown to be the required interaction to promote early T cell development *in vivo* (Hozumi et al., 2008; Koch et al., 2008a; Andrawes et al., 2013). *In vitro* stromal feeder cells engineered to express

either DLL1 or DLL4 (Jaleco et al., 2001; Schmitt and Zúñiga-Pflücker, 2002; Mohtashami and Zúñiga-Pflücker, 2006; Schmitt and Zúñiga-Pflücker, 2006) as well as feeder-free systems with Fc-fusion protein ligands coupled to the plastic surface (Ohishi et al., 2002; Delaney et al., 2005; Ikawa et al., 2010) enabled the expansion and differentiation of haematopoietic progenitors into T cells.

1.3.3 The Notch signalling cascade

1.3.3.1 Activation of Notch receptors

Membrane-bound Notch receptors are resistant to proteolysis until ligand binding-induced unfolding of the NRR domain occurs. This receptor conformation is an accessible substrate for a disintegrin and metalloprotease (ADAM). The family members ADAM10 (also referred to as Kuzbanian and sup17) and ADAM17 (also referred to as TNF- α converting enzyme or TACE) are implicated to catalyse shedding of the Notch receptor ectodomain at S2 (Brou et al., 2000; Delwig and Rand, 2008; Bozkulak and Weinmaster, 2009; van Tetering et al., 2009). Juxtamembrane cleavage at S2 produces the membrane-bound Notch extracellular truncation (NEXT) fragment, which is the substrate for a secondary activating cleavage at S3 by the γ -secretase complex. S2 proteolysis is the rate-limiting step in Notch receptor activation and the NRR can be targeted with antagonistic or agonistic antibodies, which either stabilise or destabilise the protease-insensitive receptor conformation (Li et al., 2008; Wu et al., 2010). The importance of the NRR is also reflected by several cancer-causing Notch-activating mutations, which lead to the disruption the NRR structure and shift the equilibrium to an 'open' state (Weng et al., 2004; Grabher et al., 2006; Aster et al., 2008).

The NEXT polypeptide is further enzymatically processed by the γ -secretase complex, a membrane-anchored multi-subunit protease complex, consisting at least of presenilin (catalytic subunit), nicastrin, APh1 and PEN2 (Kaether et al., 2006). Cleavage occurs progressively within the TMD of Notch (Kopan and Ilagan, 2009). It is initiated at S3, close to the inner leaflet of the plasma membrane and releases the ICN into the cytoplasm (Qi-Takahara et al., 2005; Zhao et al., 2005a). The last cleavage takes place in the middle of the TMD at site 4 (S4) and generates the N β peptide (named after the A β product of γ -secretase-mediated amyloid precursor protein cleavage, which is associated with Alzheimer's disease; Okochi et al., 2002). Mouse Notch1 is predominantly cleaved at the carboxyl side of Gly-1743 (S3-cleavage), which frees the ICN with its N-terminal Val-1744. There are also ICN variants with other N-termini, however, due to their instability (N-end rule) the more stable Val-1744 ICN is likely to mediate the bulk of Notch1 signalling (Kopan and Ilagan, 2009).

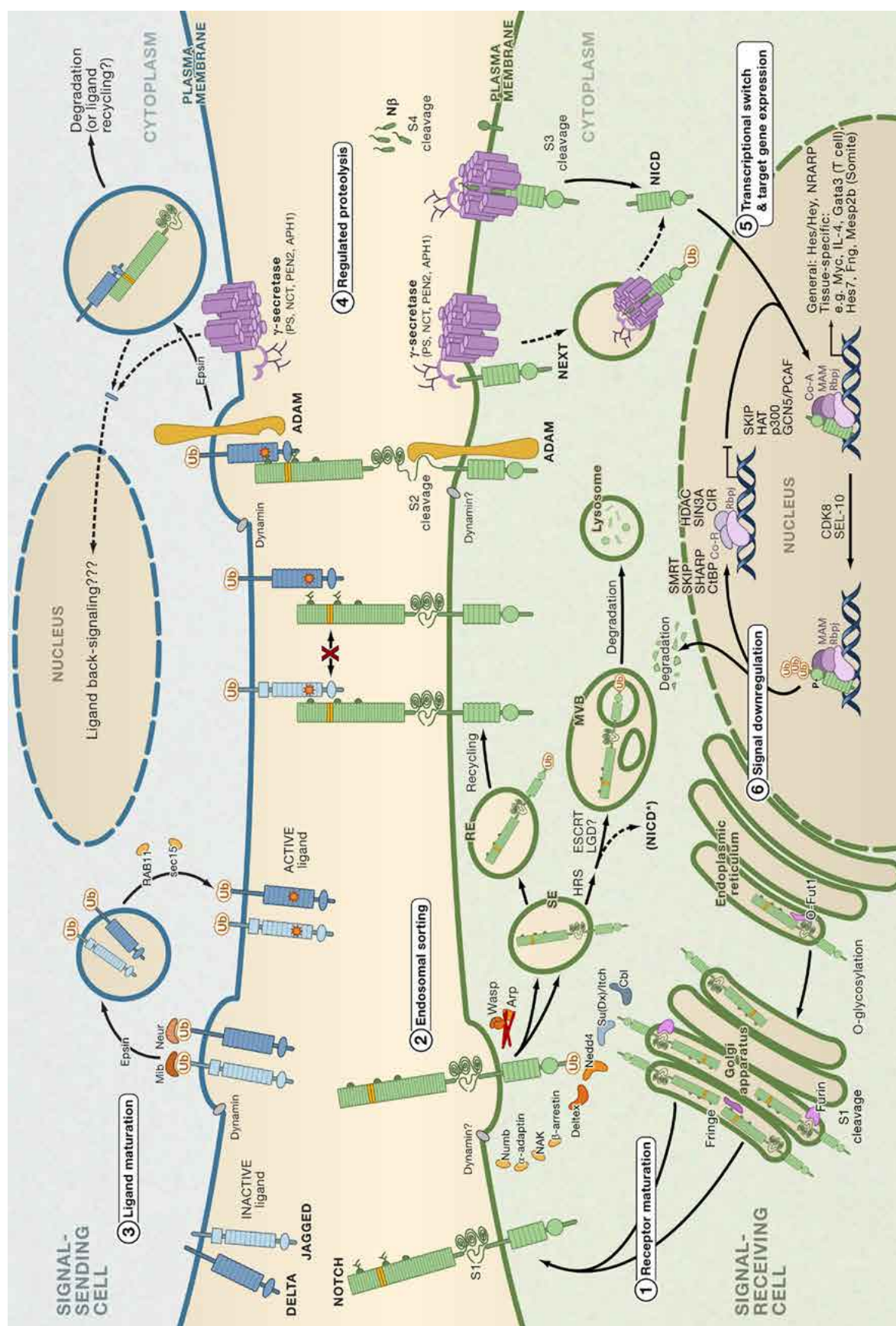
Very little is known about the nuclear translocation of cytoplasmatic ICN, as proteins and processes regulating this step remain unclear. ICN contains two nuclear localisation domains and it was recently reported that the importins $\alpha 3$, $\alpha 4$, and $\alpha 7$ are involved in nuclear import of mouse and fly ICN1 (Huenniger et al., 2010; Sachan et al., 2013).

1.3.3.2 Transcriptional regulation through the Notch pathway – the repressor complex

ICN cannot bind to DNA on its own to regulate gene expression, since it does not contain any domains with DNA-binding capacity. However, binding to CSL/Rbpj—the transcriptional effector of Notch signalling—targets ICN to its downstream genes. The Rbpj (recombination signal binding protein for immunoglobulin kappa J region) transcription factor was found by the Honjo group by its specific binding to the J region of the immunoglobulin κ locus (Hamaguchi et al., 1989; Matsunami et al., 1989). Subsequently, Rbpj was shown to recognise mostly the sequence GTGGGAA and does not bind to the V(D)J recombination signal without the flanking sequence (Tun et al., 1994).

The protein is comprised of N- and C-terminal Rel homology domains and the central β -trefoil DNA-binding domain (Chung et al., 1994; Kovall and Hendrickson, 2004; Nam et al., 2006). The recognition of Rbpj targets was reported to be independent of Notch (Kopan

Figure 1.6 (following page) The Notch signalling pathway and its modifiers (adapted from Ilagan and Kopan 2007). Schematic depiction of the Notch signalling cascade with its six main steps indicated. The names of the modifying proteins and structures are shown. Question marks indicate current questions in the field. Dashed arrows indicate potential alternative routes of receptor or ligand processing. See Sections 1.3.1, 1.3.2, 1.3.3.1, 1.3.3.2, 1.3.3.5 and 1.3.3.6 as well as Ilagan and Kopan (2007) for further details. 1) The receptors mature in the secretory pathway of the signal-receiving cell, where they are modified by Fringe-glycosyltransferases and nicked at the S1 site by furin-like convertases. 2) Endosomal sorting of Notch receptors is mediated by multiprotein complexes. The Notch receptors can be either recycled or degraded to control the steady state level of the receptors—a process, which is controlled by several E3 ubiquitin ligases such as Deltex and Nedd4. SE - sorting endosome; RE - recycling endosome; MVB - multivesicular body. 3) Ligand maturation takes place during endocytic trafficking and enhances their signalling activity. The E3 ubiquitin ligases Neur and Mib trigger epsin-mediated endocytosis, which might promote ligand cluster formation. 4) Regulated proteolysis of the Notch receptors (as well as of the ligand/NECD complex) upon receptor ligand interaction. The red X indicates the distinct specificities of Delta and Jagged ligands for Fringe-modified and non-modified receptors, respectively. The 'lift-and-cut' model proposes a structural conformation change upon ligand endocytosis. The generated pulling-force on the receptor unfolds the NRR and allows activating proteolysis by ADAM metalloproteases at S2. The NEXT product is the substrate of the γ -secretase complex, which frees the ICN. Note that the ligand/NECD complex can also be processed and endocytosed into the signal sending cell. 5) The ICN translocates into the nucleus and acts as a transcriptional switch. Rbpj is initially associated with several transcriptional co-repressors, which are displaced upon ICN recruitment. The ICN/Rbpj complex is bound by MAML proteins, which results in recruitment of additional co-activators and transcriptional activation of general and tissue-specific Notch target genes. 6) Cdk8 is recruited upon transcriptional activation and phosphorylates the PEST domain of ICN. Phospho-ICN is the substrate of the Sel10/Fbw7 E3 ubiquitin ligases and the ubiquitinated ICN is finally degraded by the proteasome, which leads to signal downregulation.



and Ilagan, 2009). Earlier studies suggested that in cells, which do not undergo active Notch signalling, Rbpj acts as a transcriptional repressor by recruiting co-repressors (reviewed by Lai, 2002a; Bray, 2006; Borggreffe and Oswald, 2009). At least seven different co-repressor complexes were described so far: *i*) SKIP (Ski-interacting protein), which in turn interacts with the co-repressor SMRT (silencing mediator for retinoic acid and thyroid hormone receptor), and thereby recruits HDAC1 (histone deacetylases 1; Kao et al., 1998). *ii*) A complex consisting of NCoR (nuclear receptor co-repressor), mSin3A and HDAC1. *iii*) CIR (CBF1 interacting co-repressor), which is thought to establish a HDAC-containing complex via with SAP30 or NKAP (Hsieh et al., 1999; Pajeroski et al., 2009; Borggreffe and Liefke, 2012). *iv*) KyoT2, which can interact with CSL/Rbpj and mediate repression via the Polycomb group (PcG) proteins Ring1 and HPC2 (Taniguchi et al., 1998; Qin, 2004; Qin et al., 2005). The crystal structure of the LIM domain-containing KyoT2 protein together with CSL/Rbpj was recently resolved and presents the first CSL/Rbpj-co-repressor structure determined (Borggreffe and Oswald, 2014; Collins et al., 2014). *v*) The human SHARP (SMRT and HDAC associated repressor protein) and murine MINT (Msx2 interacting nuclear target) proteins are members of the heterogeneous SPEN-homology (Split-ends) domain family and directly interact with CSL/Rbpj (Oswald et al., 2002; Yabe et al., 2007; Tsuji et al., 2007). SHARP also associates with other HDAC-recruiting co-repressors such as ETO, SMRT/NCoR (Salat et al., 2008; Ariyoshi and Schwabe, 2003), CtBP (C-terminal binding protein) and CtIP (CtBP interacting protein; Oswald et al., 2005). *vi*) A complex containing CtBP and as well as the histone H3K4 demethylase LSD1/KDM1A, the NAD-dependent deacetylase SIRT1, which is thought to remove H4K16ac marks from chromatin and CoREST1 (Mulligan et al., 2011). *vii*) The demethylase KDM5A (also known as RBP2 or JARID1A) was shown to interact with CSL/Rbpj and to regulate H3K4 methylation (Liefke et al., 2010). Besides recruitment of chromatin modifiers, Rbpj-mediated repression also induces the destabilisation of the general transcription factor IID (TFIID)/TFIIA interaction (Olave et al., 1998).

1.3.3.3 Transcriptional regulation through the Notch pathway – the activator complex

Nuclear ICN is thought to compete with the co-repressors for CSL/Rbpj-binding sites and mediate a 'transcriptional switch'. As ICN levels increase upon Notch receptor activation, they outcompete the co-repressors and establish transcription-activating complexes (Figs. 1.6 and 1.7). One of the first steps to activate gene expression is the formation of the ternary ICN/Rbpj/MAML complex, the structure of which has been solved by the Kovall and Blacklow labs (Fig. 1.8; Nam et al., 2006; Wilson and Kovall, 2006; Arnett et al., 2010; Choi et al., 2012).

The pivotal role of MAML in the activation complexes is reflected by the use of a dominant-negative form of MAML (Maillard et al., 2004) or SHAM (stapled α -helical peptides derived from MAML1) peptides resembling the MAML structure (Moellering et al., 2009). Both forms are incapable of recruiting additional co-activators, and therefore, fail to activate Notch target gene expression. Besides the function as platform to recruit additional co-activators, MAML proteins were reported to stabilise the ICN/Rbpj interaction (Choi et al., 2012).

Notch-activating complexes were reported to have a size of ~ 1.5 megadalton, implying that they contain additional co-activators (Jeffries et al., 2002). Recently, a mass spectrometry approach aimed to describe the Notch1 nuclear interactome in SupT1 cells, a human Notch1-dependent T cell acute lymphoblastic leukaemia (T-ALL) cell line (Yatim et al., 2012). Among other factors associated with various biological processes, the authors identified: *i*) AF4p12 (also known as FRYL), a protein that exhibits transcriptional activation properties likely through RNA polymerase II recruitment at several Notch-target genes; *ii*) the SWI/SNF nucleosome remodelling complex PBAF (Polybromo- and BRG1-associated factor), which hydrolysis ATP to disrupt DNA and histone interactions to make the nucleosomal DNA more accessible, which allows the histone octomers to 'slide' on or to dissociate from DNA (Yan et al., 2005); *iii*) members of the cohesion complex, which are involved in sister chromatid cohesion during mitosis as well as in transcriptional regulation, most likely by organising the 3D chromatin structure through looping of the chromatin, thereby juxtapositioning *cis*-regulatory elements and promoters (Peters et al., 2008); *iv*) the histone demethylases LSD1 and PHF8. Interestingly, LSD1, a known CSL/Rbpj co-repressor (see above), acts on H3K4me2 in the absence of ICN and contributes to CSL-mediated repression. However, in the presence of ICN1, LSD1 triggered demethylation of the repressive histone modification H3K9me2. PHF8 removes multiple transcriptional repressive marks, such as H3K9me1/me2, H4K20me1, as well as H3K27me2—a histone modification that was shown to increase upon inhibition of Notch signalling (Horton et al., 2010; Liu et al., 2010c; Loenarz et al., 2010; Qi et al., 2010).

The SKIP protein, a known CSL/Rbpj associated co-repressor (see above), was also shown to have a co-activator function in Notch signalling, as it bridges and stabilises the ICN-Rbpj interaction (Zhou et al., 2000b,a). Hence, the interaction of the SKIP-Rbpj complex with either the HDAC-associated co-repressors SMRT or the ICN co-activator might be the molecular explanation for the transcriptional switch. SKIP was also reported to be required for the multimerisation of ICN monomers into non-chromatin-associated complexes with MAML prior to CSL/Rbpj interaction (Vasquez-Del Carpio et al., 2011). These pre-activation complexes are thought to resolve upon binding to CSL/Rbpj into the ternary ICN/Rbpj/MAML activation complex at promoters and a ICN monomer as well as a SKIP/co-repressor complex, which both dissociate.

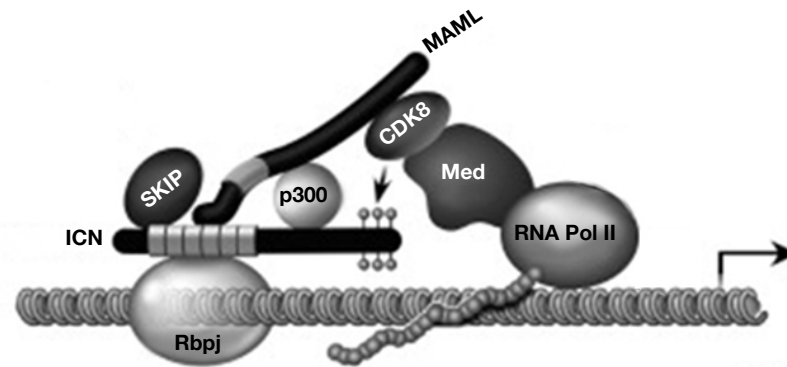


Figure 1.7 The transcription-activating complex (adapted from Fryer et al. 2004). Schematic depiction of the ICN-dependent transcription-activating complex bound to DNA in a promoter region (bent arrow). ICN binds to Rbpj, which results in recruitment of MAML1. p300 associates with MAML1 as well as ICN, and its HAT activity introduces active chromatin. MAML1 also recruits CDK8, which promotes hyperphosphorylation of the C-terminal PEST domain of ICN (grey protrusions on ICN). This triggers ICN turnover through ubiquitination followed by proteasomal degradation. Interaction with the mediator complex is thought to stabilise the recruitment of RNA polymerase II and enhance transcription. For simplicity, other co-activators, except for SKIP, that associated with ICN-dependent transcription-activating complexes are not depicted.

Recently, two research groups identified the putative ATP-dependent DEAD-box RNA helicase DDX5/p68 as a positive regulator of Notch signalling (Jung et al., 2013; Lin et al., 2013). RNA helicases can unwind RNA molecules and alter their secondary structure, which affects translation and splicing. DDX5 interacts with Rbpj, most likely via MAML1. Additionally, the non-coding RNA SRA, a cofactor of DDX5, recruits the histone acetyltransferases (HATs) p300.

The HATs p300, GCN5 and PCAF (p300/CBP-associated factor) are also recruited by the Notch-co-activator complex via interaction with ICN or MAML, whereas p300 is the key co-activator of the Notch cascade (Kurooka and Honjo, 2000; Oswald et al., 2001; Fryer et al., 2002; Wallberg et al., 2002). Since p300 can acetylate MAML (Saint Just Ribeiro et al., 2007), and since MAML enhances p300 autoacetylation as well as the histone acetyltransferase activity of p300 (Hansson et al., 2009), it is likely that both proteins act cooperatively (Borggreffe and Liefke, 2012). The two highly homologous paralogues GCN5 and PCAF are members of in the multi-subunit mammalian SAGA and ATAC complexes, which specifically acetylate nucleosomal histone H3 (Wang et al., 2009, 2008b). p300 and CBP (CREB-binding protein) belong to another class of the mammalian HAT families (Bedford et al., 2010). p300-binding to the DNA is widely used to describe regulatory enhancer elements (Visel et al., 2009). While GCN5/PCAF acetylate H3K9, p300/CBP is required for H3K18 and H3K27 acetylation (Jin et al., 2011).

1.3.3.4 Dynamic binding of CSL/Rbpj to the DNA

The most common model suggests that Rbpj is constitutively associated with DNA and acts as a repressor in the absence of active Notch signalling (Barolo et al., 2002; Bray, 2006). However, two earlier studies challenged this model and, in turn, propose a more dynamic model of Notch-mediated gene regulation. Using a human cervix carcinoma cell line, Fryer et al. (2004) showed that the ICN appeared transiently at the *Hes1* promoter one hour after Notch activation. Recruitment of ICN was accompanied by an increase in CSL/Rbpj binding.

Krejci and Bray (2007) studied the enhancer of split [*E(spl)*] Notch target gene cluster in two fly cell lines following EDTA-mediated Notch activation. Depletion of Ca^{2+} by divalent metal ion chelators, such as EDTA, compromises the function of the CA^{2+} -binding Lin12 repeats of Notch and results in spontaneous shedding of the Notch ectodomain. Subsequently, the γ -secretase-mediated cleavage of the generated transmembrane fragment produces transcription-activating competent ICN (Rand et al., 2000; Ilagan et al., 2011). Upon EDTA treatment, the CSL/Rbpj occupancy was significantly and transiently increased at some of the functional CSL/Rbpj-binding sites of the 11 *E(spl)* genes. As shown by a time course, target gene expression as well as CSL/Rbpj occupancy was maximal approximately 30 minutes after the EDTA pulse and correlated with the presence of ICN, implying that the kinetics of binding differ when Su(H) is complexed with ICN.

These two studies of selected Notch target genes were supported by a genome-wide binding study of CSL/Rbpj and ectopically-expressed ICN1, in a murine myogenic cell line (Castel et al., 2013). Binding of the factors was investigated after 6 h and 24 h of plate-bound DLL1 stimulation or in the presence of a γ -secretase inhibitor (GSI). The authors identified two distinct classes of binding sites. In the first class—the class of Notch-dependent Rbpj regulatory elements—binding Rbpj is hardly detected in the presence of GSI. However, after Notch-activation, Rbpj is dynamically recruited to its targets accompanied by co-binding of ICN and p300. While in the second class—the class of Notch-independent Rbpj regulatory elements—binding of Rbpj to DNA is constitutive and unaffected by fluctuations of Notch activity, as they are not co-occupied by ICN in this cellular context. As shown by transcriptional profiling, many of the genes with Notch-dependent Rbpj regulatory elements in their proximity are expressed upon Notch induction. In contrast, hardly any of the genes with Notch-independent Rbpj regulatory elements in their proximity respond to Notch activation, showing the functional relevance of the classification of the Rbpj-sites. Upon genetic removal of Rbpj followed GSI treatment, 50% of the genes with inducible Rbpj-binding sites were derepressed, suggesting that Rbpj can act as a repressor even if loosely bound to DNA. In contrast to this, only 10% of the genes with constant sites showed the same trend, questioning a repressive function

of Rbpj at those sites. The observation that binding of Rbpj to DNA is stabilised by ICN was substantiated in murine and human T-ALL cell lines, in which Rbpj and Notch1 occupancy were higher at sites, where both protein were detected (Wang et al., 2011). The same group could also identify Notch-dependent Rbpj-binding sites in a human T-ALL cell line after 4 h of GSI wash-out mediated Notch activation (Wang et al., 2013).

Mechanistically, stronger recruitment and/or more stable binding to DNA can explain the increased CSL/Rbpj occupancy at sites where it is part of the Notch-activating complex. An increased affinity for DNA of one activating complex itself is unlikely, as ternary ICN/Rbpj/MAML formation does not introduce novel interactions between CSL/Rbpj and DNA (Nam et al., 2006; Wilson and Kovall, 2006). This is further supported by an *in vitro* protein-binding microarray study, in which no differences in the DNA binding site preference were detected for either CSL/Rbpj alone or in complex with ICN and/or MAML (Del Bianco et al., 2010).

It is also possible that activating complexes have increased affinity to DNA due to cooperative mechanisms. Along this line, several Notch target genes contain dual 'sequence-paired' binding sites (SPS), in which two head-to-head orientated CSL/Rbpj motifs are separated by a 16 or 17 nt spacer (Fig. 1.8; Bailey and Posakony, 1995; Nellesen et al., 1999). The Notch transcriptional activation complex can bind in a cooperative fashion to SPSs, and biochemical as well as structural analyses showed that dimerisation depends on ANK domain interactions of two ICN proteins, recruitment of MAML and presence of CSL/Rbpj at both sites (Nam et al., 2007; Arnett et al., 2010; Choi et al., 2012). Dimeric Notch signalling is required for leukemogenesis and T cell development (Liu et al., 2010a). However, only a small fraction of the identified ICN-dependent CSL/Rbpj-binding sites contains paired motifs (Krejčí and Bray, 2007; Castel et al., 2013). This might be partially explained by SPSs being difficult to identify on a sequence level, as one of the two motifs can be cryptic (Arnett et al., 2010). Alternatively, combinatorial mechanisms with other nearby binding cofactors could determine the ICN-responsiveness of CSL/Rbpj-binding sites. The formation of productive transcription complexes and/or cooperative interactions with other proteins could increase the residence time of CSL/Rbpj on DNA, whereas CSL/Rbpj might rapidly exchange on and off the DNA, where it is associated with repressive complexes. A similar mechanism was observed for nuclear receptor proteins (Agresti et al., 2005; Métivier et al., 2006).

1.3.3.5 Transcription-activating-coupled turnover of ICN

Fryer et al. (2004) reported that MAML recruits cyclin C-dependent kinase Cdk8 and Med220 to the ternary ICN/Rbpj/MAML complexes, while recruitment of the transcription elongation-enhancing CycT1/Cdk9 complex upon Notch activation was independent of MAML. Cdk8 can

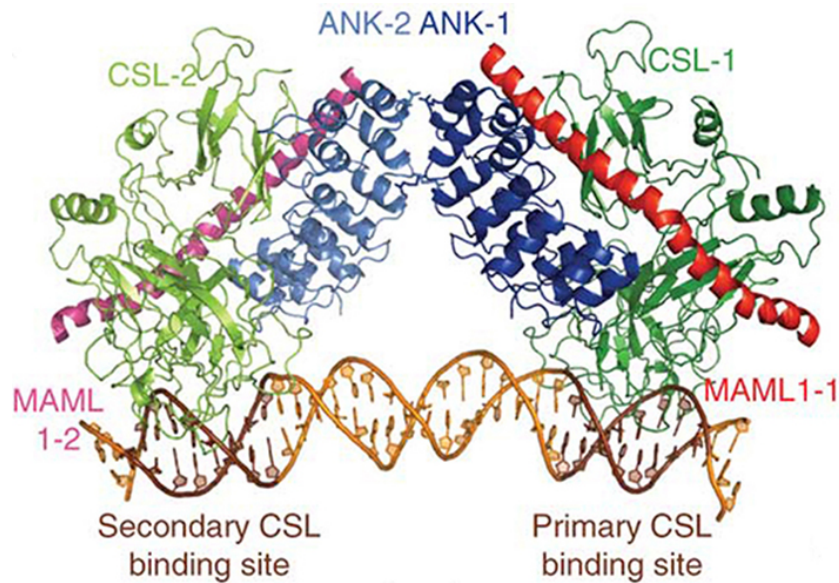


Figure 1.8 Crystal structure of a dimeric Notch transcription-activating complex on a paired-site DNA (Arnett et al., 2010). A ribbon representation based on the X-ray crystal structure of the human Notch1 ANK-CSL-MAML1 dimeric complex bound to the promoter of the human *HES1* gene. The promoter contains a two CSL-binding sites, which are separated by a spacer region and are orientated head-to-head. The primary CSL-binding site contains a high-affinity Rbpj motif (TGTGGGAA), while the motif of the secondary site is cryptic (CGTGTGAA) and bound with lower affinity (Arnett et al., 2010). CSL structures are shown in green, Notch1 ANK in blue and MAML1 in red. The subunits of the secondary complex are represented in faint colours. Note that the two complexes interact via the Notch1-ANK domains and formation of the dimeric complex promotes bending of the DNA, which partly unwinds the double helix (Arnett et al., 2010).

act as positive regulator of transcription and, like Med220, is a member of the mediator complex, which is thought to act as a molecular bridge between DNA-binding transcription factors and RNA polymerase II (Fig. 1.7; Galbraith et al., 2010). Cdk8's kinase activity establishes a transactivation-coupled turnover of Notch by hyperphosphorylation within the TAD and PEST domains of ICN, making it a substrate for rapid degradation. The hyperphosphorylated PEST domain is recognised by the E3 ubiquitin ligase Fbw7 (also known as Sel10), which polyubiquitinates ICN and Fbw7, ultimately leading to proteasome-mediated degradation within the nucleus (Fig. 1.6; Gupta-Rossi et al., 2001; Oberg et al., 2001; Wu et al., 2001; Lai, 2002b; Moretti and Brou, 2013). However, this view remains partially controversial, since Chiang et al. (2006) showed that a dominant-negative Cdk8 fails to affect the difference in phosphorylation of wild-type ICN and ICN mutated at all putative Cdk8 phosphorylation sites (Chiang et al., 2006). Regardless of these contradicting results, the efficient turnover of transcriptionally active ICN is critical for appropriate physiological Notch signalling levels, since extended signalling leads to severe pathologies such as T-ALL (Weng et al., 2004; Grabher et al., 2006; Aster et al., 2008).

1.3.3.6 Post-transcriptional modifiers of the Notch pathway activity

The Notch protein is a potential oncogene, and the output of Notch signalling needs to be tightly regulated. The Notch signalling pathway does not contain any secondary messengers and has a simple framework compared to other signalling pathways. In addition to the mechanisms described above, amounts, localisation and activity of the core components of the pathway, in particular of ICN, are regulated at multiple levels (Fig. 1.6).

The AU-rich element containing 3' UTR of the *Notch1* mRNA is targeted by the RNA-binding proteins ZFP36L1 and ZFP36L2 (also known as TIS11b and TIS11d) during T cell development leading to mRNA degradation and translational repression (Hodson et al., 2010; Nedjic and Aifantis, 2010). Furthermore, several microRNAs, such as miR-1, miR-34a, miR-146, miR181a-1/b-1, miR-199b-5p and miR-200 target transcripts of Notch pathway components as well as Notch downstream targets or components of regulatory feedback-loops of Notch signalling (Wang et al., 2010; Fragoso et al., 2012; Bu et al., 2013; Won et al., 2013).

After translation, the extracellular domains of the Notch receptor can be modified by different forms of O-glycosylation (O-fucose and O-glucose; reviewed by Haines and Irvine, 2003; Vodovar and Schweisguth, 2008). The mammalian O-fucosyltransferase Pofut1 fucosylates EGF-like repeats of the Notch extracellular domain. These structures are the substrate for the Fringe family of mammalian glycosyltransferases (Lfng, Lunatic fringe; Mfng, Manic fringe; and Rfng, Radical fringe), which catalyse the addition of N-acetylglucosamines onto specific fucosylated EGF-like repeats. This elongation of sugar moieties can determine the strength of receptor-DSL-ligand interactions, which alters the probability of activation, and consequently, modulates signal strength. During early T cell development, Lfng modifies the Notch1 receptor, resulting in enhanced Delta-to-Notch signalling and limited Jagged-to-Notch signalling (Visan et al., 2006; Yuan et al., 2011). For marginal zone B cell development, Lfng and Mfng need to cooperatively modify Notch2 to enhance DLL1-induced activation (Tan et al., 2009).

While still at the membrane, the prolyl-isomerase Pin1 potentiates Notch1 cleavage by γ -secretase (Rustighi et al., 2009). This leads to an increased ICN1 level and ultimately enhances Notch1 transcriptional and tumorigenic activity in breast cancer.

Cytoplasmic ICN1 can interact with the product of the Notch target gene *Numb* shortly after the S3 cleavage (Rebeiz et al., 2011). Numb initiates ICN1 ubiquitination via the E3 ubiquitin ligase Itch, which marks ICN1 for proteasomal degradation (McGill and McGlade, 2003). A later study demonstrated that, high levels of Numb promote sorting of the Notch1 receptor through late endosomes for degradation, while Numb depletion facilitates Notch1 re-

cycling (McGill et al., 2009). Consequently, Numb is a negative regulator of Notch signalling, as it reduces ICN1 levels, and hence, the transcriptional output.

The potential E3 ubiquitin ligase Deltex1 is either described as an inhibitor or activator of Notch signalling. Deltex1 binds to the ankyrin repeats of the ICN and can direct Notch receptor trafficking towards lysosomal degradation or towards recycling, thereby impacting receptor half-life both positively or negatively (Matsuno et al., 1998; Izon et al., 2002). In addition, Deltex1 recruits the translation initiation factor eIF3f to activate Notch on endocytic vesicles, where eIF3f deubiquitinates Notch. This is a step required prior to receptor processing by the γ -secretase complex (Moretti et al., 2010). However, *Dtx1*-mutant mice develop normally (Storck et al., 2005). Furthermore, *Dtx1* and *Dtx2* double-mutant mice have only a mild phenotype, in which the T cell compartment is not affected (Lehar and Bevan, 2006). However, this might be explained by the redundancy in the system through the paralogues Deltex3 and Deltex4 (Borggreve and Liefke, 2012).

The protein encoded by the direct Notch target gene *Nrarp* (Notch related ankyrin repeat protein; Krebs et al., 2001; Pirot et al., 2004) physically interacts with ICN. The *Nrarp* protein contains two ankyrin repeats, which function in a negative feedback loop to attenuate Notch signalling by interference with ICN/Rbpj complex formation (Lamar et al., 2001). While *Nrarp*-mutant mice show normal T cell development (Krebs et al., 2012), *Nrarp* overexpression in HCSs is able to block Notch-induced gene activation and results in a profound block of T cell development (Yun and Bevan, 2003b).

Similar to the disruption of the ternary ICN/Rbpj/MAML complex through CycC/Cdk8 and Fbx7 (see above), phosphorylation of ICN close to its ankyrin repeats by nemo-like kinase (NLK; Ishitani et al., 2010), Down syndrome-associated kinase-1A (DYRK1A; Fernandez-Martinez et al., 2009) and casein kinase-2 (CK2; Ranganathan et al., 2011) is associated with negative regulation of Notch-dependent transcriptional activation. In contrast to this, the glycogen synthase kinase-3 β (GSK3 β) phosphorylates ICN to protect it from proteasome-dependent degradation (Foltz et al., 2002; Guha et al., 2011). Similarly, ICN1 itself can be acetylated by p300 and PCAF, enhancing its stability. This process is counter-balanced by the deacetylase SIRT1, a member of the SIRT1-LSD1 co-repressor complex (see above), which removes the acetylation marks on ICN1 and histones (Guarani et al., 2011; Mulligan et al., 2011). Hence, establishment of the activator complex might be prevented through SIRT1-mediated deacetylation, resulting in immediate degradation of ICN1.

It was recently shown, that the cellular localisation of CSL/Rbpj is controlled through an Rbpj-interacting and tubulin associated (RITA) protein (Wacker et al., 2011). RITA exports CSL/Rbpj out of the nucleus through rapid shuttling between cytoplasm and nucleus, and thus, acts as a negative regulator of Notch signalling, in conditions where CSL/Rbpj amounts are

limiting. The nuclear localisation of CSL/Rbpj also depends on interactions with the SMRT co-repressor complex, as an interaction-deficient mutant form of CSL/Rbpj was restricted to the cytoplasm. This suggests that, in the absence of signalling, the CSL/Rbpj complex entering the nucleus is pre-committed to a transcriptional repression function (Zhou and Hayward, 2001).

1.3.4 Transcriptional regulators of *Notch1* expression in the lymphoid system

Notch1 expression is partially controlled by the E-box transcription factor E2a in early haematopoietic developmental stages such as HSC and LMPP. In *Tcf3* (E2a) mutant mice, *Notch1* transcript levels are reduced in HSCs as well as LMPPs, and these mice exhibit a partial arrest in adult T cell development at the DN1-to-DN2 transition (Bain et al., 1997; Kee et al., 2002; Dias et al., 2008). Ikawa et al. (2006) observed an even more severe defect upon *Tcf3* deletion during fetal T cell development. However, the reduced ETP numbers in E2a-deficient adult animals are in part caused by developmental defects and homing defects of bone marrow-resident thymocyte progenitors such as LMPPs (Dias et al., 2008). At later intrathymic T cell stages prior to β -selection, Notch1 and E2a directly regulate *Notch1* expression. Upon pre-TCR signalling, the level of the E2a antagonist Id3 increases, which inhibits E2a activity and results in a downregulation of *Notch1* expression in post- β -selection thymocytes (Yashiro-Ohtani et al., 2009).

B cell lineage development is incompatible with Notch1 activity. Hence, expression of *Notch1* is repressed in B cells through the transcription factor Pax5 (Souabni et al., 2002; Pridans et al., 2008). Since Pax5 is the B lineage commitment factor and essential for B cell development, Pax5-deficient pro-B cells lack B lineage potential, but instead differentiate into non-B lineage cells. Furthermore, *Pax5*-mutant progenitor cells express the Notch1 receptor, which, when activated *in vitro* or *in vivo*, triggers efficient T cell lineage differentiation of these cells. Thus, Pax5-deficient progenitor cells are an elegant system to study T cell development (Höflinger et al., 2004).

1.3.5 Alternative promoters of *Notch1*

The sequence of the fully mature Notch1 mRNA is encoded by 34 exons. Transcription of *Notch1* is mainly initiated at the canonical promoter, which is located just upstream of exon 1. Tsuji et al. (2003) identified three additional *Notch1* exons, when the authors examined the frequency and the sites of *Notch1* rearrangements in radiation-induced mouse thymic lymphomas. The newly identified exons 1a, 1b as well as 1c are located 5' of the canonical *Notch1* promoter and are transcribed by an alternative promoter (Fig. 1.9). In addition, spontaneous, irradiation-induced or Cre-*loxP*-mediated deletions encompassing the canonical *Notch1* pro-

motor and ATG start codon activate 'cryptic' internal *Notch1* promoters (Ashworth et al., 2010; Guidos, 2010; Jeannet et al., 2010). The observed breakpoints suggested illegitimate V(D)J recombination as Rag2 binds to the 5' end of *Notch1*, close to the breakpoints (Ashworth et al., 2010).

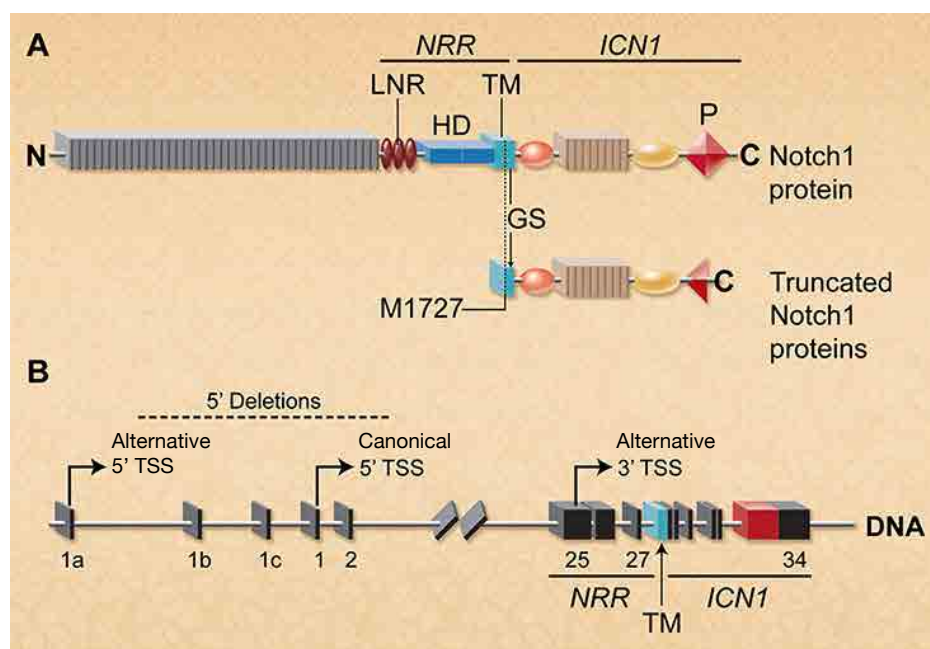


Figure 1.9 Alternative *Notch1* promoters (adapted from Guidos 2010). (A) Schematic representation showing the functional domains of the full-length Notch1 receptor (upper panel) and of truncated Notch1 variants (lower panel; see Section 1.3.1 for details about the domains). (B) Schematic representation showing the 5' and 3' regions of the *Notch1* locus with the numbered exons. The transmembrane (TM) domain encoding exon 1 is shown in blue and the PEST domain encoding exon in red. Promoters and transcriptional start sites (TSS) are depicted by arrows on the top. The canonical promoter is upstream of exon 1. Loss of Ikaros as well as 5' deletions of the gene result in activation of alternative promoters upstream of exon 1a as well as of cryptic 3' promoters. Translation of the transcripts originating from the 3' promoters starts at the Met-1727. The resulting proteins lack the ectodomain including the negative regulatory region (NRR) consisting of Lin12-Notch repeats (LNR) and the heterodimerisation domain (HD). These proteins are constantly processed by the γ -secretase complex. Note that the PEST domain is also often truncated in the case of T-ALL. Alternative splicing of the transcripts originating from the 5' alternative promoter and the resulting truncated proteins are not depicted.

Deletion of the canonical *Notch1* promoter facilitates epigenetic remodelling over the entire 3' end of *Notch1*, which starts abruptly around exon 25 (Jeannet et al., 2010). Furthermore, remodelling of this region is dramatically enhanced in the absence of the transcriptional repressor Ikaros, suggesting that Ikaros represses alternative promoter activity (Gómez-Del Arco et al., 2010; Jeannet et al., 2010). The cryptic promoters initiate transcription of truncated mRNAs in exons 25-27. Translation mainly starts at the internal initiator methionine (Met-1727), which generates highly-active leukemogenic Notch1 proteins lacking most of the Notch1 ectodomain. Transcripts initiated by the alternative 5' E1a *Notch1* promoter are processed differently compared to the canonical promoter transcripts. Exon 1 and 2 are spliced

out in E1a transcripts, resulting in a protein that lacks the signal peptide that is present in the canonical unprocessed form. This receptor isoform can undergo constitutive activation—even in the absence of CSL ligands—by S2 and subsequent S3 cleavage (Gómez-Del Arco et al., 2010; Haydu et al., 2012).

Both, the alternative 5' as well as internal 3' promoters are partially used during non-malignant T cell development in a stage-specific manner (Gómez-Del Arco et al., 2010). Their activity is normally restrained though Ikaros, and loss-of-function mutations of this regulator promote T-ALL development (Winandy et al., 1995; Sun et al., 1999; Dumortier et al., 2006; Jeannet et al., 2010). The stage-specific use of additional promoters allows regulating Notch1 signalling output through altered transcript levels as well as through the generation of isoforms with differential ligand requirement. As Notch1 autoregulates its own expression (Yashiro-Ohtani et al., 2009), this process facilitates a feed-forward mechanism that augments Notch signalling (Gómez-Del Arco et al., 2010).

1.3.6 Ikaros-mediated repression of Notch activity

The regulation of Notch signalling output through Ikaros is not only controlled at the level of *Notch1* transcription by Ikaros. By studying Notch-dependent T cell lymphomas, Beverly and Capobianco (2003) reported that CSL/Rbpj and Ikaros recognise a similar core DNA consensus sequence, namely TGGGAA. This suggested that Ikaros antagonises Notch signalling by competing with CSL/Rbpj for common target sites—a hypothesis that was further substantiated in different T cell lymphoma models (Dumortier et al., 2006; Kathrein et al., 2008). Those studies show that Ikaros represses Notch-induced transcription from the *Hes1* promoter via the CSL/Rbpj target sequence. The Ikaros-mediated repression of Notch target genes peaks at the DN4 stage of T cell development, where Ikaros protein level increases and ICN1 level decreases (Kleinmann et al., 2008). In subsequent DP and SP cell stages, Ikaros maintains repression of Notch target genes, as the loss of Ikaros leads to their derepression even in the absence of ICN (Chari and Winandy, 2008). ICN-independent activation of these genes might be facilitated by the loss of H3K27me3—the histone mark associated with PcG protein-mediated silencing—upon deletion of *Ikzf1*. Consequently, the loss of the repressive chromatin modification might allow gene activation by other regulators (Kleinmann et al., 2008; Zhang et al., 2012a; Geimer Le Lay et al., 2014). The derepression upon loss of Ikaros might also indicate that CSL/Rbpj has a limited role in Notch target gene repression in DP thymocytes. This was further substantiated by showing that *CD4*-Cre-mediated deletion of *Rbpj* does not lead to derepression of Notch target genes in DP cells, while the level of derepression upon *CD4*-Cre-

mediated co-deletion of *Rbpj* and *Ikzf1* is identical to the effect seen upon *Ikzf1* deletion alone (Chari et al., 2010).

In addition to this single-gene model, a recent study in a T-ALL cell line suggests that Ikaros directly represses the expression of most genes that are activated by Notch (Geimer Le Lay et al., 2014). On one hand, Ikaros terminates the Notch response by silencing the expression of Notch target genes required for development and leukemogenesis during the DN to DP transition. On the other hand, Ikaros efficiently represses the induction of Notch targets that are normally not expressed in T cells, thereby 'shaping' the Notch target gene repertoire in thymocytes. This study also revised the mechanism of competitive binding of CSL/Rbpj and Ikaros to the same regulatory elements. Instead of binding to the same consensus, Ikaros binds in most cases adjacent to the CSL/Rbpj-binding site, and therefore, might interact and interfere, with the transcriptional activity of the Rbpj/ICN1 complex.

Crosstalk between Notch and Ikaros was also shown to exist in Notch3-mediated and pre-TCR-dependent T cell leukaemia. In this case, Notch3 induces the expression of the gene encoding HuD, which is an RNA-binding protein of the ELAV/Hu family. HuD induces an alternative splicing process of the *Ikzf1* transcript, which generates a dominant-negative form of Ikaros (Bellavia et al., 2007). *Ptcra* expression is not restrained by the generated Ikaros isoform, as it fails to bind to the CSL/Rbpj-sites in the *Ptcra* promoter.

1.4 The functions of Notch signalling during haematopoiesis

The Notch pathway guides numerous cell fate decisions throughout the development of a multicellular organism. Besides its function in the immune system, these developmental processes comprise somitogenesis, neurogenesis or vascularisation—to name just a few of them (reviewed by Liu et al., 2010b; Andersson et al., 2011). Consequently, the straight deletion of *Notch1* (Conlon et al., 1995; Swiatek et al., 1994) or *Rbpj* (Oka et al., 1995; Krebs et al., 2004) is embryonic lethal. Even within the limits of the immune system, Notch signalling is required for the differentiation of marginal zone B cells, some DC subsets, several innate lymphocyte populations, as well as T lymphocytes (reviewed by Radtke et al., 2013).

1.4.1 Notch signalling and haematopoietic stem cells

Notch signalling is involved in the generation and maintenance of several stem cell populations such as neuronal stem cells, muscle stem cells, intestinal stem cells and HSCs (reviewed by Koch et al., 2013). However, its function in HSCs was controversial for a long time. It is now clear that Jagged1-mediated Notch activation is required for the generation of embryonic

HSCs (Robert-Moreno et al., 2008). Functionally, Notch is thought to induce the expression of the transcription factor Runx1 in embryonic HSCs (Nakagawa et al., 2006). In adult HSCs, loss- and gain-of-function studies led to contradicting results. While *in vitro* Notch activation induces the expansion of undifferentiated HSCs (Varnum-Finney et al., 1998, 2003, 2011), conditional deletion of either *Jag1*, *Notch1*, *Notch1* and *Notch2* together, or *Rbpj* in adult HSCs do not affect HSCs (Radtke et al., 1999; Mancini et al., 2005; Maillard et al., 2008). Hence, there is no physiological role for Notch signalling in adult HSCs.

1.4.2 The role of Notch signalling in the development and homeostasis of non-T cell lineages of the immune system

Early B cell progenitors are highly intolerant towards Notch signals, as signals below the level, which is required for the induction of T cells, are sufficient to interfere with B cell development (De Smedt, 2005; Schmitt et al., 2004b; Lehar et al., 2005). Because Jagged ligands are expressed in the bone marrow, B cell progenitors repress Notch activity through several mechanisms, such as: *i*) the direct repression of *Notch1* expression through Pax5 (Souabni et al., 2002; Pridans et al., 2008; Revilla-I-Domingo et al., 2012); *ii*) the expression of Lunatic fringe to redirect the receptor specificity away from Jagged ligands towards Delta ligands, which are relatively scarce in the bone marrow (Koch et al., 2001); *iii*) an incompletely understood process that depends on the transcription factor LRF (lymphoma-related factor; also known as ZBTB7A or POKEMON; Maeda et al., 2007)

By contrast, the development of marginal zone B cells, which reside in the spleen and play a role in early T cell-independent antibody responses, is Notch2-dependent (Martin and Kearney, 2002). Consequently, deletion of *Notch2* or *Rbpj* in immature B cells results in loss of marginal zone B cells (Saito et al., 2003). Notch2 receptor activation on marginal zone B cells might require DLL1 ligands presented by DCs (Kuroda et al., 2003).

The homeostasis of subsets of DCs—the cells which are specialised for the presentation of antigens—in the spleen and intestine depends on Rbpj-mediated signals through the Notch2 receptor (Caton et al., 2007; Lewis et al., 2011). The DC-specific deletion of *Notch2*, but not the deletion of *Notch1*, results in reduced activation of CD4⁺ cells, particular T_H17 cells (Caton et al., 2007; Lewis et al., 2011). However, the responsible Notch ligand for this process has not yet been identified (Radtke et al., 2013).

Recently, Notch signalling was implicated in the differentiation of type 2 innate lymphoid cells (ILC)—a newly discovered population that resembles T cells in its functional properties and transcription factor dependence (Neill et al., 2010). These cells, which are also termed nuocytes, are derived from CLPs in the bone marrow and can also develop from DN1 and

DN2 cells *in vitro* when cultured on OP9-DL1 cells in the presence of IL7 and IL33 (Wong et al., 2012b). This differentiation is reduced by co-culture in the absence of Notch ligands. Furthermore, Notch signalling was shown to be involved in the development of NKp46⁺ IL22-secreting ILC3 cells (Lee et al., 2012).

1.4.3 The role of Notch signalling in T cell development

The first report about a role of Notch1 in haematopoiesis was the identification of a translocation of the *Notch1* gene, which fused the ICN1-encoding 3' part of the gene to the *Tcrb* locus and caused T-ALL in a small cohort of children (Ellisen et al., 1991). Since then, numerous loss- and gain-of-function experiments point to an instructive role of Notch signalling in T cell differentiation. The requirement for the Notch pathway was seen upon inducible inactivation of *Notch1* (Radtke et al., 1999) or *Rbpj* (Han et al., 2002) in bone marrow progenitors, which results in a complete block of T cell development and ectopic B cell development in the thymus. In contrast, activation of Notch in bone marrow progenitors through overexpression of ICN is sufficient to induce ectopic T cell development in the bone marrow on the expense of B cell development (Pear et al., 1996; Pui et al., 1999). TSPs encounter a high density of Notch ligands presented by thymic epithelial cells. The activation of Notch signalling by DLL4 then leads to the initiation of the T cell lineage program. While Notch signalling is important throughout T cell differentiation, its role during this process changes substantially.

Both, the generation as well as the maintenance of ETPs depend on Notch signalling (Sambandam et al., 2005; Tan et al., 2005). CLPs, one of the potential TSP populations, as well as ETPs express Notch1 and low levels of Notch2 on their surface, while *Notch3* and *Notch4* are not expressed at these cell stages (IMMGEn database and Fiorini et al., 2009). Even though both receptors are structurally similar (Kraman and McCright, 2005), Notch1 is the essential receptor to drive T cell development, since the deletion of *Notch2* does not affect T cell development (Radtke et al., 1999; Besseyrias et al., 2007). The multipotent progenitors that enter the thymus have the potential to develop into non-T lineage cells and Notch1 is required to suppress these non-T lineage potentials and promote T cell lineage development. Consequently, ETPs as well as DN2a cells, when deprived of Notch signalling, divert to the B cell (ETPs only), myeloid, and NK lineages (Wilson et al., 2001; Balciunaite et al., 2005; Bell and Bhandoola, 2008; Wada et al., 2008; Feyerabend et al., 2009; Yui et al., 2010; Luc et al., 2012; De Obaldia et al., 2013a). Similarly, the overexpression of Notch modulators, such as Lfng, Deltex1, Nrarp or dnMAML1, causes a block of thymocyte development and induces ectopic B cell development in the thymus (Koch et al., 2001; Izon et al., 2002; Yun and Bevan, 2003b; Maillard et al., 2004).

In T cell-committed DN2b and DN3a cells that rearrange their T cell receptor loci, Notch is required for cell survival (Ciofani and Zúñiga-Pflücker, 2005), *Tcrb* rearrangement (Wolfer et al., 2002) and expression of the pre-TCR α chain (Reizis and Leder, 2002). *In vitro* studies with OP9-DL1 and control cell lines suggest that Notch-DL1 interactions promote DN3 cell survival by maintaining cell size, glucose uptake and metabolism through regulation of the PI3K/Akt pathway. Consequently, loss of Notch signalling could be rescued through overexpression of constitutively active Akt (Ciofani and Zúñiga-Pflücker, 2005). Conditional deletion of *Notch1* with the T cell-specific *Lck*-Cre line, which starts to delete in the DN2/DN3 compartment, partially arrests T cell development at the DN to DP transition (Wolfer et al., 2002). Notch1-deficient mice accumulate icTCR β ⁻ DN4 cells, with normal D β -to-J β rearrangements, but strongly reduced V β -to-DJ β rearrangements. Furthermore, this study suggested that $\gamma\delta$ -lineage development is not perturbed upon loss of Notch1.

At the β -selection checkpoint, Notch signalling cooperates with pre-TCR signalling, and both pathways act in a non-redundant manner (Ciofani et al., 2004; Tussiwand et al., 2011). Indeed, T cell development is partially blocked at the DN3a stage upon *Lck*-Cre-induced expression of dnMAML, and this phenotype is not rescued by TCR β or TCR $\alpha\beta$ transgenes (Maillard et al., 2006). Conversely, Rag-deficient thymocytes, which lack a pre-TCR, do not progress to the DP stage, even upon overexpression of ICN1 (Allman et al., 2001). The role of Notch during β -selection seems to be mainly mitogenic, as a block in proliferation leads to a complete arrest of differentiation and its ectopic activation—through the overexpression of *Myc*—can partially rescue DP progression in the absence of Notch signalling (Kreslavsky et al., 2012). The Notch3 receptor, which is upregulated in DN3a cells, is largely dispensable for early T lymphopoiesis, including β -selection (Shi et al., 2011; Suliman et al., 2011).

Upon β -selection, *Notch1* expression is downregulated though the pre-TCR-induced expression of Id3. Id3 heterodimerises with E2a, and thereby interferes with the DNA-binding capacity of E2a. Consequently, the E2a-mediated induction of *Notch1* expression is lost (Engel and Murre, 2001; Miyazaki et al., 2011). This downregulation of Notch signalling in DP T cells is required to avoid the oncogenic properties of Notch signalling and its targets such as *Myc* (Weng et al., 2006; Palomero et al., 2006).

Gain-of-function studies suggested a role for Notch1 signalling in intrathymic CD4/CD8 lineage commitment, maturation and survival. The transgenic overexpression of ICN1 inhibits the differentiation of CD4SP thymocytes and results in a skewing of the CD4SP vs. CD8SP ratio in the thymus (Robey et al., 1996). However, no influence on CD4/CD8 lineage commitment was seen in additional experiments using a similar strategy of ICN1 overexpression. Instead, these experiments showed an enhanced maturation or survival of DP T cells (Deftos et al., 1998, 2000; Izon et al., 2001). It was suggested that these discrepancies were due to the

tumorigenic activity of ICN1 overexpression and differences in the propensity of the transgenic mouse lines to develop tumours (Fowlkes and Robey, 2002; Laky and Fowkes, 2008). However, the conditional deletion of either *Notch1* (Bakker et al., 2001) or *Rbpj* (Tanigaki et al., 2004) using the *Cd4-Cre* line showed that Rbpj-mediated Notch signalling is dispensable for intrathymic CD4/CD8 lineage commitment, maturation and survival.

The differentiation of CD4⁺ T_H cells is also affected by Notch signalling (Bailis et al., 2013). While DLL-mediated activation of Notch by APCs favours the development of IFN γ -secreting T_H1 cells, the engagement of Jagged ligands preferentially induces the development of T_H2 and T_{Reg} cells (Amsen et al., 2009).

Studies of T_H1 development in response to different infections suggested that Notch via Rbpj regulates the expression of *Tbx21*, which encodes the 'master-regulator' for T_H1 development (Minter et al., 2005). However, follow-up studies showed that Notch controls rather the effector functions of T_H1 cells than their development, as Notch1-deficient CD4⁺ T_H1 cells produce IFN γ but can not release it (Auderset et al., 2012). Interestingly, this process seems to be independent of Rbpj and might be mediated through interactions of Notch with the transcription factor NF κ B (Palaga et al., 2003; Shin et al., 2006, 2014).

Various experimental models clearly showed a regulation of key factors that control T_H2 differentiation through the Notch pathway (Tanaka et al., 2006; Amsen et al., 2007). Notch signalling controls the expression of *Il4*, the gene encoding IL4—the main cytokine produced by T_H2 cells. It was suggested that this regulation happens through an Rbpj-binding sites in the conserved non-coding sequence 2 (CNS2) enhancer of the *Il4* gene (Amsen et al., 2004; Tanaka et al., 2006). As IL4 stimulates the differentiation of T_H2 cells, Notch signalling might control their differentiation through cell-intrinsic and -extrinsic mechanisms (Radtke et al., 2013). The Notch-dependent expression of *Il4* also depends on Gata3—another key factor for T_H2 lineage differentiation that is directly regulated by Notch1/Rbpj (Amsen et al., 2007; Fang et al., 2007). Both, Notch1 and Rbpj were reported to bind to the *Gata3* promoter and induce the expression of the exon 1b transcript (Zheng and Flavell, 1997).

T_{Reg} cells are critical for the maintenance of immune cell homeostasis. Depending on their site of development, T_{Reg} cells are subdivided into thymic T_{Reg} cells and peripherally induced T_{Reg} cells. The development of both T_{Reg} subsets depends on the transcription factor FOXP3 (Campbell and Koch, 2011). Notch signalling was shown to regulate the expansion of thymic T_{Reg} cells as well as the differentiation of peripherally induced T_{Reg} cells and the maintenance of both of these T_{Reg} cell populations (reviewed by Radtke et al., 2013). These effects are partially mediated through a Notch3-dependent upregulation of FOXP3 expression (Asano et al., 2008), which, in the case of peripheral T_{Reg} cells, might be induced by Jagged

ligands and, in the case of thymic T_{Reg} cells, is enhanced through blocking of DLL4 ligands (Vigouroux et al., 2003; Billiard et al., 2012).

The encounter of naïve CD8⁺ T cells with a cognate antigen triggers their proliferation and differentiation into cytotoxic T lymphocytes. Besides the secretion of IFN γ , these effector cells release perforin and granzymes to lyse target cells and induce apoptosis of their target cells through Fas receptor activation by presenting Fas ligands. DLL1-mediated activation of Notch on CD8⁺ T cells affects their differentiation, as it induces granzyme B and IL10 production, while IFN γ production is decreased (Wong et al., 2003; Maekawa et al., 2008). Both, the Notch1 and Notch2 receptors are thought to impact differentiation of cytotoxic T lymphocytes (Kuijk et al., 2013). Furthermore, the increased expression of the gene encoding granzyme B (*Gzmb*) is mediated directly through a Notch2/Rbpj-dependent complex on the *Gzmb* promoter (Maekawa et al., 2008).

1.4.4 Notch target genes

The widely used tools to study Notch-dependent changes in gene expression are activation of Notch signalling through overexpression of ICN or ligation of Notch receptors *in vitro* with Notch ligands, while both approaches can be further controlled with GSI compounds. Several attempts aimed to characterise the Notch-regulated transcriptome in mammalian non-lymphoid tissues, such as murine embryonic stem (ES) cells at different stages of mesodermal differentiation (Meier-Stiegen et al., 2010), mouse myogenic cells (Castel et al., 2013) or cortical neural stem cells (Li et al., 2012). Within the lymphoid system, Notch-mediated genome-wide effects on gene expression were studied in murine DP T cells (Huang et al., 2004), E2a-deficient haematopoietic progenitors (Ikawa et al., 2006), human CD34⁺ umbilical cord blood cells (Weerkamp et al., 2006b), murine ES cells that were differentiated *in vitro* into haematopoietic progenitors (Ganapati et al., 2007; Hamidi et al., 2011) as well as several T-ALL models (Palomero et al., 2006; Sharma et al., 2006; Weng et al., 2006; Chadwick et al., 2009; Riz et al., 2010; Wang et al., 2013). In fact, most known Notch-regulated genes were described in microarray profiling studies with T-ALL cell lines that were treated with GSI. In this context, Notch1 is thought to control cell growth and metabolism, and thus, GSI-treatment results in the repression of genes, which are involved in anabolic processes such as protein translation as well as the biosynthesis of ribosomes, amino acids or nucleotides (Palomero et al., 2006; Sharma et al., 2006; Weng et al., 2006).

To investigate if the detected gene expression changes might be mediated directly through ICN1/Rbpj bound to *cis*-regulatory elements, the genome-wide occupancy of ICN1 or Rbpj was studied. However, these studies were largely limited to T-ALL cell lines, where the bind-

ing of both proteins to DNA was investigated under supra-physiological ICN levels (Palomero et al., 2006; Wang et al., 2011, 2013; Geimer Le Lay et al., 2014). One of the first genome-wide occupancy studies detected binding of ICN1/Rbpj mainly within promoter regions of genes (Wang et al., 2011). Besides the Rbpj consensus motif, Znf143, Ets and Runx motifs were enriched within ICN1/Rbpj-binding sites. However, the authors noted that a large proportion of Notch-regulated genes lack promoter binding and are presumably regulated by enhancers (Wang et al., 2011). Indeed, a follow up study described binding sites that are dynamically bound by ICN1 (Wang et al., 2013). Furthermore, these sites are mainly located within distal enhancers and frequently overlap with Runx1-binding sites. Several of these enhancers exhibit exceptionally broad and high levels of H3K27ac. This feature is reminiscent of super-enhancers, which control the expression of genes with key roles in development and cancer (Hnisz et al., 2013; Lovén et al., 2013; Whyte et al., 2013). Gene regulation via such distal *cis*-regulatory elements might be achieved through looping of the chromatin. Along this line, dynamic ICN1 sites were often found to be co-occupied by CCCTC binding factor (CTCF; Wang et al., 2013), which is known to be involved in chromatin looping and gene insulation (Peters et al., 2008; Phillips and Corces, 2009).

In addition to the genome-wide studies, targeted approaches were carried out to determine Notch-regulated genes in fetal T cell progenitors (Franco et al., 2006; Taghon et al., 2005, 2007) and Pax5-deficient progenitors (Höflinger et al., 2004). Among the Notch-regulated target genes in developing T cells, *Gata3* (Amsen et al., 2007; Fang et al., 2007), *Tcf7* (Germar et al., 2011; Weber et al., 2011), *Bcl11b* (Li et al., 2010b,a), *Hes1* (Jarriault et al., 1995; Beatus et al., 2000), *Myc* (Weng et al., 2006), the cytokine receptor genes *Il2ra* (Maillard et al., 2006) and *Il7r* (González-García et al., 2009), *Notch1* itself (Yashiro-Ohtani et al., 2009), *Ptcra* (Reizis and Leder, 2002) and few others were reported to be directly regulated through Notch signalling. Some of the Notch target genes are specific to the T cell lineage (*Gata3*, *Tcf7*, *Bcl11b*, *Ptcra*), the lymphoid lineage (*Il2ra*, *Il7r*) or are regulated by Notch signals in a lineage-unspecific manner (*Dtx1*, *Hes1*, *Nrarp*).

1.5 Aims of this work

Despite the aforementioned importance of Notch signalling during early T cell development, no systematic approach for the identification of direct Notch target genes in developing thymocytes was reported so far. Hence, the aim of this study is to elucidate the genome-wide program of Notch signalling during different stages of early T cell development. Although the known Notch targets can explain some of the Notch functions in thymocytes, several biological processes in which Notch was implicated, lack precise molecular explanation. For example,

the exact mechanism of T cell lineage commitment remains obscure. Although several Notch targets were implicated in shutting down alternative lineage fates in either gain- (*Tcf7*; Weber et al., 2011) or loss-of-function (*Gata3*; García-Ojeda et al. 2013 and *Bcl11b*; Li et al. 2010b,a; Ikawa et al. 2010) experiments, none of them alone or in combination were shown to be required and sufficient for this process. Therefore, it is very likely that additional unidentified Notch targets are involved in the commitment to the T cell lineage.

Several molecular tools will be applied to identify the directly and indirectly Notch-regulated genes. Binding of ICN1 and Rbpj to the chromatin will be investigated by chromatin immunoprecipitation followed by deep sequencing with biotin-tagged knock-in alleles (Bio-ChIP-seq) of *Notch1* and *Rbpj* (developed in the Busslinger laboratory). As uncommitted progenitor populations are extremely scarce in mice, these experiments will be performed in uncommitted progenitor cell lines to provide into potential directly Notch-regulated target genes. The Notch-regulated transcriptome will be studied in freshly isolated multipotent precursor cells, which represent physiological T cell progenitors. *In vitro* Notch stimulation of these progenitors in OP9-DL1 co-cultures will allow the identification of Notch-dependent gene expression changes in a genome-wide manner by RNA-seq.

Notch signalling is required for the survival of DN3a cells (Ciofani and Zúñiga-Pflücker, 2005; Kreslavsky et al., 2012) and the rearrangement of the *Tcrb* locus (Wolfer et al., 2002), which is another example of a critical Notch-dependent process lacking molecular explanation. Notch-dependent DN3a cells can be enriched to a nearly homogenous population *in vivo* in *Rag2*^{-/-} mice. This genetic model will be used to identify direct Notch-regulated target genes in committed T cells prior to β -selection. Binding ICN1 and Rbpj to the chromatin will be investigated at physiological protein levels by Bio-ChIP-seq. In addition, the genome-wide *cis*-regulatory landscape of DN3a cells will be defined to provide a comprehensive map of promoter and enhancer elements of genes. The Notch-dependent transcriptome will be determined by RNA-seq using *in vivo* loss-of-function (*Lck*-Cre-mediated deletion of *Notch1* and *Rbpj*) as well as *in vitro* Notch stimulation approaches.

Even though Notch signalling activity is known to cease in post- β -selection DN3b cells, it is required for the proliferation of these cells (Ciofani et al., 2004; Maillard et al., 2006; Kreslavsky et al., 2012). The genome-wide Rbpj occupancy as well as Notch-dependent gene regulation will be analysed to understand how Notch signalling regulates this process.

To my knowledge, this approach is the first systematic characterisation of the Notch-dependent molecular program at different stages of T cell development. This work will provide valuable insights into the molecular mechanism of Notch1 function for a better understanding of molecular circuits underlying T cell differentiation.

2 Results and Discussion

2.1 Identification of ICN1- and Rbpj-binding sites in DN3 thymocytes

2.1.1 The *Notch1*^{Bio} and *Rbpj*^{Bio} alleles

Although the importance of Notch signalling in early T cell differentiation is long-established, so far only a hand full of direct Notch target genes, which are important in this process, were identified. Chromatin immunoprecipitation coupled with high-throughput sequencing (ChIP-seq) provides a valuable method to study protein-DNA interactions and thus to identify genes that are directly controlled by transcriptional regulators via *cis*-regulatory elements (Johnson et al., 2007; ENCODE Project Consortium et al., 2012). However, this method critically depends on the availability of high-quality antibodies against the protein of interest. In the beginning of this study, such ChIP-grade antibodies were not commercially available for the chromatin-associated domain of Notch1, ICN1, or for Rbpj. The quality of antibodies against ICN1 and Rbpj generated in the meantime remains a matter of debate. Notably, an antibody used for an Rbpj ChIP-seq study with T-acute lymphoblastic leukaemia (T-ALL) cell lines (Wang et al., 2011) was shown to cross-react with the transcription factor REST (Castel et al., 2013). Another Rbpj ChIP-seq study in murine cells of the neural cortex yielded highly unusual broad peak profiles for a transcription factor and provided a degenerate Rbpj consensus motif (Li et al., 2012). Due to the lack of appropriate tools, a systematic search for Notch targets in any system was largely restricted to studies in cell lines and conditions of artificially overactivated Notch signalling.

To bypass these problems and to allow the identification of high-confidence Notch target genes at physiological conditions, two murine knock-in alleles of *Notch1* (Dorninger, 2008) and its DNA-binding cofactor *Rbpj* (unpublished) were generated in the Busslinger lab (Fig. 2.1). Transgenic *Notch1*^{Bio} and *Rbpj*^{Bio} mice carry a tag at the carboxyl (C)-terminus of either Notch1 (Notch1-Bio) or Rbpj (Rbpj-Bio), respectively. The tag inserted at the 3' end of the last exon of the respective gene encodes several functional units: epitopes for FLAG and V5 antibodies, recognition sites for the TEV protease as well as a biotin acceptor sequence, which is recognised and biotinylated by the *Escherichia coli* biotin ligase BirA (de Boer et al., 2003). To biotinylate Notch1-Bio or Rbpj-Bio *in vivo*, *Rosa26*^{BirA/BirA} knock-in mice, which express the BirA ligase from the ubiquitously active *Rosa26* locus (Driegen et al., 2005), were crossed to tag-harboursing *Notch1*^{Bio/Bio} and *Rbpj*^{Bio/Bio} mice. The high-affinity interaction of streptavidin (SA) and biotin can be used for antibody-independent ChIP followed by high-throughput sequencing (Bio-ChIP-seq) to investigate the genomic occupancy of Rbpj and ICN1. In addition to the Bio-tag, which renders both proteins available for biochemical approaches, an

internal ribosomal entry sequence (IRES) followed by either a GFP reporter in the case of *Notch1*^{Bio} or a truncated human *Cd2* (*hCD2*) reporter in the case of *Rbpj*^{Bio} was inserted. This additional feature allows to monitor the expression of both genes at the single cell level.

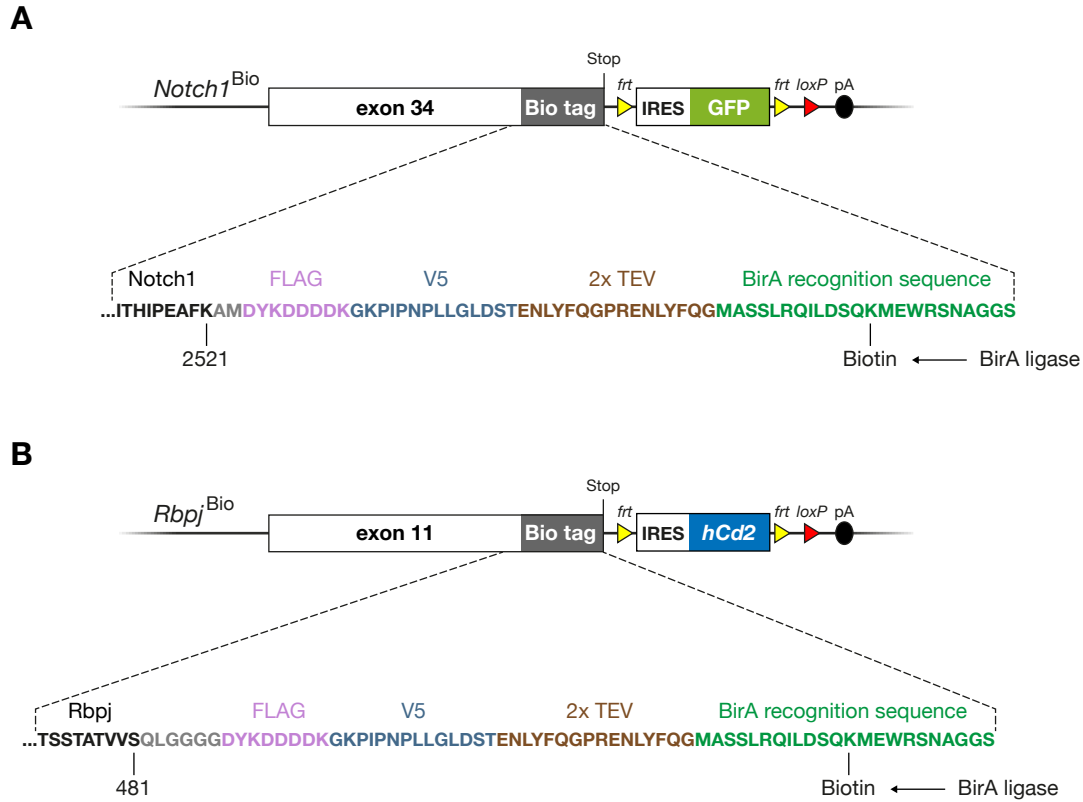


Figure 2.1 The *Notch1*^{Bio} and *Rbpj*^{Bio} alleles. (A,B) Schematic representations of the *Notch1*^{Bio} (A) and *Rbpj*^{Bio} (B) knock-in alleles (top). The last exon of the respective gene is shown followed by the inserted Bio tag with a 3' end stop codon. Yellow and red triangles represent *frt* and *loxP* sites, respectively. Black circles indicate an inserted polyadenylation (pA) sequence for termination of transcription. An IRES-GFP reporter allows to monitor *Notch1*^{Bio} expression (A) by FACS, while *Rbpj*^{Bio} expression can be traced by IRES-*hCd2*. (A,B) The last nine C-terminal amino acids (aa) from Notch1 (A) and Rbpj (B) are shown in black (the position of the last aa in the wild-type protein is indicated) followed by the C-terminal tag sequence with the functional units (bottom): Linker sequence (grey), FLAG epitope (pink), V5 epitope (blue), TEV protease recognition sequence (brown) and BirA recognition sequence (green) with the indicated lysine residue to which biotin is covalently added. Note: Figure is not drawn to scale.

2.1.2 Bio-tagged Notch1 and Rbpj proteins are efficiently biotinylated *in vivo*

To confirm efficient *in vivo* biotinylation of the Notch1-Bio and Rbpj-Bio proteins by the BirA ligase, SA-mediated pulldown and supershift experiments were performed. To this end, c-Kit⁺ thymic progenitors from *Rbpj*^{Bio/Bio} *Rosa26*^{BirA/BirA} *Rag2*^{-/-} (thereafter referred to as *Rbpj*^{Bio} *Rag2*^{-/-}), *Notch1*^{Bio/Bio} *Rosa26*^{BirA/BirA} *Rag2*^{-/-} (thereafter referred to as *Notch1*^{Bio} *Rag2*^{-/-}) and control *Rosa26*^{BirA/BirA} *Rag2*^{-/-} mice were expanded *in vitro* on OP9-DL1 feeder cells to DN2-

DN3-like pro-T cells (Fig. 2.2A). Nuclear extracts of these cells were then used in pulldown experiments with SA-coated beads. Separation of the resulting fractions by SDS-PAGE followed by immunoblot with an V5 antibody showed efficient depletion of the approximately 70 kDa Rbpj-Bio protein (Fig. 2.2B) and the approximately 120 kDa ICN1-Bio protein (Fig. 2.2C) from the supernatant. Furthermore, incubation of nuclear extract from *Notch1*^{Bio} *Rag2*^{-/-} pro-T cells with SA resulted in an uniform supershift of ICN1-Bio protein complexes due to reduced motility of the SA-conjugated complex in the SDS-PAGE (Fig. 2.2D). Therefore, *in vivo* biotinylation of both Notch1-Bio and Rbpj-Bio in pro-T cells is efficient, and both proteins remain stable in SA-mediated pulldown experiments.

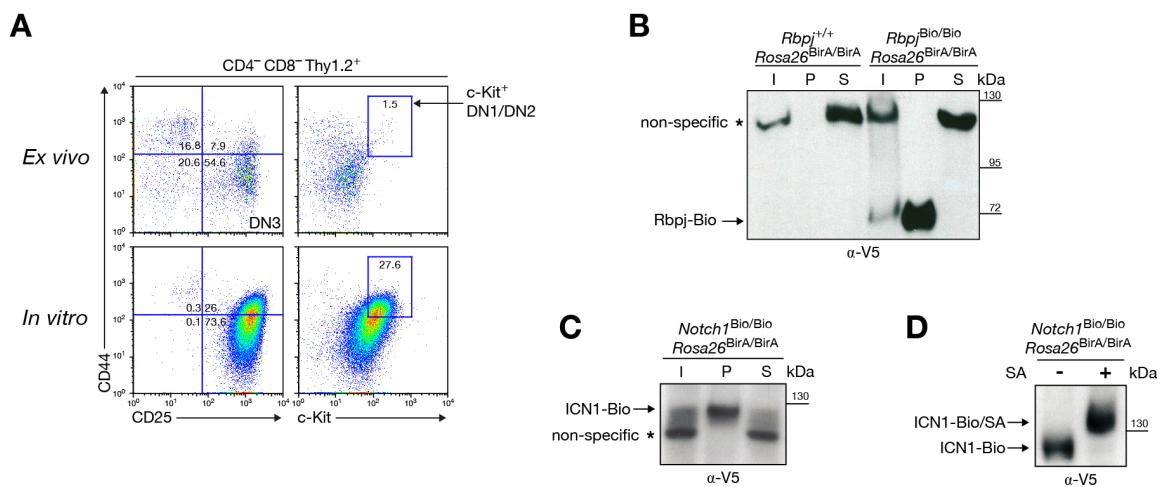


Figure 2.2 Efficient biotinylation of Bio-tagged Notch1 and Rbpj proteins. (A) FACS analysis of *in vivo* DN cells (top) and *in vitro* expanded pro-T cells (bottom) from *Rbpj*^{Bio} *Rag2*^{-/-} mice showing the DN2-DN3-like surface marker profile of the *in vitro* expanded cells. (B-D) Shown are immunoblots using an V5 antibody to detect Bio-tagged Notch1 or Rbpj. Arrows indicate the respective protein complexes and asterisks mark a non-specific band. Molecular weight markers (kDa) are shown on the right side of each blot. (B,C) SA pulldown experiments with nuclear extracts from control *Rosa26*^{BirA/BirA} *Rag2*^{-/-} and *Rbpj*^{Bio} *Rag2*^{-/-} (B) or *Notch1*^{Bio} *Rag2*^{-/-} (C) pro-T cells. Nuclear extracts of the respective cell type were incubated with SA-coated beads. 10% of the input (I) and supernatant (S) fractions together with the 100% of the precipitated (P) fraction were analysed for the presence of the Bio-tagged proteins. (D) A supershift assay with nuclear extracts from *Notch1*^{Bio} *Rag2*^{-/-} pro-T cells shows that ICN1-Bio/SA complexes have reduced motility during SDS-PAGE.

2.1.3 Bio-ChIP-sequencing of ICN1 and Rbpj in Rag2-deficient DN3a cells

I aimed to determine the genome-wide occupancy of ICN1 and Rbpj under physiological Notch signalling conditions in freshly isolated *ex vivo* thymocytes. However, Notch-independent DP, CD4SP and CD8SP cells make up more than 90% of all thymocytes in wild-type (wt) mice, while Notch-dependent progenitor populations are of very low frequency. This is problematic, since the generation of high-quality ChIP-seq libraries for most transcription regulators requires abundant cell quantities. Consequently, purification of sufficient Notch-dependent

progenitors from wt mice is accompanied by extensive cell sorting. However, chromatin-associated ICN1-Rbpj-complexes are short-lived as ICN1 is rapidly turned over upon transcription initiation (Fryer et al., 2002, 2004). Therefore, lengthy cell sorting inevitably results in a loss of ICN1 ChIP-seq signal, since Notch receptor - ligand interactions are disrupted and chromatin-associated ICN1 levels are diminished. To circumvent this limitation, I used *Notch1*^{Bio} *Rag2*^{-/-} and *Rbpj*^{Bio} *Rag2*^{-/-} mice. In *Rag2*-deficient mice, V(D)J recombination of TCR loci, and thus, the assembly of a functional pre-TCR is impaired, resulting in a complete block of thymopoiesis at the Notch-dependent DN3a stage (Shinkai et al., 1992). A *Notch1*^{Bio} *Rag2*^{-/-} thymus contains about 2-3 million cells, of which approximately 90% are DN3a cells, while DN1 and DN2 make up a very small proportion of cells that undergo active Notch signalling (Suppl. Fig. 7.1A). Hence, thymus suspensions from *Rag2*-deficient mice can be used without prior cell sorting to determine genomic ICN1- and Rbpj-binding sites by ChIP in DN3a cells under physiological conditions.

The binding of ICN1 to Rbpj leads to the recruitment of other co-activators and finally results in the generation of a large multiprotein complex (Kopan and Ilagan, 2009). Capturing the interaction of such multiprotein complexes with chromatin by ChIP is often difficult due to instability of the complex or accessibility of epitopes (Wang et al., 2011). Therefore, I tested different ChIP conditions in T cell progenitors followed by real-time quantitative PCR (qPCR) to determine the enrichment of known genomic ICN1/Rbpj-binding sites. The preparation of a nuclear fraction from whole thymocytes of *Notch1*^{Bio} *Rag2*^{-/-} mice followed by lysis of nuclei and sonication of the chromatin in the presence of 0.25% SDS reduced the background and increased the recovery of known ICN1-binding sites compared to a whole cell lysis ChIP protocol with 1% SDS (Suppl. Fig. 7.1B). ChIPs with an antibody against the V5 epitope (Suppl. Fig. 7.1C), or against an epitope that is exposed on the N-terminus of ICN1 after the S3 cleavage (Suppl. Fig. 7.1D), did not outperform the SA-mediated ICN1-Bio-ChIP. When Rbpj-Bio was precipitated with SA from nuclear lysates of whole thymocytes from *Rbpj*^{Bio} *Rag2*^{-/-} mice using the 0.25% SDS protocol, the enrichment of known Rbpj-binding sites was even higher compared to ICN1-Bio-ChIP (Suppl. Fig. 7.1E).

In chromatin-associated ICN1-Rbpj complexes, ICN1 is not in direct contact with the DNA (Nam et al., 2006; Wilson and Kovall, 2006; Choi et al., 2012). Capturing the binding of such co-regulators by ChIP is often improved by additional use of long-distance spanning crosslinking agents like disuccinimidyl glutarate (DSG) during the crosslinking step (Nowak et al., 2005). Formaldehyde (FA), which is usually used in ChIP assays, spans 2 Å while DSG spans about 7 Å and is an efficient protein-protein crosslinker. However, ChIP efficiency of both ICN1-Bio and Rbpj-Bio was reduced in dual-crosslink conditions (Suppl. Fig. 7.1F).

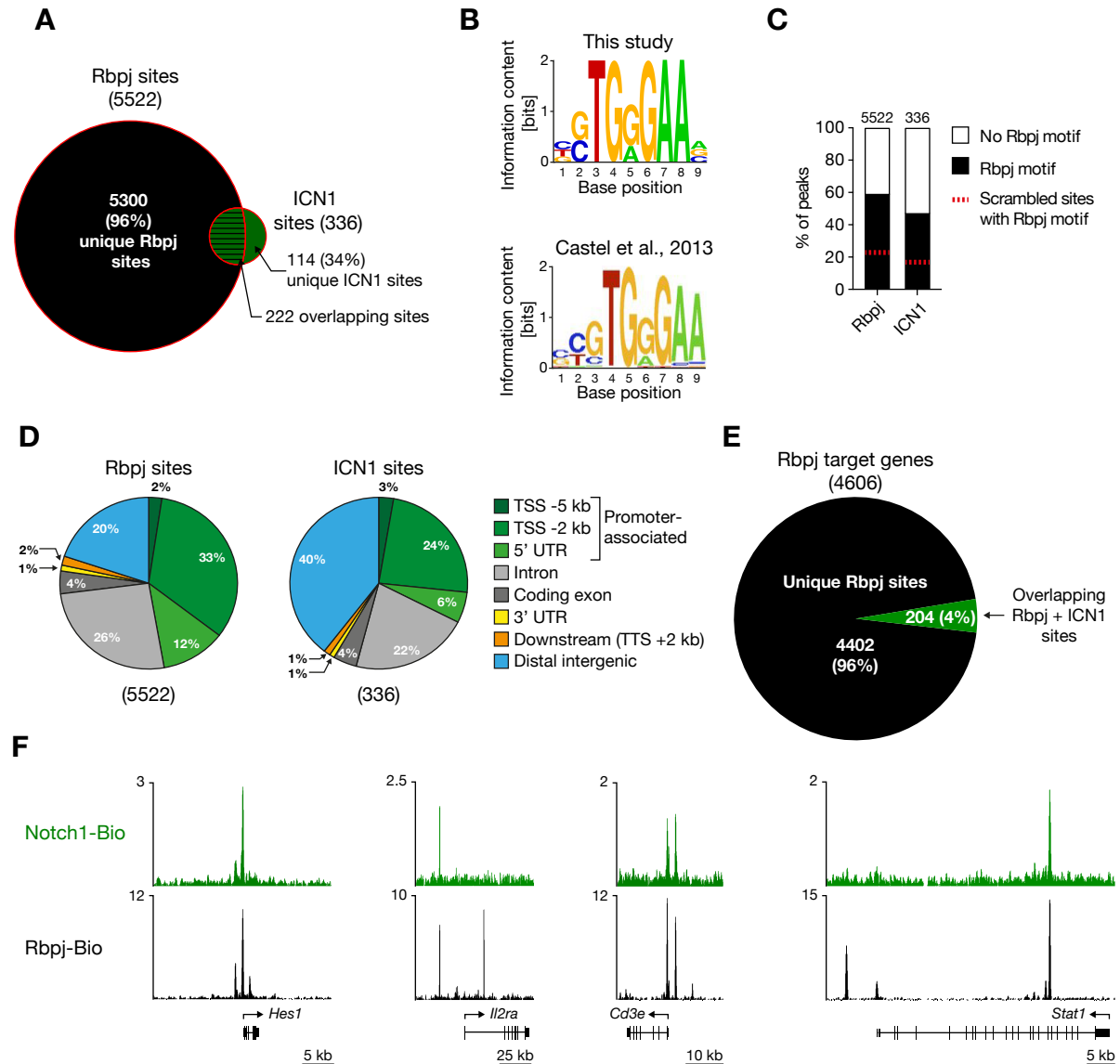


Figure 2.3 ICN1 and Rbpj Bio-ChIP-seq in Rag2-deficient DN3a cells. (A) Venn diagram representing the overlap of the common ICN1-Bio (green) and Rbpj-Bio (black) binding sites identified by Bio-ChIP-seq with total thymocytes from *Notch1^{Bio} Rag2^{-/-}* and *Rbpj^{Bio} Rag2^{-/-}* mice, respectively. (B) The position weight matrixes (PWMs) of the *de novo* identified Rbpj motif within the common Rbpj-Bio-binding sites in DN3a cells (top) and from a ChIP-seq experiment with an Rbpj-specific antibody in a myoblast cell line (Castel et al., 2013; bottom). (C) Bar chart representing the ratio of the 5522 common Rbpj-Bio and 336 common ICN1-Bio-binding sites containing the *de novo* identified Rbpj motif. The same motif was found in random DNA sequences at the frequency indicated by a dashed red line. (D) Pie charts representing the ratios of the common ICN1-Bio (left) and Rbpj-Bio (right) binding sites based on their distance to different features (see legend) of annotated genes. Promoter-associated regions are defined by their proximity to the TSS. The '-' and '+' signs indicate upstream or downstream sequences, respectively. (E) A linear distance-based peak-to-gene assignment resulted in 4,606 Notch target genes. Absolute numbers and percentages of genes with unique Rbpj-Bio (black) or co-occurring ICN1-Bio/Rbpj-Bio (green) occupancy are shown in a pie chart. (F) Genome browser screenshots of ICN1-Bio (green) and Rbpj-Bio (black) Bio-ChIP-seq read density tracks in Rag2-deficient DN3a cells at indicated genomic loci. The value on the y-axis represents the RPM-normalised read density of the given track. Arrows mark the TSS and filled black boxes represent exons. The scale in kilo bases (kb) is shown at the bottom right of each panel.

Next, I determined the genome-wide occupancy of ICN1 and Rbpj in Rag2-deficient DN3a cells by Bio-ChIP-seq of total thymocytes from *Notch1*^{Bio} *Rag2*^{-/-} and *Rbpj*^{Bio} *Rag2*^{-/-} mice. I generated three Solexa sequencing libraries for each transcriptional regulator with different enrichment qualities of known ICN1/Rbpj-binding sites (Suppl. Fig. 7.2A). To determine high-confidence ICN1-Bio and Rbpj-Bio-binding sites, I selected only the peaks that were identified by MACS peak calling, in at least two different experiments (common sites, Suppl. Fig. 7.2B).

Sequence analysis of the common Rbpj-Bio-binding sites by *de novo* motif finding identified a consensus Rbpj-motif strikingly similar to a previously described Rbpj-motif (Fig. 2.3B; Castel et al., 2013). Using a FDR < 0.85 as cutoff, the *de novo* identified motif was present in 58% of the common Rbpj-Bio-binding sites and in 49% of the common ICN1-Bio-binding sites, respectively (Fig. 2.3B). After randomised shuffling of the underlying sequences, the same motif was found only in approximately 20% of the common binding sites.

Based on the distance of the Rbpj-Bio peaks to the closest annotated gene, Rbpj-Bio binds in a promoter-focused fashion since 47% of the common Rbpj-Bio sites were found within 5 kb upstream of the transcription start site (TSS) or mapped to the 5' UTR (Fig. 2.3D). 26% of all Rbpj-Bio-binding sites were found within introns of genes and 20% within distal intergenic regions, which are defined as > 5 kb upstream of a TSS as well as > 2 kb downstream of a transcription termination site (TTS). ICN1 interacts with Rbpj in order to regulate transcription, since it cannot bind directly to DNA (Tanigaki and Honjo, 2010). Thus ICN1 should only be present at Rbpj-binding sites. Surprisingly, only one third of the identified ICN1-Bio-binding sites from single ChIP-seq experiments overlapped with common Rbpj-Bio-binding sites (Suppl. Fig. 7.2C). Using the aforementioned overlap filter, I found 336 common ICN1-Bio-binding sites (Suppl. Fig. 7.2B), of which two thirds overlapped with the common Rbpj-Bio-binding sites (Fig. 2.3A). ICN1-Bio exhibits an overall similar binding profile like Rbpj-Bio. However, 40% of all ICN1-Bio peaks were within distal intergenic sites. This relative increase is mostly attributed to the 114 unique ICN1-Bio-binding sites (Suppl. Fig. 7.2F). I took a closer look to see whether these sites could originate from real and potentially even Rbpj-independent ICN1-Bio-binding to the DNA. Only 22% of the sites contain an Rbpj consensus motif (Suppl. Fig. 7.2D), a value close to random mappability of the motif. The read density profile of the individual ICN1-Bio-ChIP-seq tracks at the unique ICN1-Bio-binding sites was similar across all three replicas (Suppl. Fig. 7.2E). In contrast, the read densities varied at high-confidence binding sites where ICN1-Bio and Rbpj-Bio co-occur. Furthermore, a sequencing track from sonicated ChIP-input control DNA showed increased read density at the unique ICN1-Bio-binding sites (data not shown), which is indicative of ChIP-seq artefact regions. Such false-positive peaks can result from PCR-artefacts during library amplification

(Waszak et al., 2014). Alternatively, artefact peaks are often found within repetitive regions of the genome that are poorly annotated such as telomeres and centromeres where reads mapping of the reads is error-prone (Bailey et al., 2013). The unique ICN1-Bio-binding sites were therefore excluded from further analysis. The remaining Rbpj-Bio-binding sites were then assigned by their linear distance to the closest gene with a maximum distance cutoff of 50 kb (see Section 4.19). This peak-to-gene assignment resulted in a total of 4,606 direct Rbpj-Bio target genes (Fig. 2.3E). The binding of ICN1-Bio was detected at 204 (4%) of those genes. ICN1-Bio and Rbpj-Bio-binding sites were found at known Notch1 target genes such as *Hes1* and *Il2ra* as well as at many genes that previously were not implicated to be regulated by Notch signalling such as *Cd3e* and *Stat1* (Fig. 2.3F).

The fact that ICN1-Bio-binding was not observed at the vast majority of the Rbpj-Bio sites was very surprising, since Notch1 signalling levels climax in DN3a cells. Thus, these cells should contain high levels of chromatin-associated ICN1-Rbpj complexes. Despite of the antibody-independent, high-affinity Bio-ChIP-seq approach, these complexes remained highly challenging to capture via ICN1-Bio-ChIP compared to Rbpj-Bio-ChIP, as ICN1-Bio read density tracks showed a much lower signal-to-noise ratio (Fig. 2.3F). This ultimately resulted in the detection of many 'unique Rbpj-Bio' binding sites (Fig. 2.3A). This could be due to several (technical) factors: *i*) the indirect binding of ICN1-Bio to the chromatin; *ii*) low stability of (crosslinked) ICN1-Bio/Rbpj/DNA complexes *iii*) the short half-life of transcription-activating ICN1-Bio complexes due to proteasomal degradation; *iv*) masking of the ICN1 Bio-tag in transcription-activating complexes.

2.1.4 Genome-wide profiling of *cis*-regulatory elements in Rag2-deficient DN3a cells

Transcription factors generally regulate expression of their target genes through interactions with *cis*-regulatory elements of the gene such as enhancers and promoters. To gain mechanistic insights about how Notch signalling regulates target gene expression, I set out to map the *cis*-regulatory landscape of DN3a cells by combining several genome-wide techniques (Revilla-I-Domingo et al., 2012).

In order to characterise the genome-wide pattern of protein-DNA interactions in DN3a cells, the DNase I hypersensitive (DHS) sites were mapped by treating freshly isolated and permeabilised whole thymocyte suspensions from *Rag2*^{-/-} mice with DNase I (Suppl. Fig. 7.3A). The released DHS 'double-hit' fragments were purified and analysed by high-throughput sequencing (Sabo et al., 2006; Hesselberth et al., 2009). This resulted in the identification of approximately 39,000 and 28,000 DHS sites (P -value < 10^{-10}) in two independent experiments

(Suppl. Fig. 7.3B). Both datasets overlapped highly (Suppl. Fig. 7.3C) and the set with more DHS sites was selected for further analysis, because of its higher quality (Suppl. Fig. 7.3C,E).

In the next step, TSSs were profiled in freshly isolated DN3a cells from *Rag2*^{-/-} mice by cap analysis of gene expression (CAGE) coupled with high-throughput sequencing. The CAGE technique allows the systematic profiling of the 5' end of capped mRNAs at single nucleotide resolution (Shiraki et al., 2003; Carninci et al., 2006). Active TSSs were defined by a cutoff of at least 2.6 RPM (reads per million mapped sequence tags) per CAGE tag cluster, which distinguishes transcriptionally active (> 2.6 RPM) from inactive (< 2.6 RPM) genes (see Section 4.26; Revilla-I-Domingo et al., 2012). Finally, annotated TSSs and CAGE-defined active TSS were overlapped with DHS sites to generate the *cis*-regulatory landscape of DN3a cells (Fig. 2.4A).

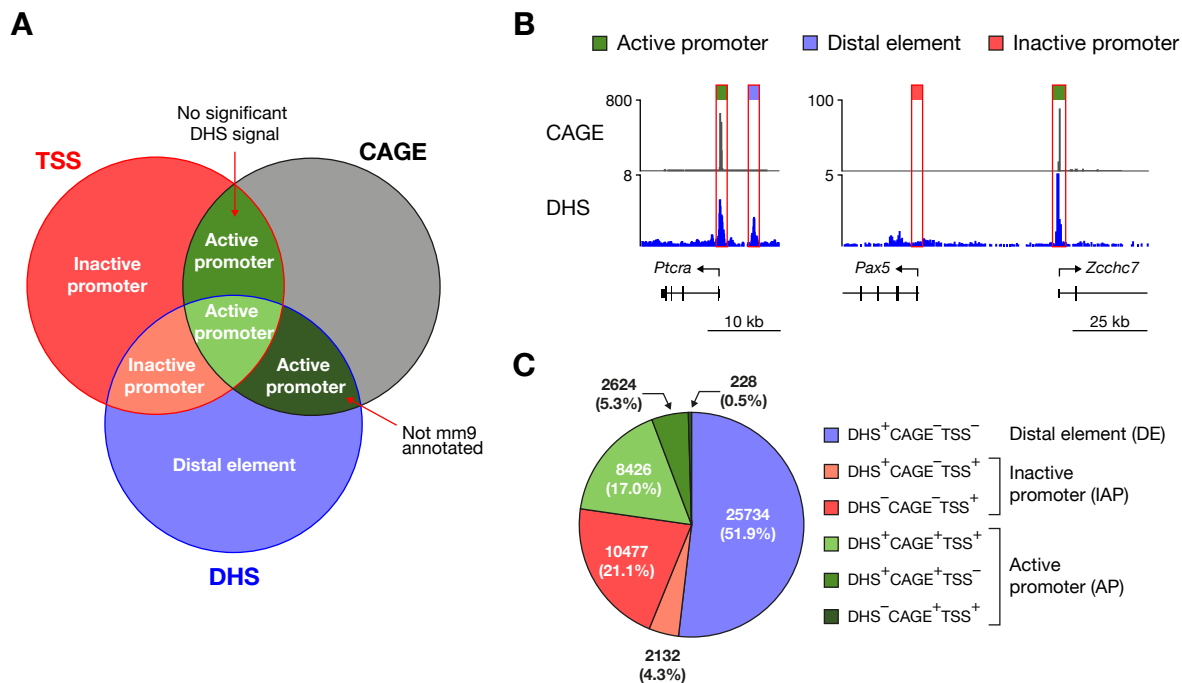


Figure 2.4 The *cis*-regulatory landscape of Rag2-deficient DN3a cells. (A) Schematic Venn diagram visualising the strategy to identify active promoters (AP), inactive promoters (IAP) and distal elements (DE). Note that the group of AP also included: *i*) Significant CAGE tag clusters overlapping with DHS but not annotated TSS, since they often represented TSS of transcripts, which were not RefSeq-annotated but in other repositories. *ii*) Significant CAGE clusters overlapping with an annotated TSS but devoid of significant DHS signal, which resulted from a stringent DHS peak calling cutoff (P -value < 10^{-10}). (B) CAGE and DHS read density tracks generated from *Rag2*^{-/-} DN3a cells at indicated genomic loci showing examples of identified *cis*-regulatory elements. Red boxes highlight colour-coded AP, IAP and DE. Numbers on the y-axis represent the RPM-normalised read density of the given track. Arrows mark the TSS and filled black boxes represent exons. The scale in kb is shown at the bottom right of each panel. (C) Pie chart with total numbers and percentages of all *cis*-regulatory elements (shown by different colours) identified in Rag2-deficient DN3a cells according to the strategy shown in panel A.

CAGE-defined active TSSs characterise active promoters (AP) at DHS sites and/or annotated TSSs, while TSSs without significant CAGE signal demarcate inactive promoters (IAP).

Distal elements (DE) are distinguished by presence of a DHS site and absence of an active TSS (no significant CAGE signal). No significant CAGE or DHS signals were detected at the B cell-specific *Pax5* gene (Fig. 2.4B). In contrast, the promoter and a well-described enhancer of the T cell-specific *Ptcra* gene (Reizis and Leder, 1999, 2001, 2002; Xu et al., 2007) were reliably identified. This confirmed the validity of the chosen approach. In total, 11,278 AP, 12,655 IAP and 25,734 DE were found in *Rag2*^{-/-} DN3a cells (Fig. 2.4C), comparable to the respective numbers of *cis*-regulatory elements found in pro-B cells using an identical approach (Suppl. Fig. 7.4A; Revilla-I-Domingo et al., 2012; Schwickert et al., 2014).

Depending on their activation or repression status, promoters and enhancers contain combinations of histone variants with specific post-translational modifications at their N-termini (Zhou et al., 2011). Consequently, mapping of these histone modifications can be used to investigate the activation status of these regulatory elements (Revilla-I-Domingo et al., 2012). To further characterise the activity of the *cis*-regulatory elements identified in DN3a cells, I performed ChIP-seq with specific antibodies against active (H3K4me2, H3K4me3, H3K9ac) and repressive (H3K27me3) histone modifications on freshly isolated *Rag2*^{-/-} thymocytes (Suppl. Fig. 7.3A). Two libraries from independent ChIP experiments for each histone modification were sequenced. MACS peak calling (P -value < 10^{-10}) resulted in peak sets with comparable peak numbers for a given histone modification (Suppl. Fig. 7.3B). The peak sets showed high overlap between biological replicates (Suppl. Fig. 7.3C). I chose the replicates with higher signal to noise ratio and more peaks for further analysis (Suppl. Fig. 7.3B,D,E). The overlap analysis of the profiled histone modifications with the identified *cis*-regulatory elements showed that almost 80% of the identified AP in *Rag2*-deficient DN3a cells carried all three profiled active histone modifications (Suppl. Fig. 7.4B). This validates that coinciding DHS sites and CAGE tag clusters reliably identified active promoters, which are known to harbour H3K4me2, H3K4me3 as well as H3K9ac modifications (Bernstein et al., 2005; Kim et al., 2005; Heintzman et al., 2007; Wang et al., 2008b; Revilla-I-Domingo et al., 2012).

In contrast, very few of the IAPs in DN3a cells contained active histone modifications and, if so, they were mainly co-occurring with H3K27me3 (Suppl. Fig. 7.4B). Tri-methylation of H3K27 is catalysed by the Polycomb repressive complex 2 (PRC2) and is a mark that is closely associated with transcriptional silencing (Ringrose and Paro, 2004; Schuettengruber et al., 2007; Mikkelsen et al., 2007). Like in the case of pro-B cells, the vast majority of the IAP did not carry any of the profiled chromatin features, while only a small fraction contained solely active chromatin marks. This indicates that this approach successfully identified transcriptionally inactive promoters.

Depending on their particular histone modification patterns, DE were further subdivided into different enhancer categories (Revilla-I-Domingo et al., 2012). Approximately 4,400 ac-

tive enhancers were found in DN3a cells, which were characterised by the presence of all three profiled active histone modifications (Suppl. Fig. 7.4B). About 50% of these enhancers were enriched for H3K4me3, a mark which is usually indicative of transcribed promoters but which can also be present at active enhancers, although generally to a lesser extent (Heintzman et al., 2007, 2009). Another group of about 3,600 DE contained only H3K4me2. This group is referred to as poised DE, since they do not exhibit significant H3K9ac and it was shown in B cells that many enhancers containing such a signature became active enhancers upon maturation (Revilla-I-Domingo et al., 2012). H3K27me3 could only be found at a small group of about 1,400 DE, which are referred to as repressed DE, since these sites are likely targeted by the PRC2 silencing complex. More than half of all DE did not harbour any of the profiled modifications. As it was shown for B cells (Revilla-I-Domingo et al., 2012), many of these sites might be CTCF- and cohesin-binding sites and thus likely to function in gene insulation, chromatin looping or chromosome cohesion (Peters et al., 2008; Phillips and Corces, 2009). In summary, the genome-wide analysis of DHS sites, TSSs, histone modifications and gene transcripts identified active as well as inactive promoters and different classes of enhancers that together constitute the *cis*-regulatory landscape of DN3a cells. This landscape can be used as a resource for any genome-wide transcription factor profiling data obtained in DN3 cells to understand through which regulatory elements a transcription factor controls gene expression.

2.1.5 Integration of the genome-wide ICN1-Bio and Rbpj-Bio occupancy into the *cis*-regulatory landscape

In the next step, the genome-wide Rbpj-Bio and ICN1-Bio occupancy in DN3a cells was integrated into the regulatory landscape (Fig. 2.5). Strikingly, 69% of all Rbpj-Bio and 64% of all ICN1/Rbpj-Bio-binding sites are located within active promoters. Less than < 5% of all binding sites coincide with inactive chromatin or are devoid of significant DHS signal (Fig. 2.5A). Roughly 25% of all Rbpj-Bio and ICN1/Rbpj-Bio-binding sites are in DE. While there were hardly any Rbpj-Bio-binding sites seen at repressed DE, more than 50% of Rbpj-Bio-occupied DE were active enhancers and 21% were poised enhancers. ICN1/Rbpj-Bio-binding was not detected at any poised DEs. However, 74% of all ICN1/Rbpj-Bio-co-occupied DE were found to be active enhancers. The fact that the vast majority of all detected Rbpj-Bio sites are within active chromatin could indicate that these sites are actually targets of active Notch signalling in DN3a cells, even though ICN1-Bio could only be detected at a small subfraction of those sites. Globally, Rbpj-Bio was bound to 30% of all identified active promoters as well as to 17% of all active enhancers, suggesting a promoter-focused binding of the ICN1/Rbpj-Bio complex (Fig. 2.5B). Finally, the combination of transcription factor occupancy and *cis*-regulatory

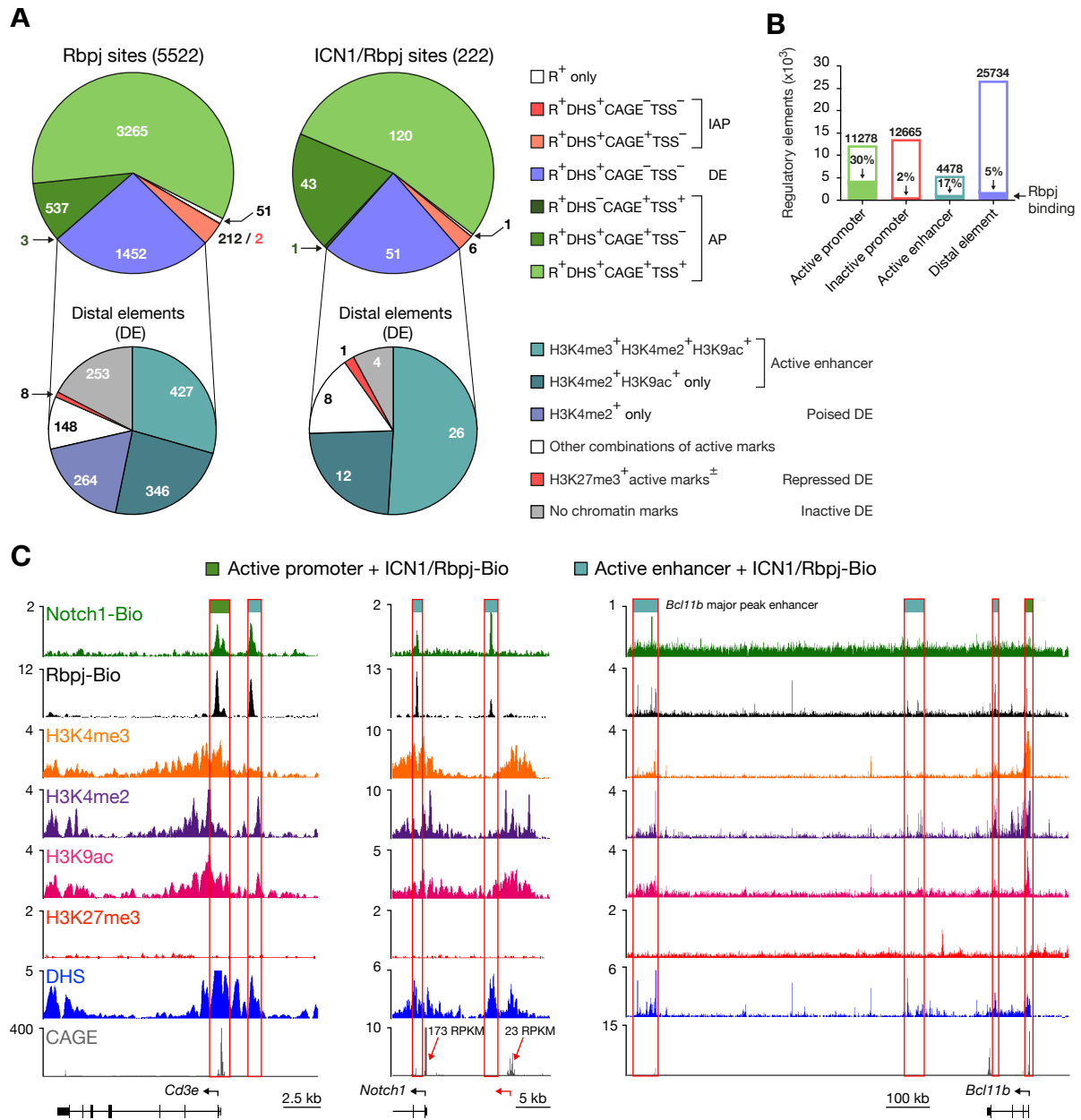


Figure 2.5 Integration of the genome-wide ICN1-Bio and Rbpj-Bio occupancy into the *cis*-regulatory landscape. (A) Pie charts representing the total numbers of all common Rbpj-Bio 'R' binding sites (left) and ICN1/Rbpj-Bio-co-occupied sites (right) at AP and IAP (top) as well as DE (bottom) in *Rag2*^{-/-} DN3a cells, as described in section 2.1.5. (B) Bar chart representing the relative contribution of Rbpj-Bio bound regulatory elements (filled bars) to all identified regulatory elements (combined open and filled bars) in *Rag2*^{-/-} DN3a cells. (C) ICN1 and Rbpj Bio-ChIP-seq, histone ChIP-seq, CAGE and DHS read density tracks generated in *Rag2*^{-/-} DN3a cells at indicated genomic loci showing examples of identified Rbpj-Bio and/or ICN1/Rbpj-Bio-co-occupied active promoters and enhancers. Red boxes highlight colour-coded Rbpj-Bio and/or ICN1/Rbpj-Bio-co-occupied AP and active enhancers. The CAGE tag signal within the DHS site at the canonical (black arrow) and an alternative (red arrow) *Notch1* promoter was quantified. The *Bcl11b* major peak enhancer (Li et al., 2013) is indicated. Numbers on the y-axis represent the RPM-normalised read density of the given track. Arrows mark TSSs and filled black boxes represent exons. The scale in kb is shown at the bottom right of each panel.

element mapping showed that Notch1 signalling might directly regulate *CD3e* transcription through the promoter and/or an upstream enhancer element (Fig. 2.5C). The autoregulation of *Notch1* could be achieved through ICN1/Rbpj co-occupied intronic enhancer elements in the vicinity of the canonical *Notch1* promoter and a described alternative promoter (Tsuji et al., 2003; Gómez-Del Arco et al., 2010). *Bcl11b*, however, could be regulated by Notch1 signalling in part through the 'major peak' enhancer, which is located over 850 kb downstream of the *Bcl11b* gene (Li et al., 2013).

2.2 Dissection of the Notch-regulated transcriptome in DN3a cells

2.2.1 Characterisation of the *Notch1^{fl/fl}* *Lck-Cre* mouse

Transcription factors often bind to hundreds or thousands of *cis*-regulatory elements within the genome. Binding of a transcription factor to *cis*-regulatory elements of a gene such as the promoter or promoter-proximal as well as intronic enhancers indicates that the gene is likely to be regulated by this transcription factor. However, in many cases transcription factor perturbation studies revealed that only subfractions of these sites seem to be functional and result in a transcription factor-dependent regulation of target genes (Revilla-I-Domingo et al., 2012; Vilagos et al., 2012; Schwickert et al., 2014). Hence, it was important to identify the genes that are regulated by Notch signalling in DN3a cells.

To study Notch1-dependent gene expression changes in DN3 thymocytes, I performed a loss-of-function experiment by T cell-specific deletion of a conditional *Notch1* allele in which the promoter region and exon 1 are flanked by *loxP* sites (Radtke et al., 1999). To this end, *Notch1^{fl/fl}* mice were crossed with mice expressing Cre-recombinase under the control of the proximal *Lck*-promoter (Lee et al., 2001), resulting in a deletion of *Notch1* from the DN2 stage onwards (Wolfer et al., 2002). To assess the effect of loss of Notch1, thymic T cell development was analysed by FACS in experimental *Notch1^{fl/fl}* *Lck-Cre* and control *Notch1^{fl/fl}* animals (Fig. 2.6).

The observed phenotype was consistent with results published by Wolfer et al. (2002). Total thymic cellularity was decreased 8-fold in experimental mice (Fig. 2.6B). The CD4/CD8 profile showed a 6.5-fold increase in the DN compartment and a mild decrease in the DP compartment, while relative CD4SP and CD8SP frequencies were slightly increased (Fig. 2.6A). FACS analysis of the post β -selection marker CD28 (Teague et al., 2010) within the CD44⁺ DN3 and DN4 stages revealed a partial block at the DN3a stage. This developmental block was also reflected in the absolute cellularity of DP cells that was reduced 12-fold as well as in CD4-SP and CD8-SP cell numbers, which both were 3.5-fold lower.

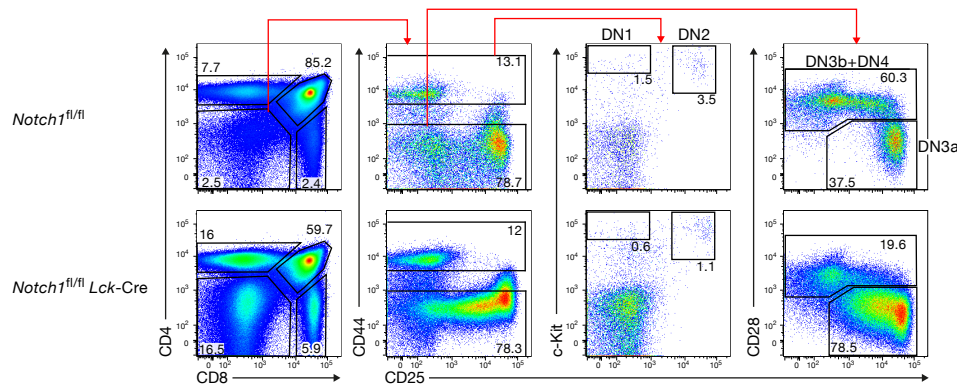
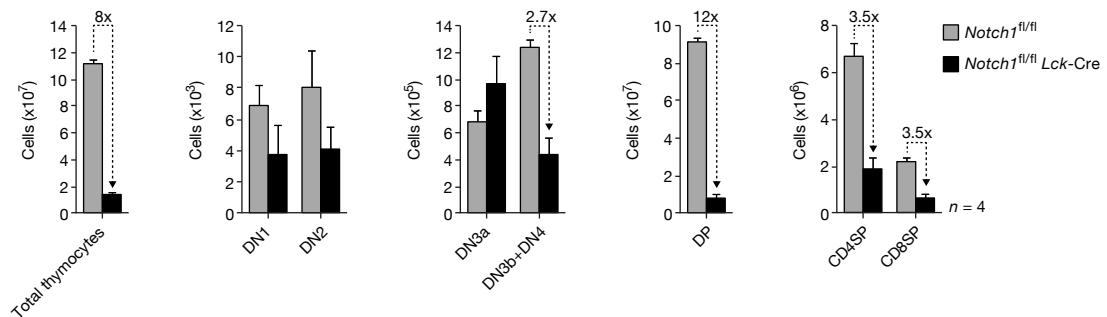
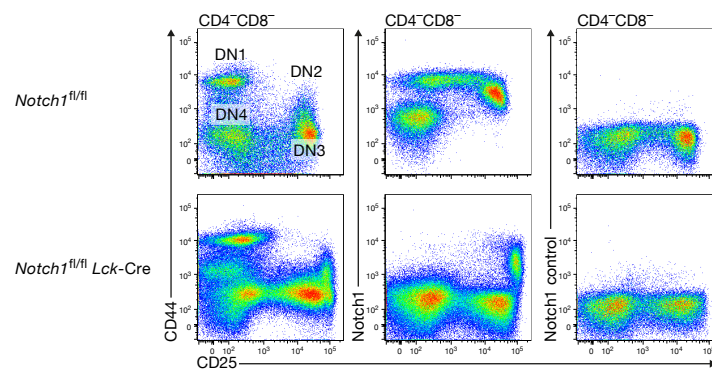
A**B****C**

Figure 2.6 Intrathymic deletion of *Notch1*. (A,B) Flow cytometry (A) and absolute numbers (B) of the indicated thymocyte subsets from 6-8 week-old *Notch1^{fl/fl}* (grey bars) and *Notch1^{fl/fl} Lck-Cre* (black bars) littermates. Numbers refer to percent of cells in the indicated gate (A), n indicates the number of mice analyzed and error bars indicate SEM (B). (C) FACS analysis of cell surface Notch1 on DN thymocytes. Notch1 was detected with a biotinylated primary antibody and a SA-PE conjugated secondary antibody. 'Notch1 control' indicates a staining without primary antibody.

To confirm the loss of the Notch1 receptor upon conditional deletion, I investigated its surface levels by FACS (Fig. 2.6C). The high specificity of the Notch1 antibody was shown earlier by Fiorini et al. (2009) as well as in a control staining. Within the DN compartment of control mice, all CD25⁺ cells had high levels of surface Notch1. However, in experimental mice there was only a small population of CD25 and Notch1 double-positive cells that com-

prised the cells with the highest CD25 surface levels. Interestingly, upon deletion of *Notch1*, a fraction of the CD28⁻ DN3a cells exhibited reduced CD25 levels compared to control DN3a cells (Fig. 2.6A). Another fraction showed an increase in CD25 on their cell surface, a phenotype that was previously reported for mice with a defect in pre-TCR signalling (Mombaerts et al., 1992a,b; Shinkai et al., 1992; Fehling et al., 1995; Malissen et al., 1995) as well as perturbed Notch signalling (Wolfer et al., 2002; Tanigaki et al., 2004). Since *Il2ra*, the gene encoding CD25, has been reported to be directly regulated by Notch1 (Deftos et al., 2000; Adler et al., 2003; Maillard et al., 2006; Pajerowski et al., 2009; Liu et al., 2010a; Wang et al., 2011), it was conceivable that reduced CD25 levels are a consequence of perturbed Notch1 signalling. Moreover, *Lck*-Cre-mediated deletion of *Notch1* leads to reduced competition among T cell progenitors for Notch ligands, as only the few undeleted cells carry full-length Notch1 receptors on their cell surface. Consequently, undeleted, *Notch1*-expressing cells can receive higher Notch signalling due to enhanced receptor-ligand interaction. This could augment *Il2ra* expression and ultimately lead to increased CD25 cell surface protein levels in non-deleted DN3 cells (Fig. 2.6C).

2.2.2 Minimal gene expression changes in *Notch1*-deleted CD25^{int} DN3a thymocytes

To identify Notch1-dependent target genes at the DN3 stage of T cell development, DN3 thymocytes from control *Notch1*^{fl/fl} and experimental *Notch1*^{fl/fl} *Lck*-Cre mice were sorted and analysed (Fig. 2.7). Since the deletion of *Notch1* is heterogeneous within the DN3 compartment (Fig. 2.6C), DN3a cells with intermediate and high levels of CD25 were sorted separately (Fig. 2.7A). Finally, CD25^{hi} and CD25^{int} DN3 cells were sorted by CD71 (transferrin receptor) and CD27 surface levels. This surface marker combination, can be used like CD28 to discriminate between pre-TCR β -selection DN3a (CD71^{lo}CD27^{lo}) and post-TCR β -selection DN3b cells (CD71^{hi}CD27^{hi}; Taghon et al., 2006; Kelly et al., 2007; Teague et al., 2010).

A PCR analysis similar to that of Feyerabend et al. (2009) was used to assess the genomic deletion of the *loxP* site-flanked region of *Notch1*. It showed that the deletion in CD25^{int} cells was complete, while a fraction of the CD25^{hi} cells did not yet fully delete the conditional *Notch1* allele (Fig. 2.7B). Additionally, the transcript levels of the Notch1 target gene *Dtx1* (Matsuno et al., 2002; Weng et al., 2006; Wang et al., 2011) were investigated by RT-qPCR (Fig. 2.7B). *Dtx1* mRNA levels were 17-fold higher in control *Notch1*^{fl/fl} DN3a thymocytes compared to DN3b cells, in which Notch signalling diminishes (Taghon et al., 2006; Yashiro-Ohtani et al., 2009). However, *Dtx1* mRNA abundance was only 2.6-fold reduced in CD25^{int} *Notch1*^{fl/fl} *Lck*-Cre DN3a cells compared to control DN3a cells.

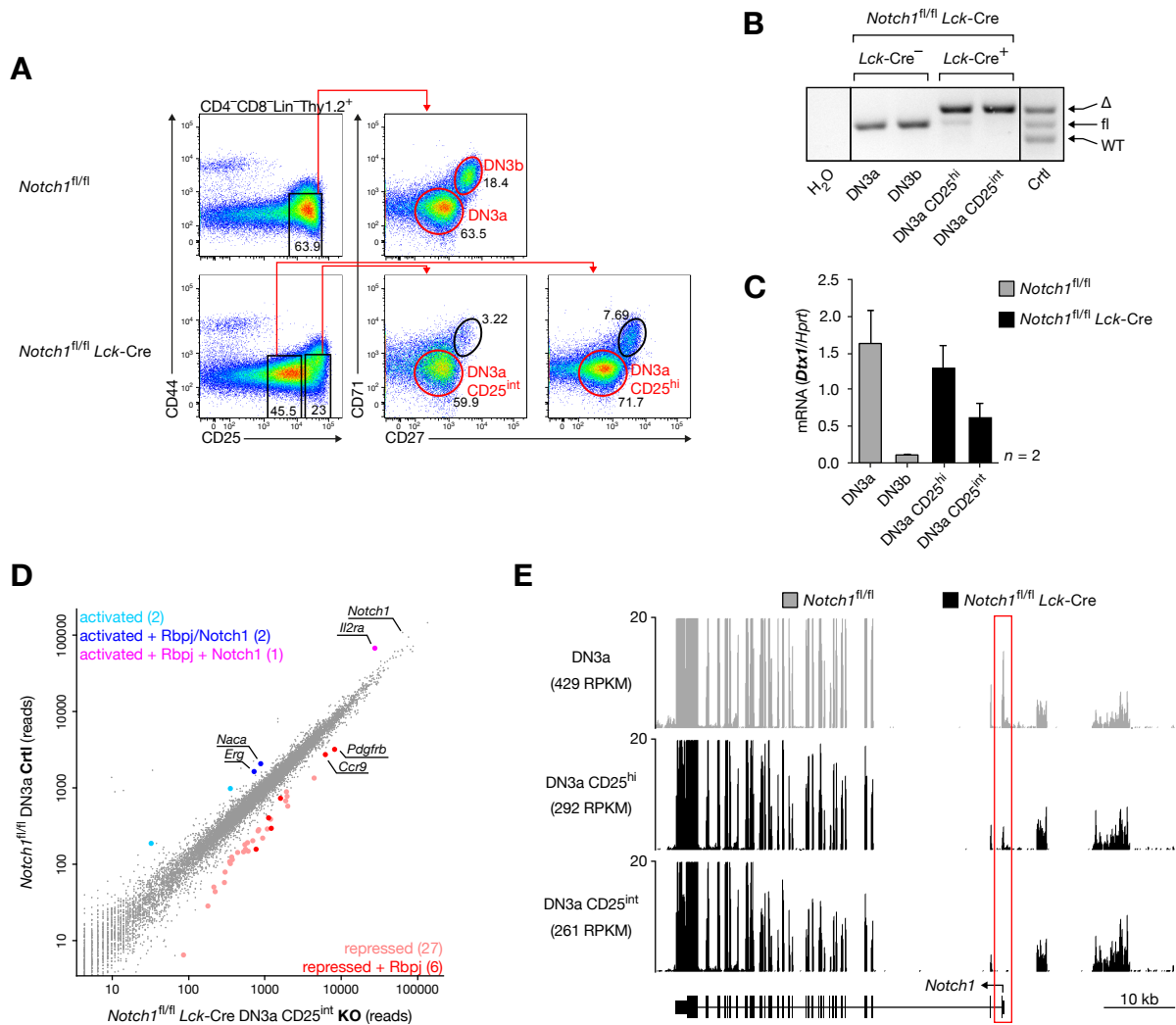


Figure 2.7 Transcriptome analysis of Notch1-deficient DN3a cells. (A) FACS analysis showing the gating strategy for the indicated thymocyte subsets from 6-8 week-old *Notch1^{fl/fl}* and *Notch1^{fl/fl} Lck-Cre* littermates. Numbers refer to percent of cells in the indicated gate. Red gates depict final sorting gates. (B) Deletion of the floxed *Notch1* allele in CD25^{hi} and CD25^{int} DN3a cells was analyzed by PCR. PCR fragments corresponding to the wild-type (wt), intact (fl) and deleted (Δ) floxed alleles are indicated. A 1:1 mix of tail DNA from *Notch1^{fl/+}* and *Notch1^{\Delta/+}* mice was used as template for the control PCR. (C) *Dtx1* mRNA abundance relative to *Hprt* mRNA in *Notch1^{fl/fl}* (grey bars) and *Notch1^{fl/fl} Lck-Cre* (black bars) DN3 cells was analyzed by reverse transcription (RT)-qPCR. *n* indicates the number of experiments. Error bars indicate the SD. (D) Scatter plot visualising gene expression differences identified by RNA-seq of control *Notch1^{fl/fl}* DN3a cells and *Notch1^{fl/fl} Lck-Cre* CD25^{int} DN3a cells. Genes, which are more than 2-fold differentially expressed and have an adjusted *P*-value of < 0.1 , are shown in blue (Notch-activated) or red (Notch-repressed). Darker colours indicate binding of Rbpj-Bio within 50 kb of the gene, identified by Bio-ChIP-seq in DN3a cells (see Section 2.1.3). Numbers in brackets correspond to the number of genes in a given group. (E) Genome browser screenshot showing RPM-normalised RNA-seq read density profiles of indicated DN3 populations from *Notch1^{fl/fl}* (grey) and *Notch1^{fl/fl} Lck-Cre* (black) mice at the *Notch1* locus. An arrow marks the TSS and filled black boxes represent exons. A red box indicates the *loxP* site flanked promoter and exon 1 region. The value on the y-axis represent the read density. The scale in kb is shown at the bottom right. Numbers in brackets indicate RPKM-normalised *Notch1* expression values.

Next, the transcriptomes of Notch1-deficient CD25^{int} DN3a cells and control DN3a cells were analysed by RNA-seq (Fig. 2.7D). By using an adjusted *P*-value of < 0.1 and a minimum of 2-fold differential expression, 38 genes were found to be significant and differentially expressed. Of those genes, 33 were up-regulated and 5 downregulated in Notch1-deficient DN3a cells. Among the 5 Notch1-activated genes, only *Il2ra* has been reported to be regulated by Notch signalling (Defetos et al., 2000; Adler et al., 2003; Maillard et al., 2006; Pajeroski et al., 2009; Liu et al., 2010a; Wang et al., 2011). The identification of *Il2ra* in this analysis is not surprising, because *Notch1*-deleted DN3a cells were sorted for intermediate CD25 levels and compared to CD25⁺ control DN3a cells. Furthermore, *Notch1* mRNA was reduced only 1.6-fold from 429 RPKM in the control to 261 RPKM in *Notch1*-deleted CD25^{int} DN3a cells (Fig. 2.7 D,E). The RNA-seq profile of CD25^{int} *Notch1*^{fl/fl} *Lck*-Cre DN3a cells at the *Notch1* locus was devoid of reads mapping the deleted exon 1, whereas a low level of exon 1-containing mRNA could still be detected in CD25^{hi} DN3a cells. This was in line with complete genomic deletion of *Notch1* (Fig. 2.7B) and a strikingly reduced Notch1 surface protein level in CD25^{int} *Notch1*^{fl/fl} *Lck*-Cre DN3a cells (Fig. 2.6C). Taken together, this analysis showed that in contrast to the profound T cell developmental defect seen upon deletion of *Notch1*, the gene expression profile of CD25^{int} *Notch1*^{fl/fl} *Lck*-Cre DN3a cells was hardly affected.

The mild changes in global gene expression upon *Notch1* deletion were surprising, since signalling through the Notch1 receptor is essential for survival of DN3 thymocytes (Ciofani and Zúñiga-Pflücker, 2005). These conflicting results could be caused by several reasons: *i*) Two studies described alternative *Notch1* promoters, which give rise to transcripts encoding non-canonical Notch1 proteins (Tsuji et al., 2003; Gómez-Del Arco et al., 2010). The activity of the alternative promoters is most likely not affected by the deletion of the canonical promoter as they are not flanked by the *loxP* sites of the *Notch1*^{fl/fl} allele. The shorter transcripts encode proteins that could lack the N-terminal epitope, which is detected by the Notch1 antibody used for FACS. Thus, ICN1 levels in *Notch1*^{fl/fl} *Lck*-Cre CD25^{int} DN3a cells might be sufficient to partially rescue the expression of many Notch1 target genes (except for *Il2ra*). *ii*) The Notch family consists of several structurally similar members. Thus, signalling through other Notch receptors might rescue Notch1 target gene expression. *Notch4* is not expressed in the entire haematopoietic system and can therefore be excluded (IMMGEN database). While pre-thymic CLP as well as thymic DN1 and DN2 cells express high levels of Notch2 on their cell surface, DN3 cells do not carry Notch2 receptors (Fiorini et al., 2009). In contrast, *Notch3* is sharply upregulated in DN3a cells. Even though Notch1 and Notch3 functions were reported to be non-overlapping in T cell development (Shi et al., 2011; Suliman et al., 2011), it is possible that Notch3 partially sustains gene expression of Notch1 target genes upon Notch1 deficiency in DN3a cells. *iii*) DN3a cells could be highly sensitive to reduced Notch1 levels, thus leading to

rapid cell death in the absence of Notch1. It is known that apoptotic cells within the thymus are rapidly engulfed and cleared by macrophages (Surh and Sprent, 1994; Shi and Petrie, 2012). Therefore, capturing Notch1-deficient DN3a cells at a stage in which deregulation of Notch target genes can be detected by RNA-seq might not be feasible.

2.2.3 *Notch1* deletion in Rag2-deficient DN3a thymocytes results in reduced expression of Notch1 target genes

Rag-deficient DN3 thymocytes exhibit higher CD25 levels compared to their wild-type counterparts (Mombaerts et al., 1992b). Consequently, the observed correlation between CD25 levels and Notch1 signalling strength implied that Rag2-deficient mice could provide a system with a higher dynamic range for the *in vivo* deletion of *Notch1*. Thymopoiesis is blocked at the DN3a stage in *Rag2*^{-/-} mice. Compared to wt mice, this block results in a roughly 100-fold reduced cellularity, while the thymus size is only ~10-fold reduced. Furthermore, Rag2-deficient thymic epithelia consists almost entirely of cells with cortical morphology (Prockop and Petrie, 2004) that express high levels of the Notch ligand Delta-like 4 (DLL4; Koch et al., 2008a). Therefore, T cell progenitors of *Rag2*^{-/-} mice have reduced competition for Notch ligands that could lead to increased Notch signalling levels.

Furthermore, signalling through both Notch1 and pre-TCR is essential for DN3 cells to transit to the DN4 stage (Maillard et al., 2006). Several studies suggested that there is crosstalk between both signalling pathways (Radtke and MacDonald, 2009; Yashiro-Ohtani et al., 2009). For instance, Notch1 signalling is required for the expression of several genes encoding components of the pre-TCR (Kueh and Rothenberg, 2012) as well as for V(D)J β -rearrangement (Wolfer et al., 2002). Hence, it was also important to discriminate between Notch1-driven effects and indirect effects mediated by pre-TCR signalling. For this purpose, I examined the effect of *Lck*-Cre-mediated deletion of *Notch1* in Rag2-deficient mice.

While thymopoiesis was blocked at the CD25^{hi} DN3a stage in *Notch1*^{fl/fl} *Rag2*^{-/-} mice, there were CD25^{int} DN3a cells in *Notch1*^{fl/fl} *Rag2*^{-/-} *Lck*-Cre animals (Fig. 2.8A). This indicated that the loss of CD25 surface expression upon *Notch1* deletion was independent of the pre-TCR signalling status. FACS-purified *Notch1*^{fl/fl} *Rag2*^{-/-} *Lck*-Cre as well as control *Notch1*^{fl/fl} *Rag2*^{-/-} DN3 cells were then analysed for deletion of the floxed *Notch1* allele and changes in gene expression levels. Deletion of *Notch1* was almost complete in CD25^{hi} DN3a cells and the floxed allele could not be detected in CD25^{int} DN3a cells by deletion PCR analysis (Fig. 2.8B). RT-qPCR analysis showed a 1.6-fold reduction of *Dtx1* mRNA in *Notch1*-deleted CD25^{hi} DN3a cells compared to control *Rag2*^{-/-} DN3a cells (Fig. 2.8C). This effect was much more pronounced in *Notch1*^{fl/fl} *Rag2*^{-/-} *Lck*-Cre CD25^{int} DN3a cells, in which the

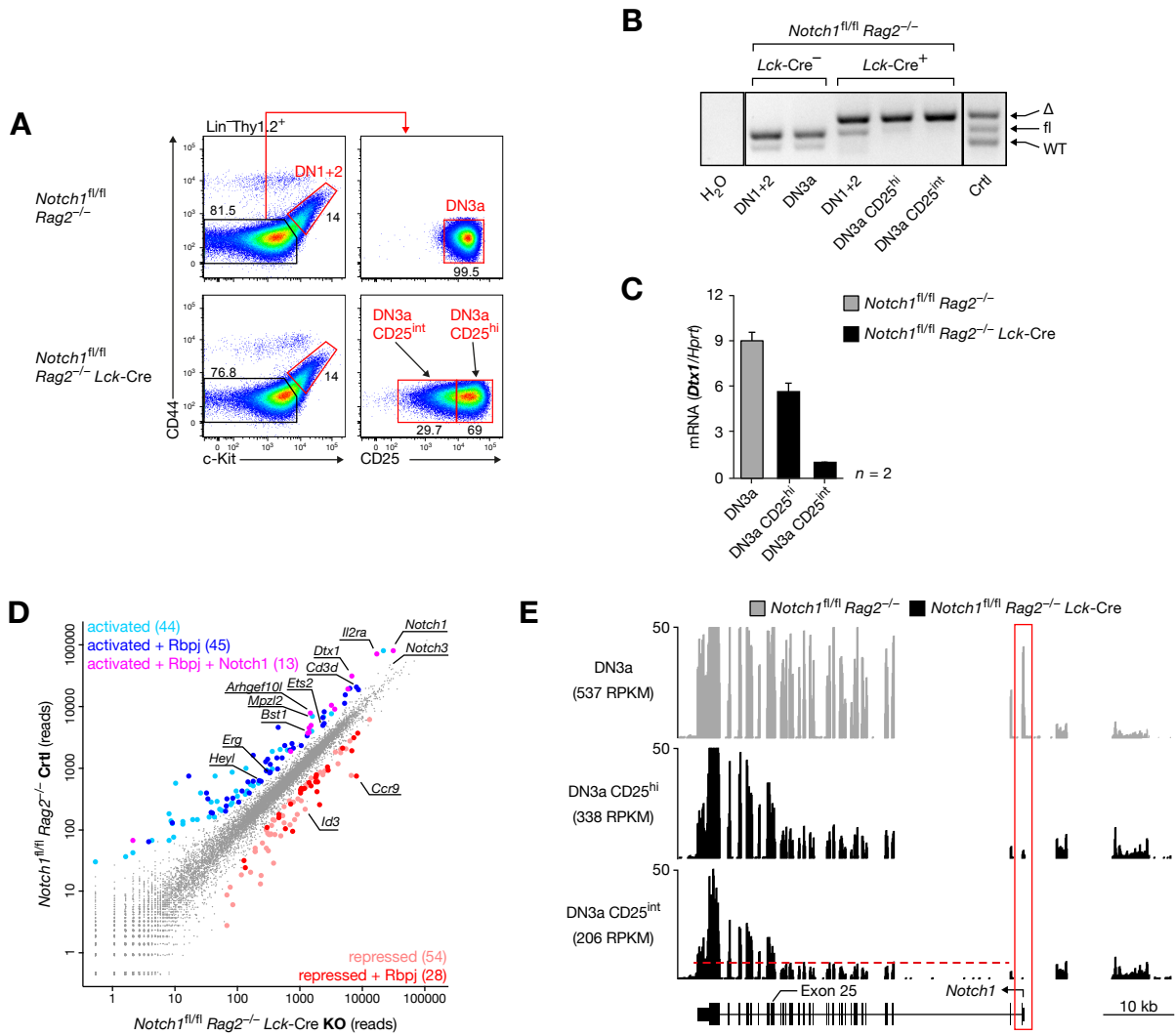


Figure 2.8 Transcriptome analysis of Notch1 and Rag2 double-deficient DN3a cells. Note that cells from *Notch1^{fl/fl} Rag2^{-/-}* and *Notch1^{fl/+} Rag2^{-/-}* mice were pooled for the *Lck-Cre⁻* control, but are referred to as *Notch1^{fl/fl} Rag2^{-/-}* for simplicity (A–E). (A) FACS analysis showing the gating strategy for the indicated thymocyte subsets from 6–8 week-old *Notch1^{fl/fl} Rag2^{-/-}* and *Notch1^{fl/fl} Rag2^{-/-} Lck-Cre* littermates. Numbers refer to percent of cells in the indicated gate. Red gates depict final sorting gates. (B) Deletion of the floxed *Notch1* allele in CD25^{hi} and CD25^{int} *Rag2^{-/-}* DN3a cells was analyzed by PCR. PCR fragments corresponding to the wild-type (wt), intact (fl) and deleted (Δ) floxed alleles are indicated. A 1:1 mix of tail DNA from *Notch1^{fl/+}* and *Notch1^{Δ/+}* mice was used as template for the control PCR. (C) *Dtx1* mRNA abundance relative to *Hprt* mRNA in *Rag2^{-/-} Notch1^{fl/fl}* (grey bars) and *Notch1^{fl/fl} Rag2^{-/-} Lck-Cre* (black bars) DN3 cells was analyzed by RT-qPCR. *n* indicates the number of experiments. Error bars indicate the SD. (D) Scatter plot visualising gene expression differences identified by RNA-seq of control *Notch1^{fl/fl} Rag2^{-/-}* DN3a cells and *Notch1^{fl/fl} Rag2^{-/-} Lck-Cre* CD25^{int} DN3a cells. See legend of Fig. 2.7 D for colour code and cutoffs. In addition, magenta dots indicate Notch1-activated genes co-occupied by Rbpj-Bio and ICN1-Bio within 50 kb of the gene, identified by Bio-ChIP-seq in DN3a cells (see Section 2.1.3). Numbers in brackets correspond to the number of genes in a given group. (E) An IGB screenshot showing RPM-normalised RNA-seq read density profiles of indicated DN3 populations from *Notch1^{fl/fl} Rag2^{-/-}* (grey) and *Notch1^{fl/fl} Rag2^{-/-} Lck-Cre* (black) mice at the *Notch1* locus. An arrow marks the TSS and filled black boxes represent exons. A red box indicates the *loxP* site flanked promoter and exon 1 region. The dashed red line demarcates the increase in the read density downstream of exon 25, which is indicated (see Section 1.3.5 and 3.2 for detail on alternative TSS of *Notch1*). The value on the y-axis represent the read density. The scale in kb is shown at the bottom right. Numbers in brackets indicate RPKM-normalised *Notch1* expression values.

Dtx1 mRNA level was reduced 8.4-fold. The relative transcript abundance of the Notch target gene *Dtx1* was higher in Rag2-deficient *Notch1^{fl/fl} Rag2^{-/-}* DN3a cells compared to Rag2-sufficient *Notch1^{fl/fl}* DN3a cells (compare Figs. 2.7C and 2.8C). This finding is in line with the hypothesis that Rag2-deficient DN3a cells might undergo higher levels of Notch signalling.

RNA-seq of CD25^{int} *Notch1^{fl/fl} Rag2^{-/-} Lck-Cre* and control *Notch1^{fl/fl} Rag2^{-/-}* DN3a cells showed that Notch1 and Rag2 double-deficiency led to a 2.6-fold reduction of *Notch1* transcript from 537 RPKM in control cells to 206 RPKM in *Notch1*-deleted cells (Fig. 2.8E). This shows that Rag2-deficient cells have an increased dynamic range for the deletion of *Notch1*, compared to the Rag2-sufficient cells, where the reduction of *Notch1* transcript was only 1.6-fold (Fig. 2.7B). Notch1 deficiency resulted in profound gene expression changes in *Rag2^{-/-}* thymocytes (Fig. 2.8D). Overall, 102 genes were found to be 2-fold or more downregulated, while 82 genes were up-regulated in Notch1 and Rag2 double-deficient CD25^{int} DN3a cells. Bio-ChIP-seq identified 4,606 Notch target genes in Rag2-deficient DN3a cells (Fig. 2.3E). Based on approximately 21,100 annotated genes, this results in a random gene-to-peak assignment with a chance of 22%. Interestingly, 58 (57%) of the 102 Notch1-activated genes are bound by ICN1-Bio and/or Rbpj-Bio in Rag2-deficient DN3a cells, while only 28 (34%) of the 82 repressed genes are bound (Fig. 2.8D). This is consistent with the notion that the function of Notch signalling is mainly transcriptional activation (Wang et al., 2013). Among the Notch1-activated genes there are many previously described Notch target genes such as *Heyl* (Buas et al., 2009), *Dtx1* (Matsuno et al., 2002), *Ptcr*a (Reizis and Leder, 2002), *Il2ra* (Deftos et al., 2000) and *Notch1* (Yashiro-Ohtani et al., 2009), which implied that the approach is valid. Furthermore, I found Notch1-activated genes known to be important for lymphocyte development, which so far were not implicated to be regulated by Notch signalling such as *Cd3d* (Tanaka et al., 1995; Wang et al., 1998), *Bst1* (Vicari et al., 1996; Itoh et al., 1998), *Ets2* (Zaldumbide et al., 2002), and *Erg* (Thoms et al., 2011). Among the genes that were derepressed upon *Notch1* deletion, *Id3* (Miyazaki et al., 2011) and *Ccr9* (Wurbel et al., 2001) are known to play important roles in T cell development. However, I did not find any prominent Rbpj-Bio peaks at *cis*-regulatory elements in the vicinity of the strongly derepressed genes such as *Ccr9* and *Id3* (data not shown). This could imply that the observed derepression is caused by indirect effects.

In a parallel approach, I wanted to study the effects of the loss of Rbpj, the DNA-binding factor of Notch signalling, in DN3 cells. *Lck-Cre* transgenic mice were crossed with wild-type (Suppl. Fig. 7.5) and *Rag2^{-/-}* (Suppl. Fig. 7.7) mice that express a conditional *Rbpj* allele in which *loxP* sites flank exon 6 and exon 7 coding for the DNA-binding domain (Han et al., 2002). On the one hand, this approach should confirm the findings made by *Notch1* deletion. On the other hand, it should provide insights concerning a potential contribution of other Notch

receptors (mainly Notch3) in transcriptional activation as well as a potential function of Rbpj as transcriptional repressor in DN3 thymocytes.

FACS analysis showed that T cell development was less compromised in *Rbpj^{fl/fl} Lck-Cre* mice compared to the observed effects upon *Notch1* deletion (Suppl. Fig. 7.5). Furthermore, DN3 cells with reduced CD25 surface levels could only be detected upon *Rbpj* deletion in *Rag2*-deficient mice and were of very low frequency (Suppl. Fig. 7.7A). The floxed *Rbpj* allele was only partially deleted in DN3a cells in both *Rbpj^{fl/fl} Lck-Cre* mice (Suppl. Fig. 7.6B) and *Rbpj^{fl/fl} Rag2^{-/-} Lck-Cre* mice (Suppl. Fig. 7.7B), while it was fully deleted in DN3b cells (Suppl. Fig. 7.6B). However, RNA-seq of *Rbpj^{fl/fl} Lck-Cre* DN3a or DN3b cell transcriptomes showed only little changes in gene expression when compared to the transcriptomes of the corresponding control cells (Suppl. Fig. 7.6). This observation might be attributed to the delayed deletion of the floxed *Rbpj* allele by *Lck-Cre* resulting in an inefficient reduction of Rbpj protein levels in DN3 cells.

2.2.4 RNA-seq of *in vitro* Notch-stimulated Vav-Bcl2 DN3a cells

To investigate the direct Notch dependency of the differentially expressed genes identified by deleting *Notch1* in DN3a thymocytes, RNA-seq of *in vitro* Notch-stimulated DN3a cells was performed. To activate Notch receptors *in vitro*, OP9-DL1, a bone-marrow derived pre-adipocyte cell line ectopically expressing the Notch ligand Delta-like 1 (DLL1), was used. It supports T cell development from bone marrow or thymic progenitors up to the Notch-independent DP and CD8SP stages (Schmitt and Zúñiga-Pflücker, 2002). The *in vitro* Notch induction system, has two main advantages compared to the *in vivo* deletion of *Notch1*: *i*) The acute manipulation of Notch signalling could reveal many direct Notch-induced transcriptome changes, because secondary Notch-indirect effects can be controlled by keeping the duration of Notch stimulation short. *ii*) Although *Notch3* expression is not required for T cell development and the thymic phenotype of *Notch1/Notch3* double-deficient mice is indistinguishable from that of *Notch1* single ko mice (Shi et al., 2011; Suliman et al., 2011), it is possible that *Notch3* partially regulates Notch-dependent gene expression in DN3a cells. Since DLL1 activates *Notch1* (Schmitt and Zúñiga-Pflücker, 2002; Mohtashami et al., 2010; Andrawes et al., 2013) and *Notch3* (Maekawa et al., 2003), this approach would assess the pan-Notch-induced molecular program.

DN3a cells are committed to the T cell lineage (Schmitt et al., 2004a; Taghon et al., 2007) and undergo high levels of Notch signalling, as they express high levels of *Notch1* receptors on their cell surface (Fig. 2.6C; Fiorini et al., 2009), and reside within the (sub-) cortical region of the thymus (Bhandoola, 2011) that has a high density of the Notch ligand DLL4 (Besseyrias

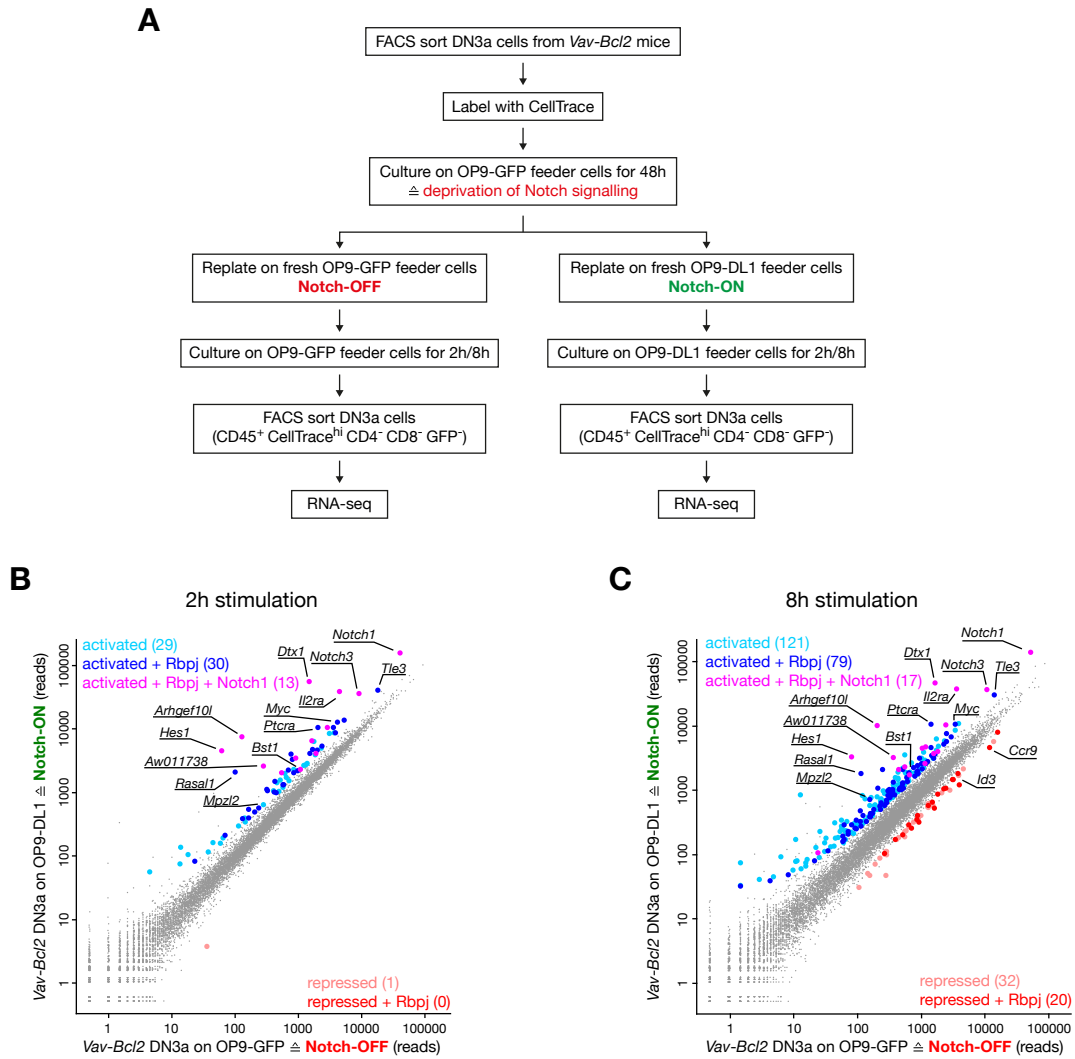


Figure 2.9 RNA-seq of Notch-stimulated *Vav-Bcl2* DN3a cells. (A) Flowchart describing the experimental design. (B,C) Scatter plot visualising gene expression differences identified by RNA-seq of *Vav-Bcl2* DN3a cells after 2 h (B) and 8 h (C) of Notch stimulation on OP9-DL1 feeder cells. Genes that are more than 2-fold differentially expressed, have an adjusted *P*-value of < 0.1 and a dispersion < 0.5, are shown in blue (Notch-activated) or red (Notch-repressed). Darker colours indicate binding of Rbpj-Bio and magenta co-occupancy of ICN1-Bio and Rbpj-Bio within 50 kb of the gene, identified by Bio-ChIP-seq in DN3a cells (see Section 2.1.3). Numbers in brackets correspond to the number of genes in a given group.

et al., 2007; Koch et al., 2008a; Yuan et al., 2011). Therefore, DN3a cells had to be deprived of Notch signalling to reduce mRNA levels of Notch-dependent target genes. It was previously shown that, in *in vitro* cultured DN3a cells, ectopic expression of *Bcl2* rescues DN3a cell survival upon loss of Notch signalling (Kreslavsky et al., 2010, 2012). Hence, *Vav-Bcl2* DN3a thymocytes were sorted, labelled with CellTrace and cultured for 48 h on OP9-GFP feeder cells (Fig. 2.9A and Suppl. Fig. 7.8A). After that, the cells were transferred either onto fresh OP9-GFP feeders (Notch-OFF) or onto OP9-DL1 feeders (Notch-ON) and co-cultured for 2 h or 8 h. Kreslavsky et al. (2010) observed that some *Vav-Bcl2* transgenic DN3a cells proliferated

and progressed to DP cells in the absence of Notch signalling in OP9-GFP co-cultures. The authors suggested that this may be the result of a sufficient level of Notch signalling, which the cells received *in vivo*. To exclude these cells, undivided CD4⁺CD8⁺ DN3a cells were sorted and prepared for RNA-seq (Suppl. Fig. 7.8C).

Transcriptome analysis of DN3a cells, co-cultured for 2 h on OP9-DL1 or OP9-GFP feeders, showed that 72 genes were more than 2-fold up-regulated upon activation of Notch signalling, while only 1 gene was repressed (Fig. 2.9B). 217 Notch-activated genes as well as 52 Notch-repressed genes were detected after prolonged stimulation of Notch for 8 h (Fig. 2.9C). Gene expression changes correlated well with increased duration of Notch activation (Suppl. Fig. 7.8D). Based on approximately 21,100 annotated genes and 4,606 potential Notch target genes identified by Bio-ChIP-seq, a random gene-to-peak assignment could occur with a chance of 22%. Strikingly, 43 (60%) of the 72 Notch-activated genes identified after 2 h of OP9-DL1 co-culture are bound by ICN1-Bio and/or Rbpj-Bio in *Rag2*^{-/-} DN3a cells. After 8 h of Notch activation, this ratio decreased to 44% (96 out of 217 genes) in the Notch-activated genes, which may indicate to potential indirect effects by other transcriptional regulators. Rbpj-Bio peaks were assigned to 38% (20 out of 53 genes) of the repressed genes. However, these Rbpj-Bio-binding sites exhibit only low occupancies in the ChIP-seq experiments and were mostly not within *cis*-regulatory elements such as promoters of the Notch-repressed genes (data not shown). This was comparable to the result obtained by the deletion of *Notch1* and could imply that the observed repression is caused by indirect regulation, for instance through the Notch-dependent activation of transcriptional repressors.

Next, I assessed the overlap of the gene sets that were identified upon the deletion of *Notch1* in *Rag2*-deficient DN3a cells and upon *in vitro* Notch stimulation of *Vav-Bcl2* DN3a cells (Fig. 2.10A). Of the total 298 Notch-activated genes, 33 genes (11%) were identified with both experimental strategies. The comparison of the fold change of gene expression between 8 h of Notch stimulation and *Notch1* deletion showed that this group contains most of the genes that show the strongest regulation upon perturbation of Notch signalling (Fig. 2.10B). The majority of the Notch-activated genes were identified either by *in vitro* Notch stimulation (196 genes; 66%) or *Notch1* deletion (69 genes; 23%; Fig. 2.10A). Many genes, especially the ones identified uniquely by *in vivo* *Notch1* deletion, exhibit a similar gene expression trend upon Notch signalling perturbation in both experiments (Fig. 2.10B). Therefore, thresholding in the RNA-seq data analysis could partially attribute to the observed discrepancy. However, genes that show a strong Notch dependency only upon *in vivo* deletion might encode stable transcripts that do not decay within 48 hours of *in vitro* Notch signalling starvation. Alternatively, their repression upon loss of Notch signalling could depend on signalling pathways or conditions that are not recapitulated in the *in vitro* culture. In contrast, genes activated only

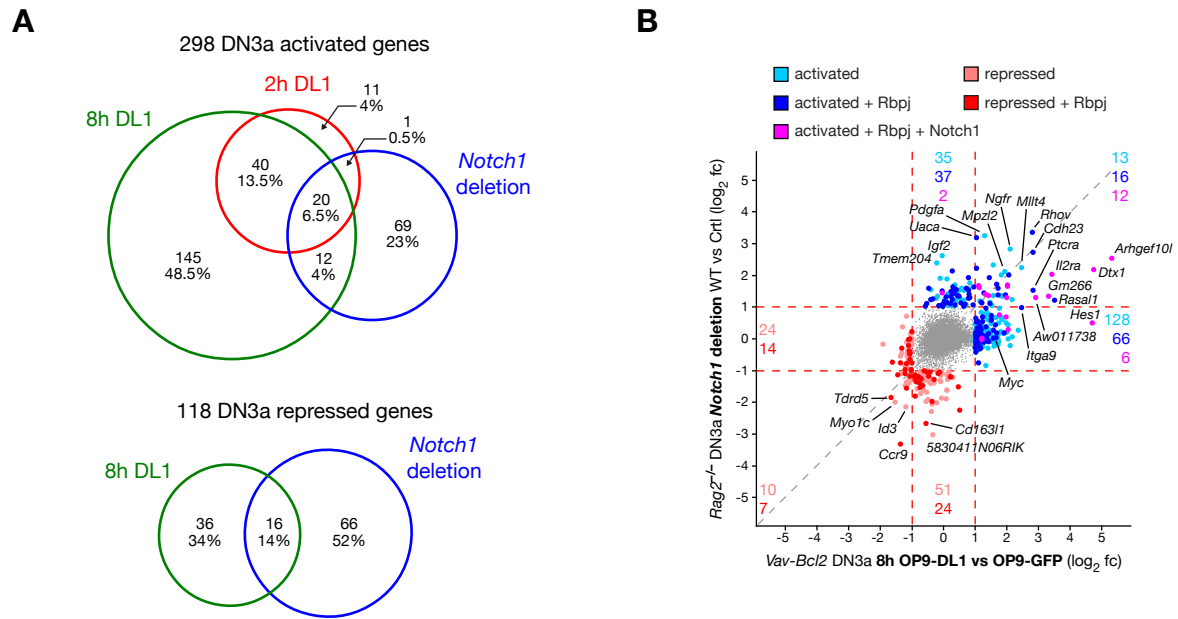


Figure 2.10 Comparison of gene expression changes in DN3a cells upon *in vitro* Notch stimulation and *in vivo* Notch1 deletion. (A) Venn diagram depicting the overlap among the activated (top) and repressed (bottom) genes identified by *Lck-Cre* mediated *Notch1* deletion in *Rag2*-deficient mice (blue) and 2 h (red) or 8 h (green) *in vitro* OP9-DL1 stimulation of *Vav-Bcl2* DN3a cells. Numbers indicate the absolute number or percentage of all regulated genes within the given subset. (B) Scatter plot visualising the fold change of gene expression identified by RNA-seq of *Rag2*^{-/-} DN3a cells after deletion of *Notch1* by *Lck-Cre* (y-axis) and 8 h Notch stimulation of *Vav-Bcl2* DN3a cell (x-axis). Genes, which are more than 2-fold differentially expressed (dashed red lines) are shown in blue (Notch-activated) or red (Notch-repressed). Darker colours indicate binding of Rbpj-Bio and magenta co-occupancy of ICN1-Bio and Rbpj-Bio within 50 kb of the gene, identified by Bio-ChIP-seq in DN3a cells (see Section 2.1.3). Numbers correspond to the number of genes in a given group. Note that the *P*-value and dispersion threshold was omitted for this correlation analysis. The dashed grey line depicts a correlation of 1.

by *in vitro* Notch stimulation might be crucial for cell survival *in vivo*. In this case, *Notch1*-deleted cells that do not bear a *Vav-Bcl2* transgene might die prior to the point where reduced transcript levels could be captured by RNA-seq. Alternatively, the high DLL1 ligand availability in culture conditions could lead to an over-activation of the Notch1 as well as the Notch3 receptor that are highly expressed in DN3 cells (Fiorini et al., 2009; Suliman et al., 2011). Consequently, DN3a cells in OP9-DL1 cell co-cultures might receive higher levels of Notch signalling than they receive *in vivo*. This could activate Notch target genes, which are not regulated at physiological Notch signalling levels at the DN3 stage. Furthermore, it has to be noted that in contrast to the *Notch1* deletion experiment, the cells used for *in vitro* Notch activation were not *Rag2*-deficient. After 8 h of Notch stimulation, some cells might have expressed a functional TCR β chain, which together with the Notch-activated expression of *Ptcra*, results in the onset of pre-TCR signalling in these cells. Genes that are detected only after 8 h of

Notch stimulation might thus be indirectly regulated by Notch signalling through activation of pre-TCR signalling.

A total of 118 genes were repressed by Notch signalling of which 16 (14%) were identified with both experimental strategies, 36 genes (34%) were only identified through *in vitro* Notch stimulation and 66 genes (52%) only by *in vivo* *Notch1* deletion (Fig. 2.10A). Derepression upon Notch signalling perturbation was stronger upon *in vivo* deletion of *Notch1* (Fig. 2.10B). In addition to the aforementioned reasons regarding the discrepancy between the results obtained by *in vitro* Notch stimulation and *in vivo* *Notch1* deletion, the stronger derepression effect observed *in vivo* can be explained by the different experimental designs. Detecting genes repressed by Notch signalling after *in vitro* OP9-DL1-stimulation depends on the reduction of a gene's mRNA, which is mainly the result of reduced gene expression in concert with mRNA decay or dilution by rounds of cell division. Hence, it is not surprising that the analysis of undivided cells after 8 h of Notch stimulation results in weaker Notch-dependent transcriptional repression compared to *in vivo* *Notch1* deletion. In addition, *Notch1* *Lck*-Cre deleted *Rag2*^{-/-} DN3a cells might have persisted for many hours or days with decreased Notch1 signalling activity. In this case, genes repressed via the Notch1 pathway would be rapidly derepressed if directly regulated by Notch1-Rbpj. Whereas, if the gene is indirectly regulated by a Notch-activated repressor, derepression would depend on the turnover of the repressor.

2.2.5 *In vivo* expression profiles across early lymphocyte development of Notch-regulated genes in DN3a cells

In the next step, I aimed to interrogate the expression of the identified Notch-regulated genes in the course of early T cell development. To this end, the transcriptome profiles of FACS-purified LMPPs from bone marrow, as well as DN1, DN2a and DN2b cells from thymi of wild-type mice were investigated by RNA-seq (Fig. 2.11A). For the subsequent thymocyte stages I used the transcriptome profiles of DN3a and DN3b cells, which were generated as controls for the conditional deletion of *Notch1* (Fig. 2.6), *Rbpj* (Suppl. Fig. 7.5) and *Gata3* (Busslinger lab, unpublished). In addition, I included transcriptome profiles of the CLP subsets ALP and BLP (Revilla-I-Domingo et al., 2012). ALPs and LMPPs are the likely candidates for the yet to be identified adult thymus-seeding cells (Ceredig, 2012; Ramond et al., 2014). To test if these datasets from different experiments can be combined to delineate the expression of Notch-regulated genes through lymphocyte development, all profiles were compared to each other in a RPKM-based principal component analysis (PCA) together with transcriptomes of pro-B and pre-B cells (Busslinger lab, unpublished; Schwickert et al., 2014) that served together with the BLP as non-T cell lineage controls (Fig. 2.11B). B and T lineages separated largely on the

principal components (PC) 1 and 2, respectively. The separation began at post ALP stages, since DN1 and BLP samples already clustered apart from each other. Subsequent T cell maturation stages clustered in a nearly linear fashion up to the DN3b stage, largely described by PC2 and 3. Taken together, this indicates that the combination of RNA-seq data from different experiments is legitimate to survey the expression profile throughout early lymphocyte development.

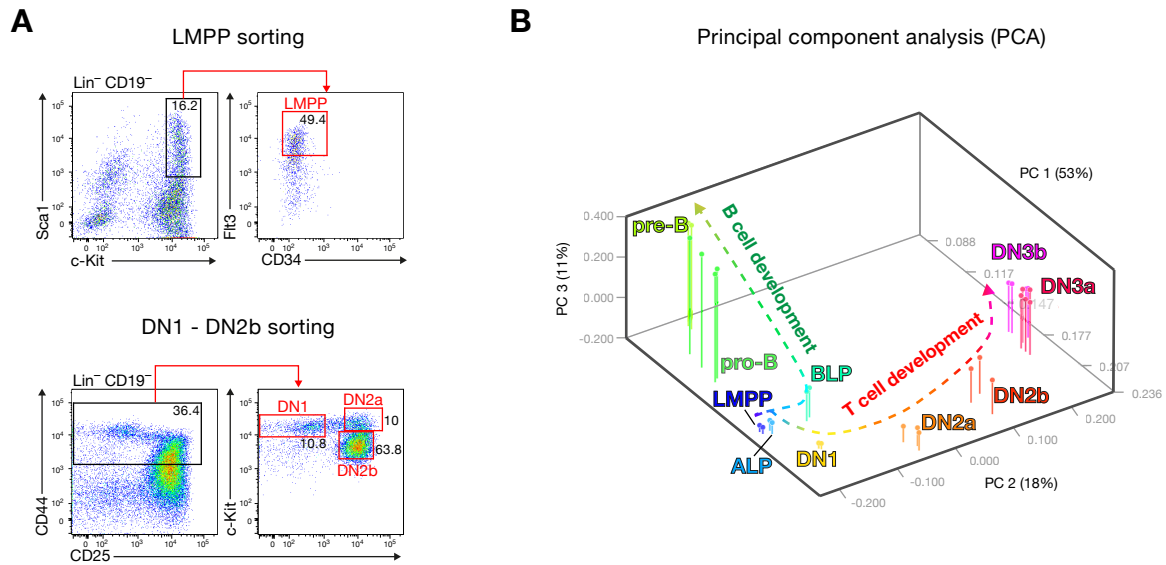


Figure 2.11 Transcriptome analysis of bone marrow and thymic progenitors. (A) FACS analysis showing the gating strategy for LMPP cells (top) and DN1, DN2a, DN2b thymocytes from 6-8 week-old wild-type mice. Numbers refer to percent of cells in the indicated gate. Red gates depict final sorting gates. (B) Principal component analysis of RNA-seq data obtained with the subsets of bone marrow and thymus populations. Arrows indicate the T and B cell lineage development. Numbers on the axis present percentage of the variance of gene expression that is captured by the indicated principal component.

In the subsequent step, the identified Notch-regulated genes were grouped into Notch-repressed or Notch-activated. The latter group was further subdivided whether or not a gene qualified as direct Notch target gene, based on the Rbpj-Bio-ChIP-seq analysis in *Rag2*^{-/-} DN3a cells (see Section 2.1.3). Next, hierarchical clustering of the genes within each group was performed. Clustering was based on the expression profile of the genes from the LMPP to the DN3b stage and the results were visualised in a heatmap. There were four main clusters among the Notch-activated and bound genes (Fig. 2.12A). Genes in cluster B1 (bound) reached their expression maximum prior to the DN3a stage and several genes within this cluster as well as in cluster B3 showed a noisy expression profile. This was due to a low overall expression within the thymic DN1-DN3b stages.

Cluster B2 (bound2) comprised most of the previously identified canonical Notch targets such as *Hes1*, *Dtx1*, *Notch1*, *Notch3*, *Il2ra* and *Ptcrca*. Most of the genes within this cluster were upregulated upon both *in vivo* *Notch1* deletion and *in vitro* Notch stimulation, which identified

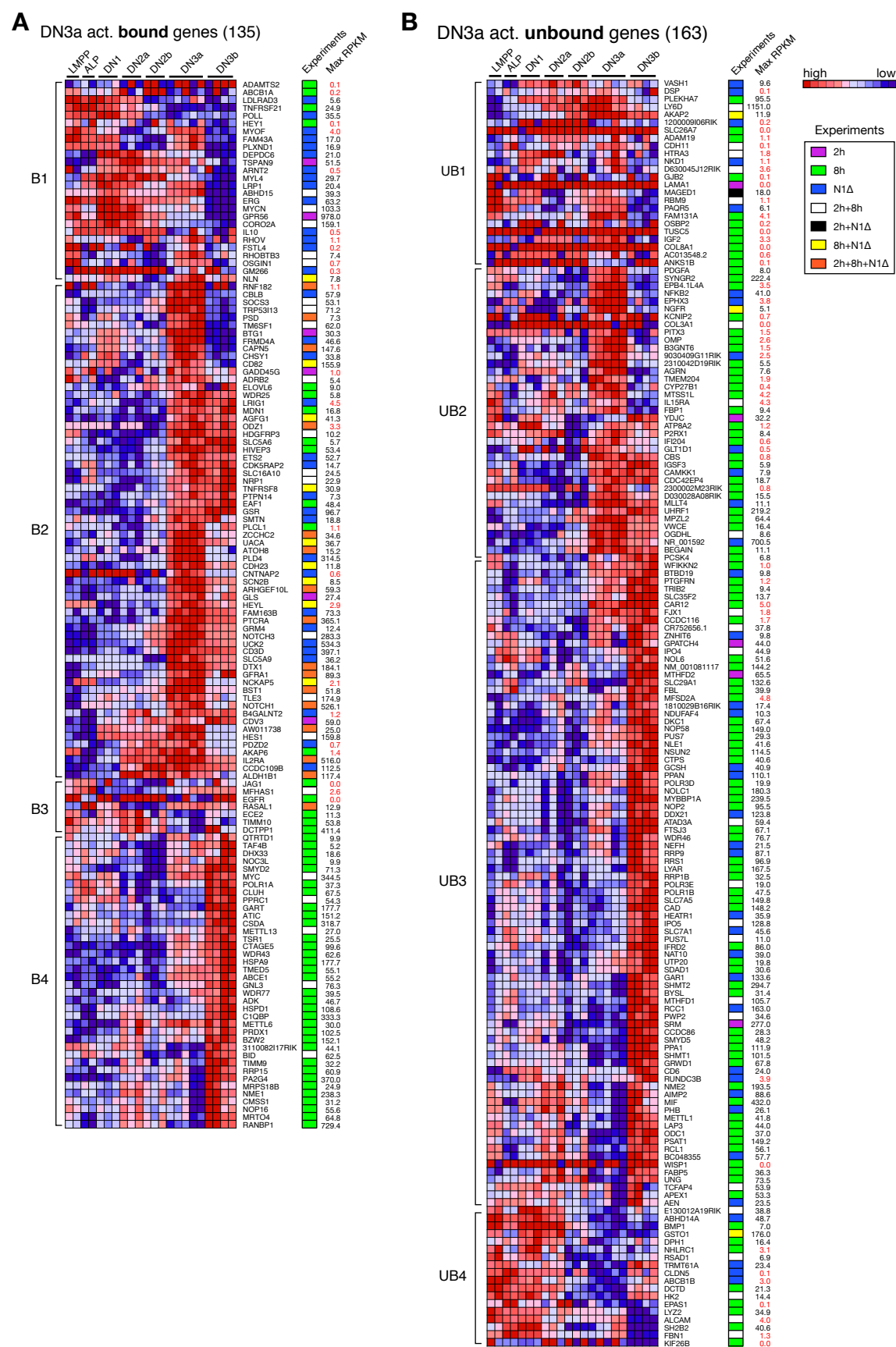
these genes as *bona fide* Notch target genes. Expression of the genes found in cluster B4 was upregulated in the DN3a to the DN3b transition, similar to the genes in the largest cluster of the unbound genes UB3 (unbound3; Fig. 2.12B). Most of the genes within these two clusters were identified only after 8 h of *in vitro* Notch stimulation and might therefore be a result of Notch signalling in cooperation with the aforementioned onset of pre-TCR signalling. *Myc* is known to be a Notch-regulated direct target gene (Weng et al., 2006) and it was detected after 2 h and 8 h of Notch activation. Hence, in particular the genes that were also identified after 2 h of Notch stimulation and/or by *Notch1* deletion qualify as direct Notch targets. The fact that these Notch targets exhibit a delayed *in vivo* expression profile compared to most of the canonical Notch target genes (cluster B2) might indicate the requirement of additional factors, such as pre-TCR signalling, in cooperation with Notch signalling to induce their expression.

Moreover, most of the non Rbpj-Bio bound genes in the clusters UB1, UB2 and UB4 are expressed at very low levels during thymic DN1-DN3b stages. Some of the genes within these clusters like *Ly6d*, *Akap2* and *Ngfr* have an expression profile reminiscent to that of canonical Notch target genes and were identified in more than one experiment. Since these genes did not qualify as direct Notch targets based on the Rbpj-Bio-ChIP-seq in DN3a cells, expression of these genes might depend on long-range interactions with Notch-controlled *cis*-regulatory elements that are not captured by our peak-to-gene assignment. Alternatively, these genes might also be regulated indirectly through a Notch-regulated transcription factor that is rapidly upregulated upon Notch activation.

Most of the identified Notch-repressed genes were expressed at low levels throughout the assessed thymocyte stages, as their expression level in thymic DN1 to DN3a stages was low (Suppl. Fig. 7.9). Several genes were downregulated upon thymus seeding (LMPP/ALP to DN1) or upon transition to later stages. This expression profile is reciprocal to the described pattern of Notch signalling strength throughout early T cell development and indicates a negative regulation of these genes by Notch signalling. However, if this is due to direct repression

Figure 2.12 (following page) Expression profiles through early lymphocyte development of genes activated by Notch signalling in DN3a cells. (A,B) Heatmaps showing the expression profiles of Notch-regulated genes that are bound by Rbpj-Bio in *Rag2*^{-/-} DN3a cells (A) and those Notch-regulated genes to which no Rbpj-Bio-binding site was assigned to (B). Hierarchical clustering by pairwise average-linkage was performed on log-transformed RPKM expression values of LMPP, ALP, DN1, DN2a, DN2b, DN3a and DN3b cells. The number of columns equals the number of independent RNA-seq samples per cell type. The distance between rows (genes) was measured by Pearson correlation. Expression values are row-normalised. Brackets on the left depict a strongly simplified clustering tree. The colour code on the right side depicts, in which experiments (Exp.) the gene was identified (2/8 h = *in vitro* Notch stimulation on OP9-DL1 feeders; N1Δ = *in vivo* *Notch1* deletion by *Lck*-Cre in *Rag2*-deficient mice). Max indicates the maximum RPKM expression value of a gene within the DN1 to DN3b stages. Values are averages of all samples of one population, and values below 5 RPKM are red.

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by Notch signalling through ICN/Rbpj complexes or due to an indirect regulation, for instance by Notch-dependent activation of transcriptional repressors, remains to be determined.

2.2.6 Functional classification of Notch-activated target genes identified in DN3a cells

To gain further insight into how Notch controls T cell differentiation at the DN3 stage, the Notch-activated target genes were grouped according to their function based on a PubMed search. Only the genes with a minimum expression of more than 2 RPKM at any of the thymic DN1 to DN3b stages were included, since these genes are more likely to be biologically significant during early T cell development. These 117 Notch-activated direct target genes comprised several different functional classes, while more than 50% of all genes belonged to the following four classes (Fig. 2.13): Transcriptional regulators represent the largest group with 20 genes. 17 of the identified Notch-activated genes encode surface receptor molecules, another 16 genes encode proteins with functions during cell metabolism and 14 encode signal transducers.

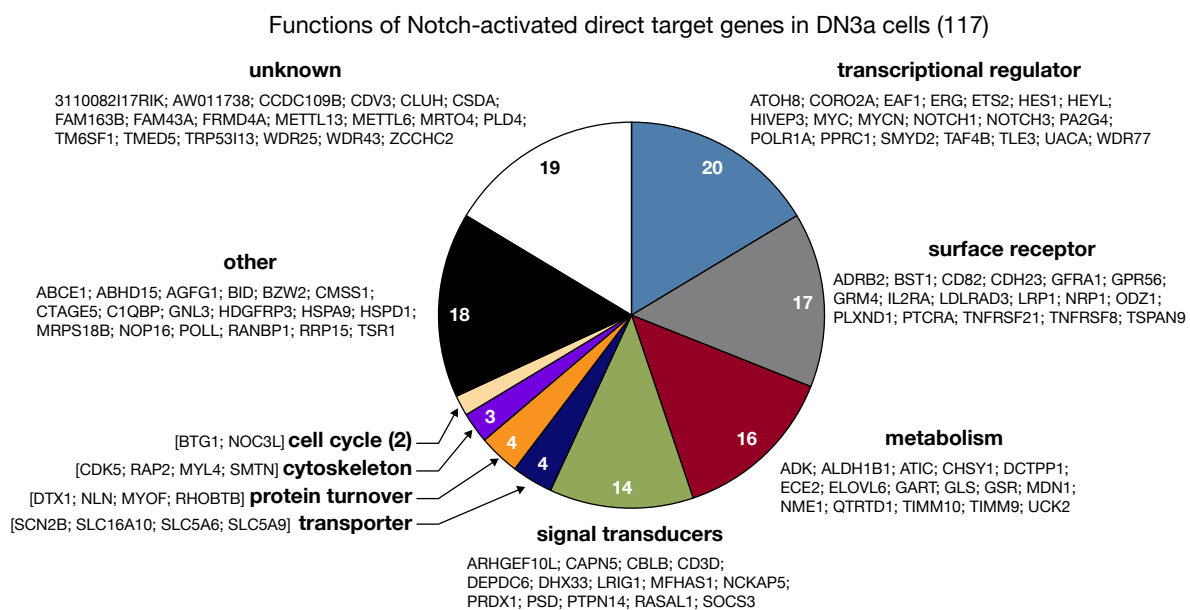


Figure 2.13 Functions of Notch-activated target genes identified in DN3a cells. A Pie diagram representing the different functional classes of the identified Notch-activated target genes in DN3a cells. Numbers indicate the absolute number of genes in the according section of the pie chart. Only Notch-activated and Rbpj-Bio bound genes with a minimum RPKM level > 2 at any of the thymic DN1-DN3b stages were included.

Several known Notch-target genes like *Hes1*, *Dtx1*, *Myc*, *Notch1*, *Notch3*, *Il2ra* and *Ptrca* were identified, which proves that this approach is valid. More importantly, among the 117 Notch-activated target genes, 93% were previously not implicated as being regulated by Notch signalling during early T cell development.

2.3 Dissection of the Notch-induced molecular programme in DN4 thymocytes

2.3.1 *In vivo* generation of a synchronous DN4 population by CD3 ϵ antibody injection

Expression of the pre-T cell receptor upon productive rearrangement of the *Tcrb* locus results in a burst of proliferation and in progression via DN3b and DN4 stages to the CD4⁺CD8⁺ double-positive (DP) stage. During this process, which is termed β -selection, Notch signalling cooperates with the pre-TCR in driving proliferation and progression to the Notch-independent DP stage (Ciofani et al., 2004; Maillard et al., 2006). Here, proliferation is the key function of Notch, as a block in proliferation leads to a complete arrest of differentiation and its ectopic activation can partially rescue DP progression in the absence of Notch signalling (Kreslavsky et al., 2012). Hence, Notch signalling plays rather distinct roles in pre-TCR⁺ DN3b and DN4 thymocytes compared to quiescent pre-TCR⁻ DN3a cells, where Notch is required for survival and rearrangement of *Tcrb* (Wolfer et al., 2002). To investigate if these specific outputs of the Notch signalling pathway are the result of the control of distinct target gene sets in pre- and post- β -selection thymocytes, I defined the genome-wide Rbpj occupancy in DN4 cells.

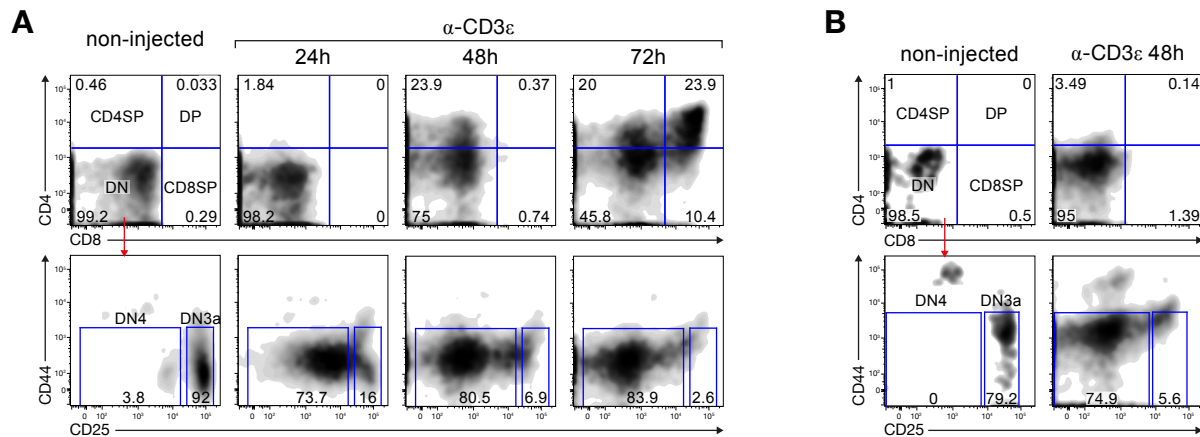


Figure 2.14 *In vivo* stimulation of *Rag2*-deficient mice with CD3 ϵ antibody. (A,B) FACS analysis of thymocytes from 6-8 week-old *Rag2*^{-/-} (A) and *Rbpj*^{Bio} *Rag2*^{-/-} (B) mice at indicated time points after intraperitoneal injection of 300 μ g CD3 ϵ antibody. Numbers refer to percent of cells in the indicated gate.

Like in the case of DN3a cells, DN3b and DN4 cells are of low frequency in wild-type mice, which renders genome-wide analysis of transcription factor-binding studies challenging in these cell types. Crosslinking of the CD3 complex by CD3 ϵ antibody into *Rag*-deficient mice efficiently mimics pre-TCR signalling and allows the generation of a largely uniform pool of DN4 cells (Shinkai and Alt, 1994). The kinetics of this process were assessed in order

to identify the time point after injection with the highest proportion of DN4 cells. *Rag2*^{-/-} mice were injected intraperitoneally with 300 µg CD3ε antibody and thymocyte compartments were analyzed 24 h, 48 h, and 72 h later by FACS (Fig. 2.14A). While 24 h post injection some of the thymocytes still had a DN3 phenotype, 48 h post injection, most thymocytes progressed to the DN4 cell stage and did not reach the DP cell stage yet.

2.3.2 Binding of Rbpj-Bio in DN4 cells is restricted to a subset of its binding sites in DN3a cells

CD3ε antibody was injected into a cohort of *Rbpj*^{Bio} *Rag2*^{-/-} mice to promote developmental progression of blocked DN3a cells to the DN4 stage. Thymocyte suspensions were prepared 48 h post injection, immediately fixed with FA and analyzed by FACS (Fig. 2.14B). The genome-wide occupancy of Rbpj-Bio was determined by Bio-ChIP-seq. Prior to deep sequencing the quality of the ChIP sample was assessed by qPCR to determine the enrichment of known Rbpj-binding sites (Fig. 2.15A). Peak calling of the aligned Bio-ChIP-seq reads identified 351 peaks (P -value < 10^{-10}). 149 (48%) of the peaks contained a motif similar to the Rbpj consensus motif identified in DN3a cells, indicating that these peaks originate from specific binding of Rbpj to chromatin (Fig. 2.15B). Most of the remaining peaks originated from ChIP-seq artefact regions or were only lowly enriched (data not shown) and were therefore excluded from further analysis. An overlap analysis of the consensus motif-containing Rbpj-Bio-binding sites in DN3a and DN4 cells showed that 94% of the binding sites identified in DN4 cells were also present in DN3a cells (Fig. 2.15C). In order to assess the differential binding of Rbpj between DN3a and DN4 cells, the read densities within all identified Rbpj-Bio-binding sites were calculated in both cell types. In line with the decreased number of binding sites in DN4 cells compared to DN3a cells, the read densities were lower in the vast majority of the detected Rbpj-Bio-binding sites (Fig. 2.15D). Furthermore, this analysis also showed that most of the DN4 unique binding sites also contained relatively high read densities in DN3a cells, even though they did not meet our peak-calling criteria in DN3a cells. While Rbpj binding was hardly detectable by Bio-ChIP-seq on *Gm266* and *Ptcra*, genes that are regulated by Notch signalling in DN3a cells, there were also prominent Rbpj-binding sites in DN4 cells like in the intronic regions of *Il2ra* or *Ms4a6d*, validating comparable qualities of the DN3a and DN4 Bio-ChIPs (Fig. 2.15F). Binding sites with higher Rbpj-Bio occupancy in DN4 cells than in DN3a cells indicated that the observed reduction in Rbpj-Bio-binding sites is not a technical artefact. Consequently, the results imply a decreased binding capacity of Rbpj-Bio after β -selection, which ultimately results in binding of Rbpj-Bio to a small subset of the sites that are also occupied in DN3a cells while no novel binding sites emerge at this developmental stage.

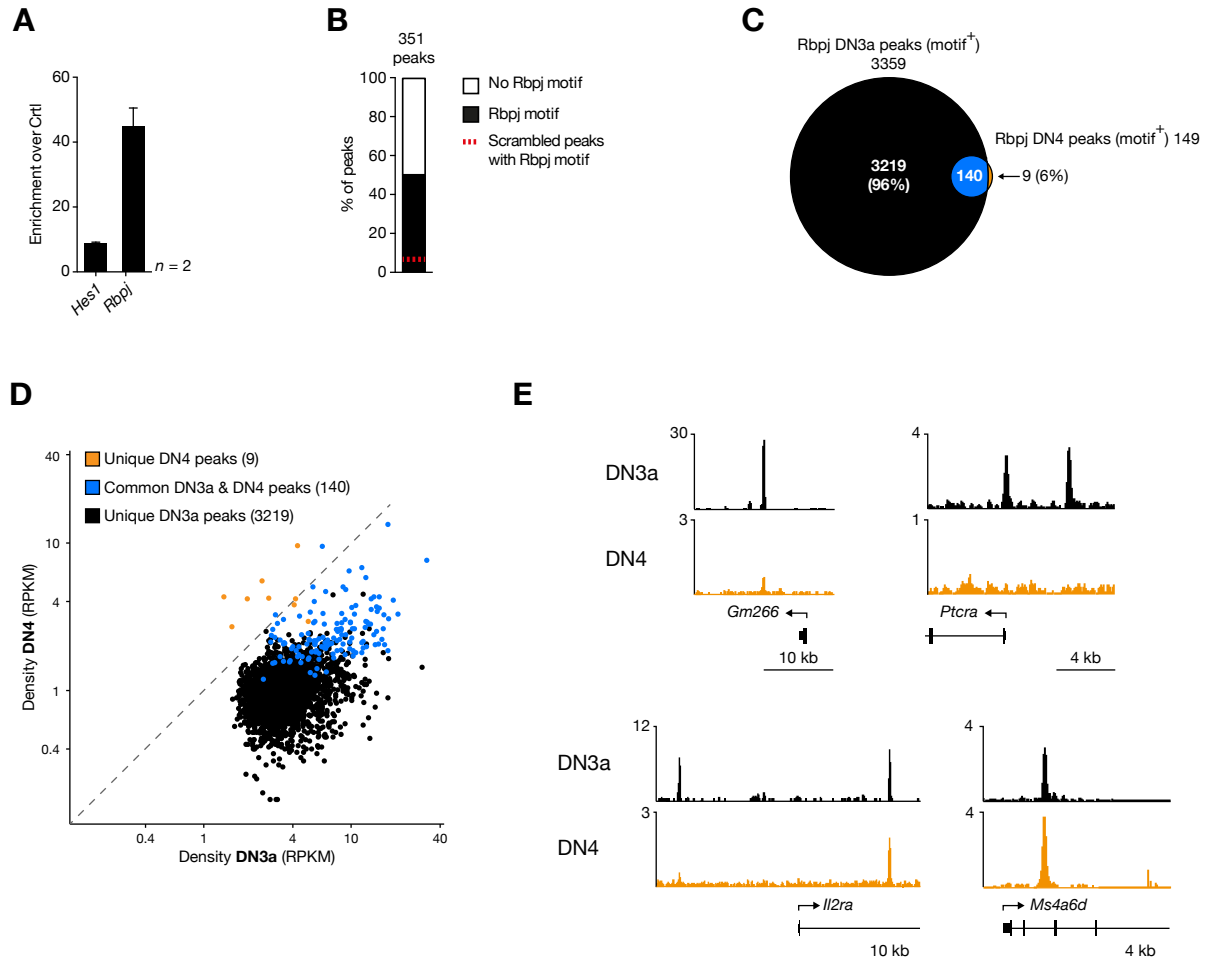


Figure 2.15 Rbpj Bio-ChIP-seq in DN4 thymocytes. (A) qPCR analysis of Rbpj-Bio-ChIP efficiency. Input and precipitated DNA were quantified by qPCR with primer pairs amplifying *Hes1* and *Rbpj* promoter regions with known Rbpj-binding sites and two negative control regions. The relative enrichment over the average of the two negative control regions is shown. n indicates the number of experiments and error bars indicate the SD. (B) Bar chart representing the ratio of the 351 identified Rbpj-Bio peaks containing the Rbpj consensus motif identified in DN3a cells (Fig. 2.3B). The same motif was found in random DNA sequences at the frequency indicated by a dashed red line. (C) Venn diagram indicating the number of Rbpj consensus motif-containing Rbpj-Bio sites that are common in DN3a and DN4 cells (blue) or unique for either DN3a (black) or DN4 cells (orange). Numbers indicate the total numbers of binding sites per subset and percentages in brackets represent the relative number of unique binding sites. (D) Scatter plot representing the RPKM-normalised read densities of all Rbpj consensus motif-containing Rbpj-Bio peaks identified in DN3a and DN4 cells. Colour code is the same as in panel C. (E) Rbpj-Bio-ChIP-seq read density profiles from DN3a (black) and DN4 cells (orange) at indicated genomic loci. The value on the y-axis represents the RPM-normalised read density of the given track. Arrows mark the TSS, and filled black boxes represent exons. The scale in kb is shown at the bottom right of each panel.

2.3.3 The Notch-regulated target genes in DN4 cells are a small subset of the targets in DN3a cells

Even though no newly emerging Rbpj-Bio-binding sites were detected in DN4 cells by ChIP-seq, the regulation of genes other than the ones controlled by Notch in DN3a cells could not be excluded so far. For instance, Notch-regulated *cis*-regulatory elements could interact with promoters of different genes in DN3a and DN4 cells resulting in the activation of genes unique for DN4 cells. To determine the Notch-regulated genes in DN4 cells, the transcriptome of *in vitro* Notch-activated post- β -selection thymocytes was determined by RNA-seq, identical to the way that it was done with DN3a cells (see Section 2.2.4). Briefly, FACS-purified DN3b and DN4 cells (thereafter referred to as DN4 cells for simplicity; Suppl. Fig. 7.8A,B) from *Vav-Bcl2* transgenic mice were 'parked' on OP9-GFP feeder cells for 48 h, before Notch signalling was either activated *in vitro* for 2 h after transfer onto OP9-DL1 cells or remained inactive in Notch signalling-deprived DN4 cells that were transferred onto fresh OP9-GFP feeders (Fig. 2.16A).

RNA-seq analysis of 2 h Notch-stimulated and control DN4 cells showed that Notch signalling activated only nine genes more than 2-fold (Fig. 2.16B). Comparison of the gene expression changes of OP9-DL1-stimulated *Vav-Bcl2* DN3a and DN4 cells showed that the Notch-regulated genes at the DN4 stage are a subset of the Notch-targets in DN3a cells and that activation of target gene expression upon Notch stimulation was weaker in DN4 cells (Fig. 2.16C). This was in line with reports describing reduced Notch signalling activity in DN4 cells (Kleinmann et al., 2008). Six of the Notch-regulated genes in DN4 cells also contained an assigned Rbpj-Bio-binding site in DN4 cells and, hence, are considered to be direct Notch target genes in this cell type. However, *Myc*, *Rasall* and *Ptcra* exhibited DN3a-specific Rbpj-Bio-binding and might be regulated in DN4 cells either via regulatory elements, at which Rbpj-Bio-binding was not detected by Bio-ChIP-seq in DN4 cells or via enhancers that are not captured by our peak-to-gene alignment algorithm.

In summary, even though pre-TCR-expressing DN4 thymocytes carry high levels of Notch1 protein on their cell surface (Fig. 2.6C; Fiorini et al., 2009), Notch signalling is known to decrease in these cells, yet Notch signalling has a mitogenic function in DN4 thymocytes (Kreslavsky et al., 2012). The combination of Rbpj-Bio-ChIP-seq and RNA-seq approaches with Notch-activated pre- and post- β -selection thymocytes showed that lower Notch signalling levels in post- β -selection cells resulted in the genome-wide reduction of Rbpj occupancy and an absence of novel Rbpj-binding sites. Together, this might explain the observed decrease in multitude and magnitude of Notch target gene regulation in DN4 cells.

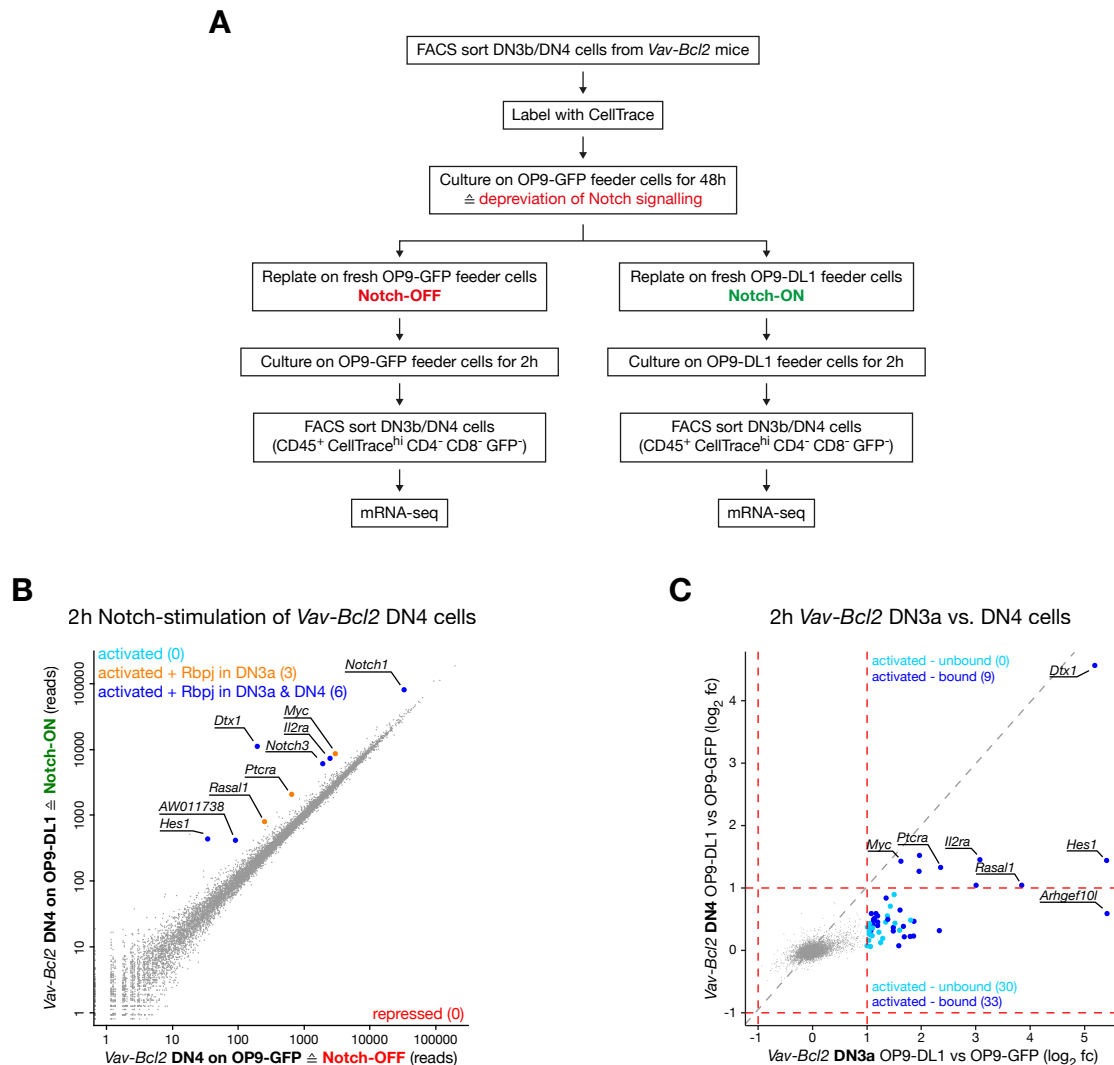


Figure 2.16 RNA-seq of Notch-stimulated *Vav-Bcl2* DN4 cells. (A) Flowchart describing the experimental design. (B) Scatter plot visualising gene expression differences identified by RNA-seq of *Vav-Bcl2* DN4 cells after 2 h of Notch stimulation on OP9-DL1 feeder cells. Genes that are more than 2-fold differentially expressed and have an adjusted *P*-value of < 0.1 and a dispersion < 0.5 , are shown in blue (Notch-activated) or red (Notch-repressed). Dark blue and orange colours indicate binding of Rbpj-Bio within 50 kb of the gene, identified by Bio-ChIP-seq in DN3a (see Section 2.1.3) and DN4 cells (Fig. 2.15). Numbers in brackets correspond to the number of genes in a given group. (C) Scatter plot visualising the moderated $\log_2$ fold change gene expression differences identified by RNA-seq of *Vav-Bcl2* DN4 (y-axis) and *Vav-Bcl2* DN3a cells (x-axis) after 2 h of Notch stimulation on OP9-DL1 feeder cells. Genes, that are more than 2-fold differentially expressed (dashed red lines), are shown in blue (Notch-activated) or red (Notch-repressed). Darker colours indicate binding of Rbpj-Bio within 50 kb of the gene, identified by Bio-ChIP-seq in DN3a cells (see Section 2.1.3). Numbers correspond to the number of genes in a given group. The dashed grey line depicts a correlation of 1.

2.4 Dissection of the Notch-induced molecular programme in uncommitted lymphoid progenitor cells

2.4.1 Notch1-dependent binding of Rbpj in Pax5-deficient pro-B cells

Notch signalling is essential during T cell lineage specification and commitment in early T cell progenitors (Radtke et al., 1999; Han et al., 2002; Feyerabend et al., 2009; Rothenberg, 2014). So far, only few genes were shown to be directly regulated by Notch signalling in uncommitted T cell progenitors (Jarriault et al., 1995; Weber et al., 2011; De Obaldia et al., 2013b; Rothenberg, 2014). Furthermore, there is no report of a systematic approach aiming to identify direct Notch targets in uncommitted progenitors. This could be explained by the lack of appropriate tools for Notch or Rbpj ChIP-seq studies. Hence, having access to the powerful Bio-ChIP-seq system for both factors, I aimed to delineate the directly Notch-induced molecular program in uncommitted T cell progenitors in a genome-wide manner. However, there were two main problems. Firstly, uncommitted T cell progenitor populations are very scarce in wild-type mice and unlike for DN3 cells, there is no system to generate sufficient amounts of uncommitted T cell progenitors for an *in vivo* ChIP-seq approach. Secondly, the usage of Cre lines to study gene expression changes upon deletion of *Notch1* in uncommitted T cell progenitors is problematic due to several factors. On the one hand, there are hardly any Cre lines available that allow an efficient manipulation of gene expression at the appropriate stage in the early T cell compartment. On the other hand, uncommitted T cell progenitors rapidly differentiate into non-T cell lineages upon disruption of Notch signalling. This was seen after the deletion of *Notch1* by *Cpa3*-Cre (Feyerabend et al., 2009) or by *Rag1*-Cre (my own observation; McCormack et al., 2003). Both Cre lines are known to delete in the earliest thymocytes (Feyerabend et al., 2009; Malin et al., 2010). Pax5-deficient pro-B cells have the potential to efficiently differentiate along the T cell lineage *in vivo* (Rolink et al., 1999) as well as *in vitro*, when cultured on OP9-DL1 feeder cells (Höflinger et al., 2004). Therefore, this system was used to determine the genome-wide occupancy of ICN1 and Rbpj. To this end, *Notch1*^{Bio/Bio} *Rosa26*^{BirA/BirA} *Pax5*^{Δ/Δ} *Vav-Bcl2* (thereafter referred to as *Notch1*^{Bio} *Pax5*^{Δ/Δ}) and *Rbpj*^{Bio/Bio} *Rosa26*^{BirA/BirA} *Pax5*^{Δ/Δ} (thereafter referred to as *Rbpj*^{Bio} *Pax5*^{Δ/Δ}) mice were generated, in which the *Pax5* gene is deleted (see Materials and Methods for details). Pax5-deficient pro-B cells proliferate *in vitro* on OP9 stromal cells in the presence of IL7 and express *Notch1*, because Pax5 is required for the repression of *Notch1* in the B cell lineage (Souabni et al., 2002; Pridans et al., 2008). The *Vav-Bcl2* transgene was used, as it was shown to partially rescue apoptosis of *Pax5*-mutant pro-B cells upon long-term induction of Notch signalling in OP9-DL1 co-culture conditions (Dorninger, 2008).

In a first step, the optimal conditions for Bio-ChIP-seq experiments were determined. To this end, the kinetics of ICN1-Bio recruitment to known ICN1/Rbpj-binding sites upon Notch1 activation were assessed in *Notch1^{Bio} Pax5^{Δ/Δ}* pro-B cells by Bio-ChIP followed by qPCR. ICN1-Bio-binding to the *Hes1* and *Notch3* promoters could be detected after 0.5 h of OP9-DL1 co-culture, reached its maximum after 1.5 h and decreased thereafter (Suppl. Fig. 7.10A). ICN1/Rbpj complexes are inherently linked to proteasomal degradation after activation of transcription. Hence, the turnover of ICN1 in combination with a synchronous wave of Notch receptor activation could result in cyclic binding of ICN1 in conditions where the availability of non-activated surface Notch receptors is limiting. Surprisingly, Rbpj-Bio-binding in *Rbpj^{Bio} Pax5^{Δ/Δ}* pro-B cells to Notch target genes showed similar dynamics (Suppl. Fig. 7.10B). This observation favours a model of Notch-induced binding of Rbpj, which was also recently put forward by studies in myoblasts (Castel et al., 2013) and T-ALL cell lines (Wang et al., 2013).

Next, potential Notch target genes as well as the dynamic binding of Rbpj in uncommitted lymphoid progenitors were investigated by genome-wide Bio-ChIP-seq with *Notch1^{Bio} Pax5^{Δ/Δ}* and *Rbpj^{Bio} Pax5^{Δ/Δ}* pro-B cell lines. MACS peak calling (P -value $< 10^{-10}$) identified 241 Rbpj-Bio peaks before Notch stimulation and approximately 10 times more peaks after short-term (0.5 h) Notch stimulation (Fig. 2.17A). ICN1-Bio ChIP-seq identified 2730 peaks after 1 h OP9-DL1 stimulation, of which one third overlapped with Rbpj-Bio-binding sites. The surprisingly small overlap between the identified ICN1-Bio and Rbpj-Bio-binding sites was largely due to thresholding in the peak calling analysis, as post-stimulation ICN1-Bio and Rbpj-Bio density profiles across all detected binding sites showed a high correlation (Fig. 2.17C). In total, ICN1/Rbpj-Bio complexes were found to occupy approximately 4,600 sites genome-wide and binding of Rbpj-Bio was largely dependent on the Notch signalling status in Pax5-deficient pro-B cells.

There were few sites, at which binding of Rbpj-Bio was independent of Notch signalling, such as at the *Rbpj* promoter (Fig. 2.17B, left). However, Bio-ChIP-seq density profiles across almost all 4,600 ICN1-Bio and Rbpj-Bio-binding sites showed increased Rbpj-Bio occupancy after Notch stimulation (Fig. 2.17C). The heatmap visualisation as well as the composite density plots of the Bio-ChIP-seq signals show that ICN1 and Rbpj co-occur at identical positions within each binding site, as it is expected for ICN1/Rbpj transcription-activating complexes. Furthermore, these profiles show that the Notch-dependency of Rbpj-Bio-binding has a wide dynamic range. Some binding sites were highly Notch-dependent such as at the 3' end of *Bcl11a*, where Rbpj-Bio was only detected by Bio-ChIP-seq upon Notch stimulation (Fig. 2.17B, right). While most sites also showed a low level of Rbpj-Bio occupancy under non-stimulated conditions that increased after Notch activation such as at the *Ddx6* gene (Fig. 2.17B, middle).

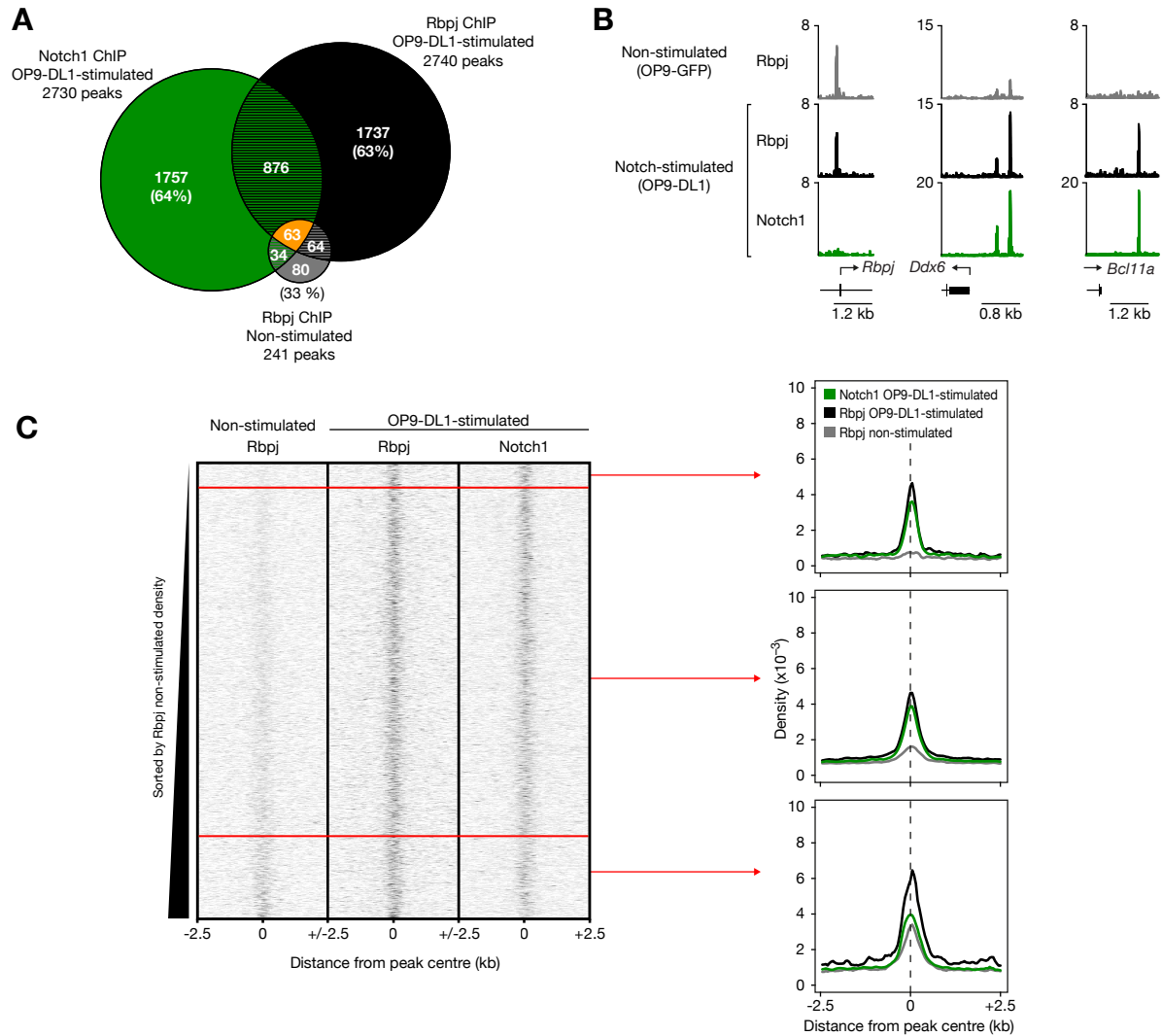


Figure 2.17 Notch-dependent binding of Rbpj-Bio in Pax5-deficient pro-B cells. (A) Venn diagram representing the overlap of the ICN1-Bio (green) and Rbpj-Bio (black) binding sites identified by Bio-ChIP seq in OP9-DL1-stimulated *Notch1*^{Bio} *Pax5*^{Δ/Δ} and *Rbpj*^{Bio} *Pax5*^{Δ/Δ} pro-B cells, respectively, together with the Rbpj-Bio-binding sites determined by Bio ChIP-seq before Notch stimulation (on OP9-GFP cells; grey). Numbers refer to identified peaks and percentages indicate the ratio of unique peaks. Note that slight peak number discrepancies between all identified peaks, overlapping peaks and unique peaks result from single peaks that overlap with two or more peaks in another sample. (B) Rbpj-Bio and ICN1-Bio read density tracks generated from *Rbpj*^{Bio} *Pax5*^{Δ/Δ} and *Notch1*^{Bio} *Pax5*^{Δ/Δ} pro-B cells cultured under the indicated conditions. The indicated genomic loci represent examples of Notch1-independent to highly Notch1-dependent Rbpj-Bio-binding sites (from left to right). The value on the y-axis represents the RPM-normalised read density of the given track. Arrows mark the TSS and filled black boxes represent exons. The scale in kb is shown at the bottom right of each panel. (C) A heatmap depicts the read densities of the Rbpj-Bio and ICN1-Bio Bio-ChIP-seq tracks shown in (B) across a 5 kb peak-centred window of all identified ICN1-Bio and Rbpj-Bio (non-stimulated and Notch-stimulated) binding sites. The sites were sorted by increasing Rbpj-Bio density under non-stimulated conditions. The black triangle represents trend of dynamic Rbpj-Bio occupancy (bottom to top), which correlates with the Notch-dependency of Rbpj-Bio-binding. The partitions were generated by the median absolute deviation (MAD) cutoffs of Rbpj-Bio density in unstimulated conditions (left). The composite plots show the ICN1-Bio and Rbpj-Bio signals across a 5 kb peak-centred window of the according partitions (right).

The peak-to-gene assignment to determine potential Notch target genes in uncommitted progenitors was based on the ICN1-Bio peak set, because of a cDNA contamination during library preparation of the Rbpj-Bio-ChIP-seq samples, which resulted in false-positive peaks over gene exon regions (data not shown). Furthermore, the ICN1-Bio peak set exhibits a better signal-to-noise ratio compared to the Rbpj-Bio peak set. The assignment with maximum peak-to-gene distance of 50 kb resulted in 2,098 direct target genes.

2.4.2 Cell type-specific binding of ICN1-Bio and Rbpj-Bio in Pax5-deficient pro-B cells and Rag2-deficient DN3a cells

Notch signalling fulfils different functions depending on the developmental stage of T cell progenitors. While Notch is the T lineage specification and commitment factor at early DN1 and DN2 stages, it is required for survival, proliferation and further developmental progression during DN3 and DN4 stages. Variable outputs of the same signalling pathway can be the result of the regulation of distinct target gene sets during different developmental stages. Such regulation is often achieved by stage-specific binding profiles of transcriptional regulators.

To assess if the binding of ICN1 and Rbpj is stage-specific, I compared the binding profiles of both transcriptional regulators in uncommitted *Pax5*-mutant pro-B cells and T cell lineage committed Rag2-deficient DN3a cells. 40% of the Rbpj-Bio peaks and 34% of the ICN1-Bio-binding sites identified in Pax5-deficient pro-B cells were also bound by Rbpj-Bio in Rag2-deficient DN3a cells, indicating a stage-specific binding of ICN1/Rbpj (Fig. 2.18A).

To obtain a more dynamic picture of context-specific binding of ICN1-Bio and Rbpj-Bio, the read density in both cell types within the unique and common peaks was determined. Due to the higher quality of the tracks (less false-positive peaks), I restricted the analysis to the ICN1-Bio peaks in *Pax5*-mutant pro-B cells and Rbpj-Bio peaks in Rag2-deficient DN3a cells. As expected, the common peaks showed high read densities in both cell types (Fig. 2.18B). The unique peaks sets indicated that the overlap analysis shown in Figure 2.18A only partially recapitulated the stage-specific binding features of the ICN1-Bio and Rbpj-Bio proteins. Even though a large fraction of peaks within each unique peak set was cell type-specific, some peaks showed a high correlation between both cell types. This shows the importance of a differential binding analysis of ChIP-seq peak to investigate cell type-specific binding of transcription factors in general and in particular for genome-wide occupancy data obtained with proteins like ICN1 and Rbpj, which are difficult to capture by ChIP.

Examples of highly *Pax5*^{Δ/Δ} pro-B cell-specific ICN1/Rbpj-Bio-binding sites are found upstream of the *FasL* gene encoding the pro-apoptotic Fas ligand, as well as at downstream regulatory elements of genes encoding transcription factors with important roles in T cell develop-

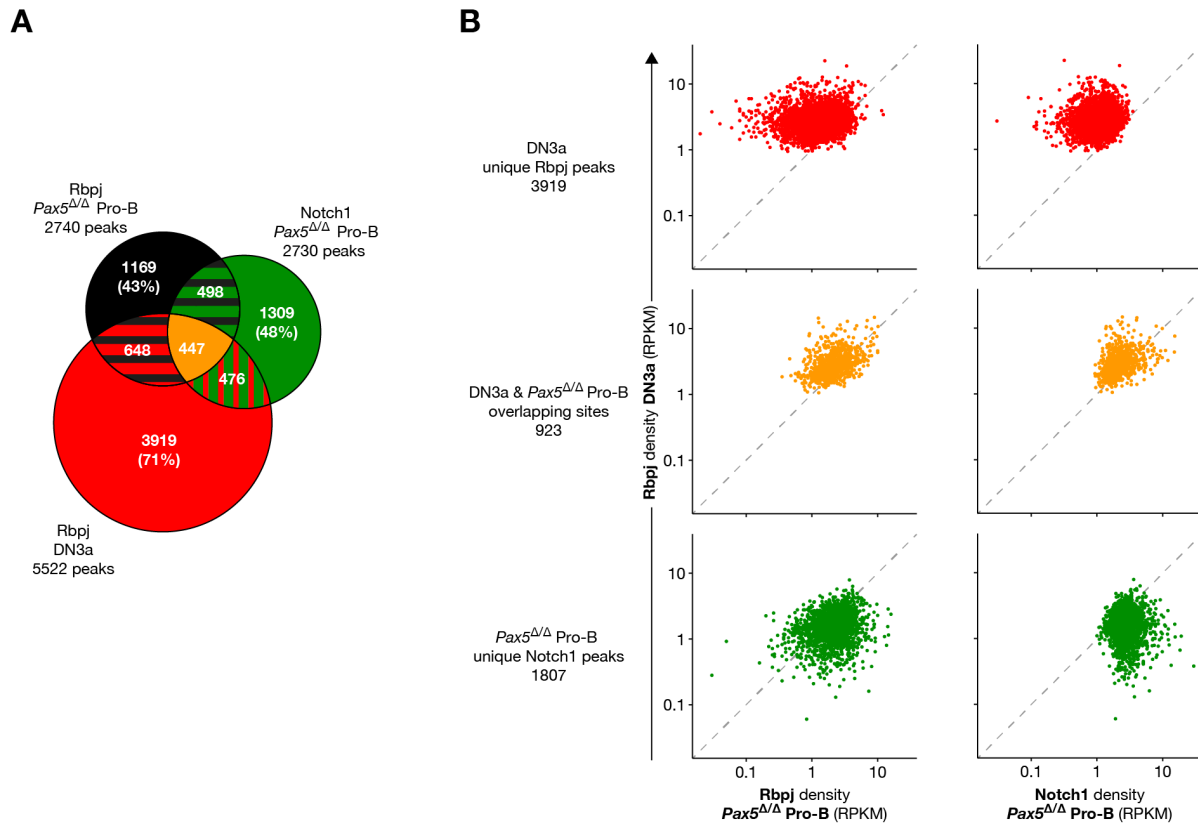


Figure 2.18 Genome-wide comparison of cell type-specific binding of ICN1-Bio and Rbpj-Bio. (A) Venn diagram representing the overlap of the ICN1-Bio (green) and Rbpj-Bio (black) binding sites identified by Bio-ChIP seq in OP9-DL1-stimulated *Notch1^{Bio} Pax5^{Δ/Δ}* and *Rbpj^{Bio} Pax5^{Δ/Δ}* pro-B cells, respectively, together with the *Rbpj*-Bio-binding sites determined by Bio-ChIP-seq in Rag2-deficient DN3a cells (red). Numbers refer to identified peaks, and percentages indicate the ratio of unique peaks. Note that slight peak number discrepancies between all identified peaks, overlapping peaks and unique peaks result from single peaks that overlap with two or more peaks in another sample. (B) Scatter plots comparing the read densities of *Rbpj*-Bio (left) and *ICN1*-Bio (right) Bio-ChIP-seq tracks from *Pax5*-deficient pro-B cells (x-axis) to *Rbpj*-Bio read densities of Rag2-deficient DN3a cells (y-axis). RPKM-normalised read densities within the identified peak regions are plotted. Peak regions refer to the overlap analysis shown in (A) of the *ICN1*-Bio peaks from *Pax5^{Δ/Δ}* pro-B cells and *Rbpj*-Bio peaks from *Rag2*-mutant DN3a cells (see Suppl. Fig. 7.2B). Hence, DN3a-unique peaks (top) refer to the red and red/black dashed regions shown in A, overlapping sites (middle) comprise the peaks within the orange and red/green dashed regions in shown in (A) and *Pax5^{Δ/Δ}* pro-B unique peaks (bottom) refer to the green and green/black dashed regions shown in A.

ment such as *Gata3* (Ho et al., 2009; Hosoya-Ohmura et al., 2011) and *Bcl11a* (Fig. 2.19A; Yu et al., 2012b; Liu et al., 2003). In contrast, DN3a cell-specific binding of ICN1 and Rbpj was seen at the *Il2ra* encoding the DN2 and DN3 cell-specific surface marker CD25 (Fig. 2.19B). DN3a stage-specific binding was also found at the *Cd3e* gene, which encodes the ϵ chain of CD3 that is required for both pre-TCR and TCR signalling. Interestingly, expression of *Cd3e* peaks at the DN3a stage compared to DN2 and DN3b stages (IMMGEn database). Therefore Notch might play a role in the regulation of *Cd3e* at this stage. Canonical Notch target genes such as *Hes1*, *Notch1* and *Notch3* exhibit similar binding profiles of ICN1-Bio and Rbpj-Bio

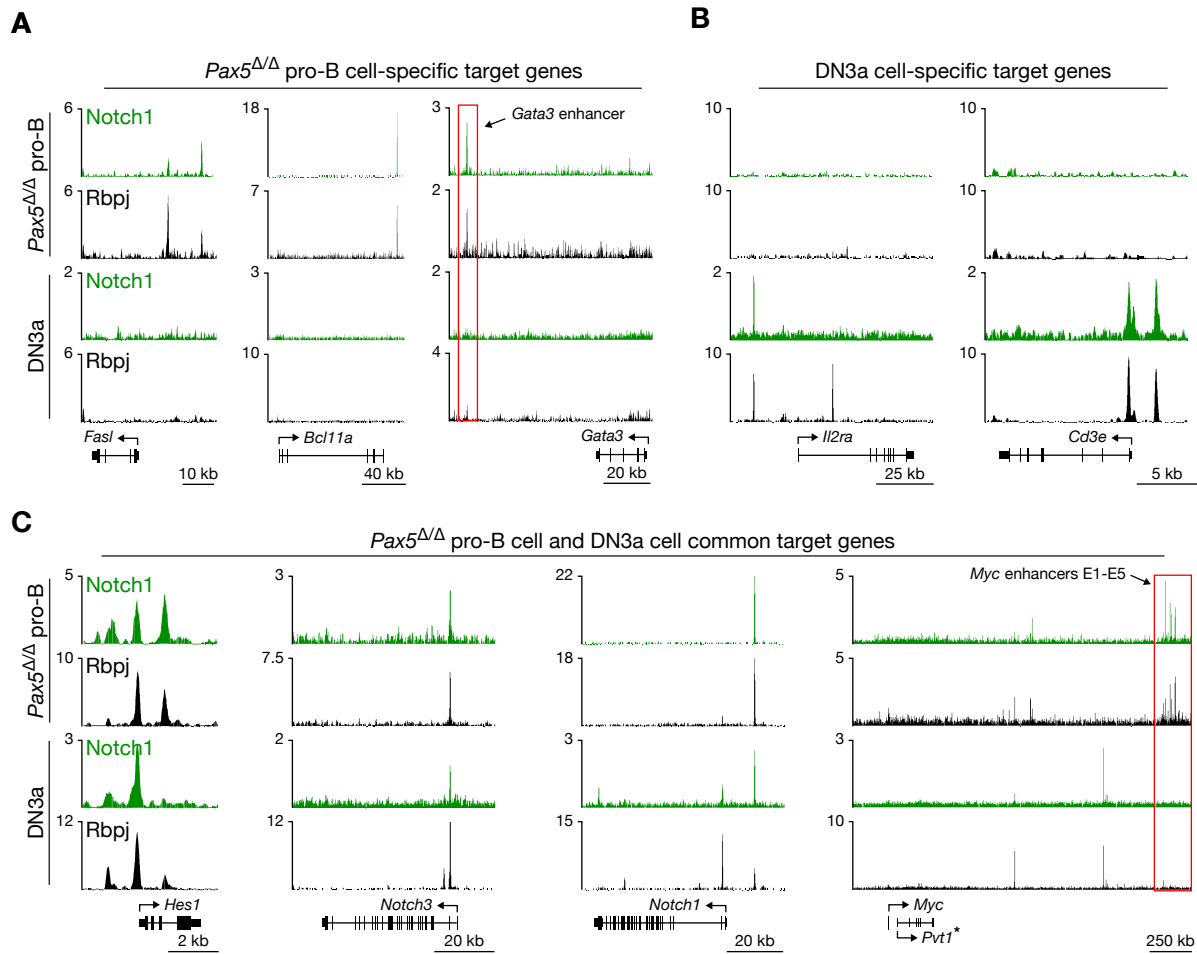


Figure 2.19 Examples of cell type-specific and common ICN1-Bio and Rbpj-Bio-binding sites. (A-C) Rbpj-Bio (black) and ICN1-Bio (green) read density tracks generated from Notch-stimulated *Pax5*^{Δ/Δ} pro-B cells (top) and *Rag2*-mutant DN3a cells (bottom) at *Pax5*^{Δ/Δ} pro-B cell-specific target genes (A), *Rag2* DN3a cell-specific target genes (B) and common target genes (C). A 280 kb downstream enhancer of *Gata3* (Hosoya-Ohmura et al., 2011) and a 1.7 megabases (mb) downstream enhancer of *Myc* (Shi et al., 2013) are highlighted. The asterisk denotes that *Pvt1* encodes a long non-coding RNA. The value on the y-axis represents the RPM-normalised read density of the given track. Arrows mark the TSS and filled black boxes represent exons. The scale in kb is shown at the bottom right of each panel.

in both cell types (Fig. 2.19C). *Myc* was of particular interest for several reasons: *i*) Its encoded transcription factor c-Myc is a critical target gene of Notch signalling in T-ALL (Weng et al., 2006; Palomero et al., 2006). *ii*) c-Myc was shown to mediate pre-TCR-induced, Notch-dependent proliferation (Dose et al., 2006). *iii*) *Myc* expression is activated within 2 h upon Notch stimulation of DN3a cells (Fig 2.9B and 2.12A), even though Bio-ChIP-seq for both ICN1-Bio and Rbpj-Bio did not show any prominent occupancy at the *Myc* promoter or anywhere nearby the genebody (Fig. 2.19C). However, Shi et al. (2013) reported the regulation of *Myc* expression in acute myeloid leukemic (AML) cells via enhancer elements E1-E5 located 1.7 mb downstream of the gene. ICN1-Bio and Rbpj-Bio are bound to E1-E5 in *Pax5*^{Δ/Δ} pro-

B cells but not in Rag2-deficient DN3a cells. However, prominent ICN1-Bio and Rbpj-Bio occupancy was seen in *Rag2*-mutant DN3a cells at two sites, which lie more proximal to the *Myc* locus and might represent likely candidates for mediating a the DN3-specific regulation of *Myc* expression by Notch1.

2.4.3 The Notch-regulated target genes in uncommitted Pax5-deficient pro-B cells

To assess which genes are regulated by Notch signalling in uncommitted progenitors, the transcriptomes of *in vitro* cultured *Pax5*^{-/-} transgenic pro-B cells (Suppl. Fig. 7.11A) as well as *ex vivo* LMPPs (Fig. 2.20 and Suppl. Fig. 7.12) were analysed by RNA-seq at different time points after *in vitro* Notch activation. RNA-seq of *Pax5*^{-/-} pro-B cells served to complement the genome-wide ICN1 and Rbpj occupancy derived from *Notch1*^{Bio} *Pax5*^{Δ/Δ} and *Rbpj*^{Bio} *Pax5*^{Δ/Δ} pro-B cells, whereas RNA-seq of Notch-stimulated LMPPs should reveal Notch-regulated genes in physiological T lymphocyte progenitors. Notch stimulation of *Pax5*^{-/-} pro-B cells resulted in the activation of 41 genes after 2 h of OP9-DL1 co-culture, 56 genes after 4 h and 136 genes after 8 h, while only 25 genes were repressed after 8 h (> 2-fold differentially expressed; adjusted *P*-value of < 0.1; Suppl. Fig. 7.11B). Gene expression changes correlated well with increased duration of Notch activation (Suppl. Fig. 7.11C). To address which of the regulated genes could be direct Notch target genes, the peak-to-gene assignment results from Bio-ChIP-seq data generated in *Notch1*^{Bio} *Pax5*^{Δ/Δ} pro-B cells (2,098 genes; see Fig. 2.17). In addition, the Rbpj-Bio targets in DN3 cells (4,606 genes; see Fig. 2.3) were included at this stage, because of the the high quality of this ChIP and due to the aforementioned cDNA contamination and resulting false-positive peaks of the *Rbpj*^{Bio} *Pax5*^{Δ/Δ} Bio-ChIP-seq dataset. Together, this leads to a total of 5,715 Notch target genes. With approximately 21,100 annotated genes, this results in a random gene-to-peak assignment of 27%. Interestingly, Notch-activated genes were enriched for binding of ICN1/Rbpj-Bio, since 48% of the genes activated after 2 h of Notch signalling were direct Notch target genes (Suppl. Fig. 7.11B; left). This enrichment increased to 71% after 4 h (Suppl. Fig. 7.11B; middle) and 57% after 8 h (Suppl. Fig. 7.11B; right) of Notch activation, respectively. While only 35% of the genes repressed by Notch signalling were potential direct Notch targets (Suppl. Fig. 7.11B). Thus, pointing towards a mainly transcription-activating function of Notch signalling. *In vivo* expression profiles showed that many of the Notch-activated genes in *Pax5*^{-/-} pro-B cells reached maximum transcript abundance during early DN1 and DN2 stages. This was equally true for approximately 50% of ICN1/Rbpj-Bio bound (Suppl. Fig. 7.13A) and unbound (Suppl. Fig. 7.13B) genes and is in contrast to the expression profiles observed for the Notch-activated genes in DN3a cells, which mainly peak at DN3 stages (Fig. 2.12).

2.4.4 The Notch-regulated target genes in LMPPs

Notch stimulation of freshly isolated LMPPs resulted in the activation of 76 genes after 4 h of OP9-DL1 co-culture and 213 genes after 12 h, respectively (> 2 -fold differentially expressed; adjusted P -value < 0.1 ; Fig. 2.20B). Gene expression changes correlated well with increased duration of Notch activation of LMPPs (Suppl. Fig. 7.12B). Considering the peak-to-gene assignment from Bio-ChIP-seq data and the resulting 5,715 Notch target genes (see Section 2.4.3 for details), 72% (55 genes) of the genes activated after 4 h qualify as direct target genes, while 57% (122 genes) of the genes activated by Notch after 12 h show significant ICN1/Rbpj-Bio occupancy (Fig. 2.20B). This decreased proportion of direct Notch target genes might represent secondary effects of Notch-activated transcriptional regulators. 28 (46% bound by ICN1/Rbpj-Bio) genes were repressed by Notch after 4 h of stimulation and 93 (28% bound by ICN1/Rbpj-Bio) genes after 12 h.

Many of the Notch-activated genes identified in *Pax5*^{-/-} pro-B cells, especially genes that exhibit strong regulation by Notch, were also regulated in LMPPs (Suppl. Fig. 7.12C). This validates the suitability of the *Pax5*-mutant pro-B cell system for dissecting the Notch-induced molecular programme of uncommitted lymphoid progenitors.

In addition to the 4 h and 12 h time points, LMPPs were cultured for 4 days (d) on OP9-DL1 feeders to determine the genes, which are activated only upon long-term stimulation of Notch signalling. *In vitro* cultures after 4 d cells were heterogeneous since only a fraction of cells differentiated to T cells, while many cells differentiated into non-T cell lineages (data not shown). RNA-seq analysis of LMPPs after 4 d of OP9-GFP or OP9-DL1 co-culture identified, besides several other genes, *Tcf7* and *Bcl11b* that are known to be highly activated during T cell differentiation (Suppl. Fig. 7.12D,E). The transcription factors TCF-1 and Bcl11b, encoded by these genes, are crucial for early T cell development (Rothenberg, 2014). Interestingly, both genes were only activated in long-term co-cultures of LMPPs on OP9-DL1 cells (Suppl. Fig. 7.12E) and have well-defined *cis*-regulatory elements (Zhang et al., 2012b; Li et al., 2013) that are bound by ICN1-Bio and/or Rbpj-Bio (Fig. 2.5C and data not shown). However, a Notch-dependent regulation of these genes is likely to happen at later developmental stages and might require the action of additional transcriptional regulators, which are not yet active in LMPPs.

Based on their *in vivo* gene expression profiles during early lymphocyte development, the Notch-activated direct target genes identified in LMPPs could be grouped into three clusters (Fig. 2.21A). Cluster B1 (bound1) comprised approximately 35% of the Notch-regulated genes in LMPPs, and expression of the genes in cluster B1 peaked at the DN3a stage. In contrast, the remaining genes in clusters B2 and B3, exhibit an expression profile with maximum transcript

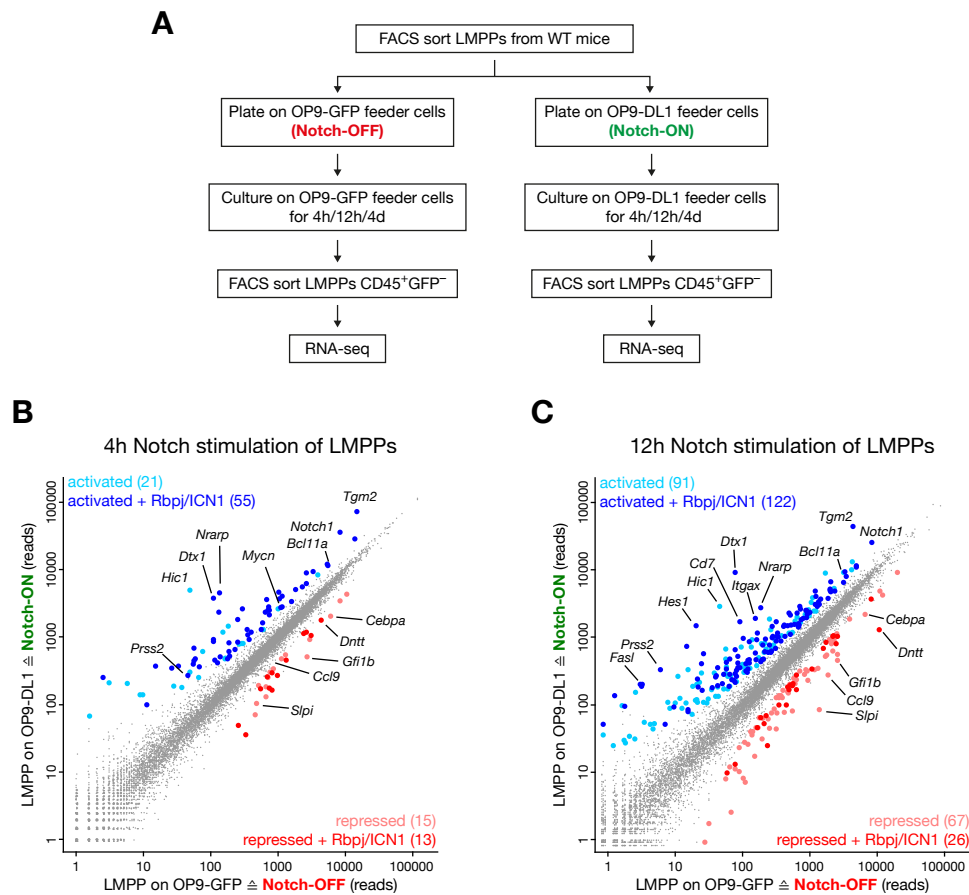


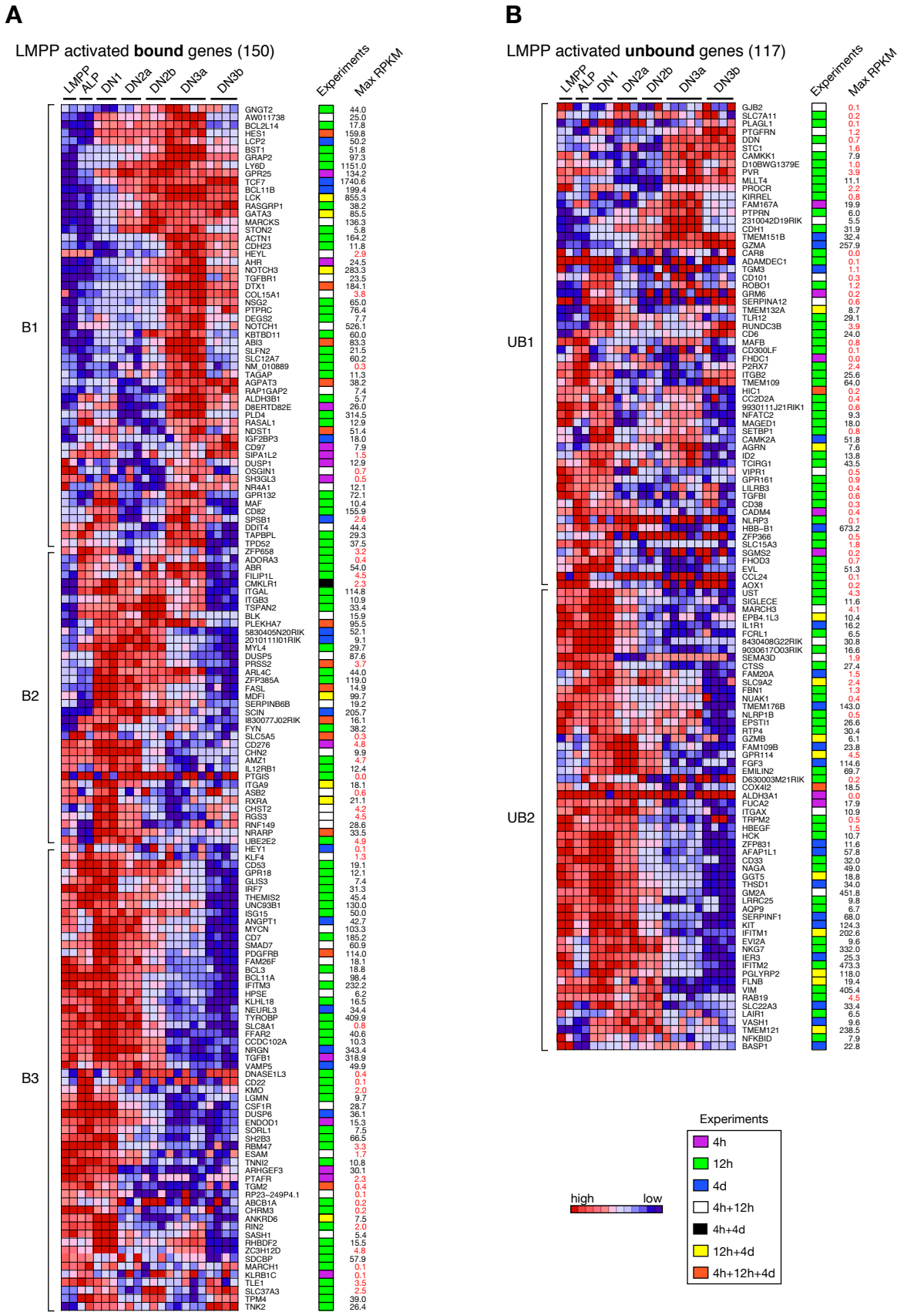
Figure 2.20 RNA-seq of Notch-stimulated LMPPs. (A) Flowchart describing the experimental design. (B) Scatter plot visualising gene expression differences identified by RNA-seq of freshly isolated LMPPs after *in vitro* Notch stimulation for 4 h (left) and 12 h (right). Genes that are more than 2-fold differentially expressed and have an adjusted *P*-value of < 0.1, are shown in blue (Notch-activated) or red (Notch-repressed). Darker colours indicate binding of Rbpj-Bio in Rag2-deficient DN3a cells (see Section 2.1.3) and/or ICN1-Bio in Pax5-deficient pro-B cells (see Section 2.4.1) within 50 kb of the gene, identified by Bio-ChIP-seq. Numbers in brackets correspond to the number of genes in a given group.

abundance during early DN1 or DN2 stages. The contribution of these transiently expressed genes was much higher in the LMPP gene set compared to the DN3a gene set (Fig. 2.12A). The Notch activated genes without an assigned ICN1/Rbpj-binding site showed an overall similar expression pattern compared to the bound genes (Fig. 2.21B), with most genes being expressed at very low levels within thymic DN1 to DN3b stages.

Many of the genes repressed by Notch signalling after *in vitro* OP9-DL1 stimulation of LMPPs were also rapidly repressed *in vivo* upon thymus entry, since these genes exhibit reduced transcript levels in DN1 or DN2 cells compared to pre-thymic stages (Suppl. Fig. 7.14). The *Cebpa* gene was repressed in LMPPs upon Notch activation already after 4 h (Fig. 2.20B,C). The gene encodes the myeloid and dendritic cell-specific transcription factor C/EBP α . *Cebpa* expression in lymphoid progenitors was recently shown to be constrained via the Notch-Hes1

axis in order to prevent diversion of early T cells into the myeloid lineage (De Obaldia et al., 2013b). *Hes1* expression in LMPPs is rapidly included upon Notch activation (Fig. 2.20B,C). Hence, this could describe a general paradigm for many of the genes repressed by Notch signalling, as they might be direct target genes of Notch-activated transcriptional repressors. This mechanism of indirect repression through the Notch pathway is also supported by the observation that most repressed genes in LMPPs were not directly bound by ICN1-Bio or Rbpj-Bio. Furthermore, the ICN1-Bio or Rbpj-Bio-binding sites that were assigned to repressed genes exhibit only weak ChIP-seq signals and were not found to be in obvious *cis*-regulatory elements such as promoters or gene-proximal regulatory elements (data not shown).

Figure 2.21 (following page) Expression profiles through early lymphocyte development of Notch-activated genes in LMPPs. (A,B) Heatmaps showing the expression profiles of Notch-activated genes identified by RNA-seq of OP9-DL1-stimulated LMPPs. Genes bound by Rbpj-Bio in *Rag2*^{-/-} DN3a cells and/or ICN1-Bio in *Pax5*-mutant pro-B cells are shown in A. The Notch-regulated genes without an Rbpj-Bio or ICN1-Bio-binding site are shown in B. Hierarchical clustering by pairwise average-linkage was performed on log-transformed RPKM expression values of LMPP, ALP, DN1, DN2a, DN2b, DN3a and DN3b cells. The number of columns equals the number of independent RNA-seq samples per cell type. The distance between rows (genes) was measured by Pearson correlation. Expression values are row-normalised. Brackets on the left depict a strongly simplified clustering tree. The colour code on the right side depicts, in which experiments (duration of Notch stimulations) the gene was identified. Max indicates the maximum RPKM expression value of a gene within the DN1 to DN3b stages. Values are averages of all samples of one population, and values below 5 RPKM are red.



2.4.5 Functional classification of directly Notch-activated target genes identified in LMPPs

To gain further insight into how Notch controls T cell differentiation in uncommitted progenitors, the directly Notch-activated target genes in LMPPs were grouped according to the function of the encoded proteins based on the PubMed database (Fig. 2.22). Genes, which were activated only upon 4 d of OP9-DL1 co-culture were excluded, as many of these might be indirect Notch targets. Furthermore, to filter for genes that are more likely to have biologically significant effects during early T cell development, a minimum expression filter of more than 2 RPKM at any of the thymic DN1 to DN3b stages was applied. From the 117 directly Notch-activated target genes in LMPPs, only 6 genes (~5%) were previously implicated to be directly controlled by Notch signalling (*Gata3*, *Hes1*, *Heyl*, *Notch1*, *Notch3*, *Nrarp*). More than 50% of all genes belonged to the four categories of transcriptional regulators (17), surface receptors (21), cell metabolism (7) and signal transducers (25). This ratio is comparable to the functional categories of the directly Notch-activated target genes in DN3a cells (Fig. 2.13). In LMPPs, however, more genes encoding proteins involved in signal transduction were activated by Notch directly (25 in LMPPs vs. 14 in DN3a). In contrast, the group of genes encoding enzymes that function in cell metabolism was more than 2-fold reduced in LMPPs compared to DN3a cells (7 in LMPPs vs. 16 in DN3a). Likewise, the group of genes encoding membrane bound transporters was reduced in LMPPs compared to DN3a cells (1 in LMPPs vs. 4 in DN3a). This may reflect differences in the functional outcome of Notch signalling in uncommitted LMPPs and pre- β -selection DN3a cells. In committed DN3a cells, which are about to undergo β -selection, the directly Notch-induced transcriptional profile primarily suggests functions in pre-TCR signalling (*Ptcra*, *Cd3d*), proliferation (*Myc*, *Mycn*) and metabolism that requires nutrient uptake (Fig. 2.13). In uncommitted cells, however, these genes, except for *Mycn*, are not regulated by Notch signalling. Instead, more signal transducers are induced by Notch in LMPPs, together with the genes encoding the transcription factors GATA3 and Hes1, which are required for T cell specification (García-Ojeda et al., 2013; De Obaldia et al., 2013b). This may suggest that besides playing a role in T cell specification, Notch signalling activates components of cell signalling pathways, which may be required during early T cell development.

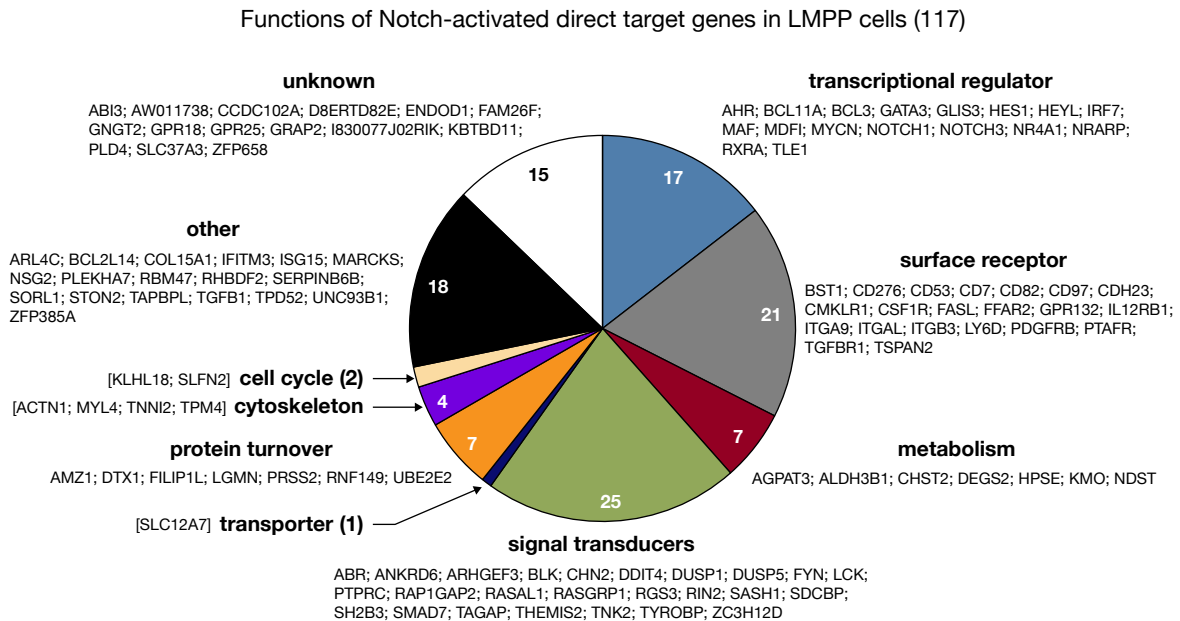


Figure 2.22 Functions of Notch-activated target genes identified in LMPPs. A Pie diagram representing the different functional classes of the identified Notch-activated target genes in LMPPs. Numbers indicate the absolute number of genes in the according section of the pie chart. Only Notch-activated and Rbpj-Bio bound genes with a minimum RPKM level > 2 at any of the thymic DN1-DN3b stages were included.

2.4.6 Notch-dependent gene expression profiles are highly cell type-specific

To assess if Notch controls different sets of genes in uncommitted LMPPs and committed DN3a cells, the overlaps of the directly Notch-activated genes (Figs. 2.12A and 2.21A) as well as the genes repressed by Notch signalling (Suppl. Figs. 7.9 and 7.14) in both cell types were analysed. The analysis shows that, from 150 Notch-activated genes in LMPPs, only 18 genes (12%) were also activated in DN3a cells (Fig. 2.23). Among other genes, this group comprised the genes encoding the Notch1 and Notch3 receptors, the regulator of the Notch signalling pathway Dtx1, members of the Hes/Hey family of basic helix-loop-helix transcription factors Hes1, Hey1 and Heyl as well as the Aw011738 gene, which encodes a non-coding RNA and has a sequence similarity to human *HES4* a non-conserved member of the Hes family (conservation analysis performed by Alexander Schleiffer; data not shown). Another group of genes that was activated by Notch in both cell types comprises genes encoding the cell surface molecules octocadherin Cdh23, CD82, BST-1, Ly6d and phospholipase D family member 4 (PLD4). Eight genes were repressed by Notch signalling in both cell types. Besides others, this group comprised the genes encoding the C-type lectin Clec12a, the cell surface antigen Ly6C, the transcription factor PU.1 (*Spib*) and thrombospondin 1 (*Thbs1*). A total of 10 genes showed inverse patterns of expression upon Notch signalling. *Egfr* and *Paqr5* encoding the

epidermal growth factor receptor *Egfr* and a progesterin receptor family member, respectively, were repressed by Notch in LMPPs and activated by Notch in DN3a cells. In contrast, eight genes including those encoding the transcription factor *IRF7*, the stem cell growth factor receptor kinase *c-Kit*, granzyme A, *RAP1* GTPase activating protein 2 and the glycerol-3-phosphate transporter *Slc37a3*, were initially activated by Notch in LMPPs and later on repressed in DN3a cells. Overall, the overlap analysis shows that Notch regulates distinct sets of genes in LMPPs and DN3a cells.

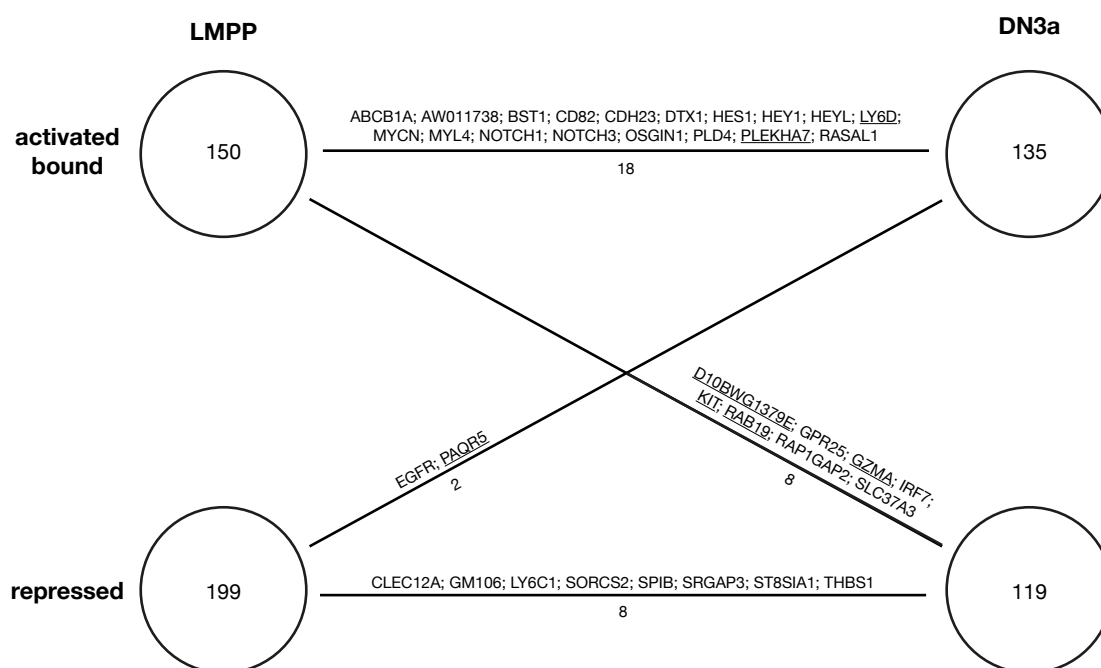


Figure 2.23 Comparison of Notch-activated and Notch-repressed genes in DN3a and LMPPs. A schematic representation of the directly Notch-activated genes (top) and the genes repressed by Notch (bottom) in LMPPs (left) and DN3a cells (right). Numbers in the circles indicate the total number of genes per group. Lines connect the two groups that were overlapped and the genes on top of the lines were present in both groups. Repressed genes were not filtered for binding of Rbpj-Bio and/or ICN1-Bio. Hence, if activated in the other cell type, these represses genes (underlined) were not necessarily bound. Two genes (*Ly6d*, *Plekha7*) of the 18 genes activated by Notch in both cell types were not directly bound in DN3a cells and are marked by underlined gene names.

2.4.7 A large set of direct Notch-activated target genes is only transiently expressed during early T cell development

In order to determine a stringent set of direct Notch-regulated target genes in LMPPs, I hypothesised that these genes had to fit the following criteria: *i*) Expression of the genes should be activated upon *in vitro* Notch stimulation of LMPPs for 4 h or 12 h . *ii*) *In vivo* activation of Notch signalling upon thymus entry should result in an increased transcript levels in DN1 cells

compared to LMPPs. *iii*) Bio-ChIP-seq with ICN1-Bio in *Pax5*-mutant pro-B cells or Rbpj-Bio in Rag2-deficient DN3a cells must identify a significant binding site within 50 kb up or downstream of the genes. Finally, this approach resulted in a set of 71 genes (Fig. 2.24A). These 71 Notch-activated target genes could be separated into two clusters based on their *in vivo* expression profile during early lymphocyte development (Fig. 2.24B).

Cluster 1 contained most of the canonical Notch target genes, such as *Hes1*, *Dtx1*, *Notch1* and *Notch3*, which reached their maximum expression level in DN3a cells followed by decreased transcript levels in DN3b cells. The expression pattern of the genes in cluster 2 was unexpected, as they were downregulated by the DN2b/DN3a stages, where commitment to the T cell lineage takes place, which coincides with the highest level of canonical Notch target gene expression. *Nrarp* (Notch-regulated ankyrin repeat protein) is the only gene within cluster 2 that was previously described to be a direct Notch target gene (Krebs et al., 2001; Pirot et al., 2004). *Nrarp* interacts with the ICN to block Notch-mediated transactivation and T lineage commitment (Yun and Bevan, 2003a). Interestingly, several genes within cluster 2 were characteristic for non-T cell lineages. The integrin $\alpha 9 \beta 1$, which is encoded by the *Itga9* gene, contributes to granulopoiesis by enhancing granulocyte colony-stimulating factor receptor (G-CSFR) signalling (Chen et al., 2006) and is highly expressed in several macrophage subsets (IMMGEN database). *Cfs1r* encodes the functional cell surface receptor for colony-stimulating factor 1 (CSF1), the macrophage-specific growth factor (Sherr et al., 1985). While *Tyrobp* encodes the Tyro protein tyrosine kinase-binding protein Tyrobp (DAP12), which is a key activating signal transducer in NK cells and monocytes (Colonna, 1998; Lanier et al., 1998). Both, NK and DC/myeloid lineage potentials exist in early DN1 as well as DN2a thymocytes, while transition to the subsequent DN2b stage coincides with *Bcl11b* upregulation and commitment to the T cell lineage.

The analysis shows that Notch on the one hand activates genes in LMPPs which are critical for T cell development such as *Hes1*, *Gata3* or *Lck*, and on the other hand, surprisingly, activates many genes, which appear to be non-T lineage-specific (*Itga9*, *Cfs1r*, *Tyrobp*) or inappropriate (*Nrarp*) for T cell development. However, expression of the latter set of genes is only transient, which could imply that these transcription signatures have to be repressed in order for T cell development to proceed. Such regulation could be achieved through activation of transcriptional repressors during DN1 as well as DN2a stages, which might be a Notch-dependent process. *Hes1* is a potential candidate, as its expression is rapidly upregulated upon thymus seeding. In addition, repression of these transiently expressed genes could depend on other (Notch-regulated) transcription factors, which can act as repressors such as GATA3, *Bcl11b* or TCF-1. However, because of the delayed expression of the genes encoding these proteins compared to the expression of *Hes1*, they are more likely to play a role in the regulation of the genes, which are repressed at the DN2b stage.

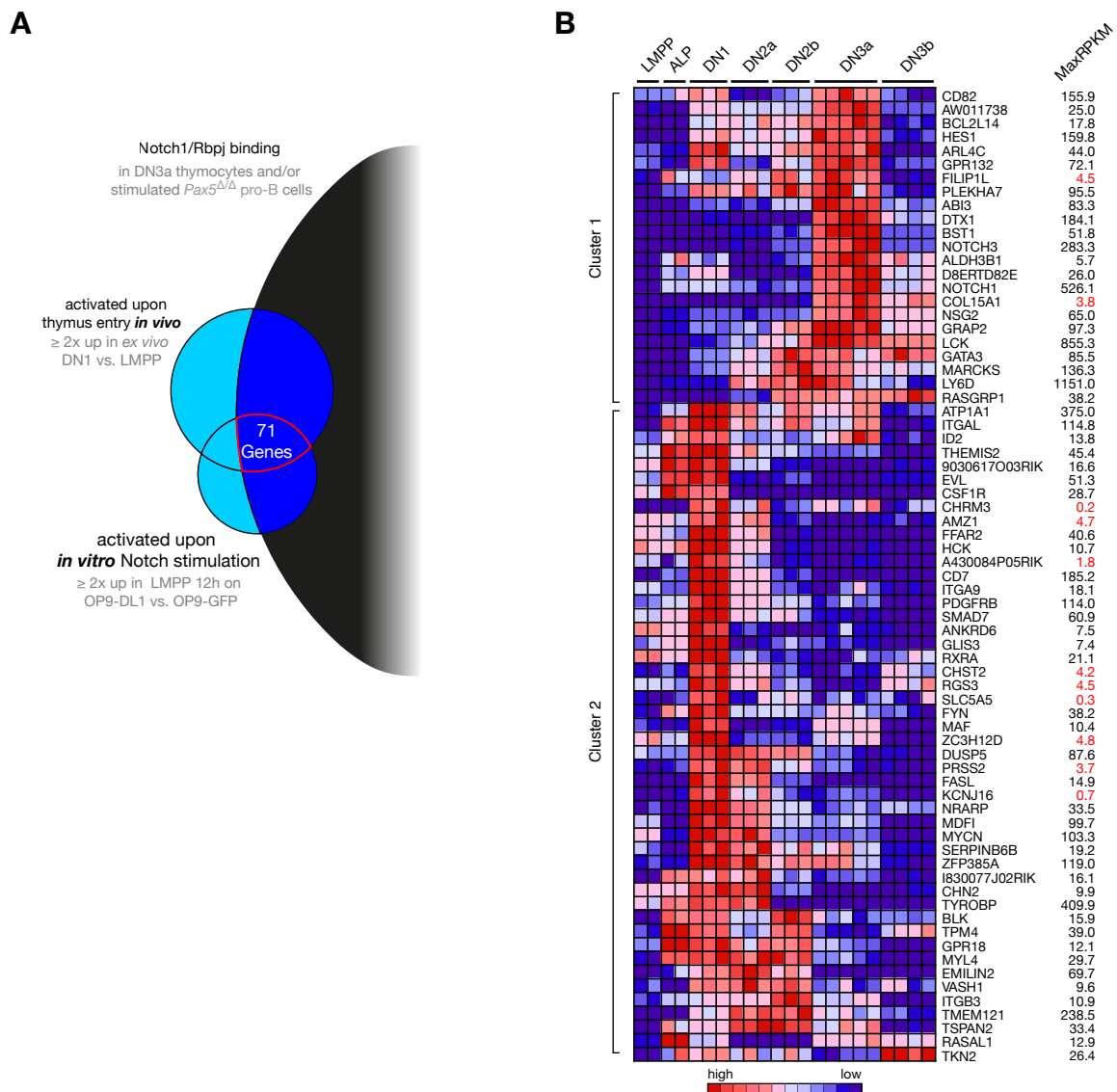


Figure 2.24 A class of transiently Notch-activated target genes. (A) Venn diagram depicting the strategy to identify a stringent set of Notch-activated target genes in LMPPs. Genes, that were more than 2-fold activated by Notch in LMPPs after 12 h OP9-DL1 stimulation, were filtered in addition for an minimum of 2-fold activation upon thymus seeding (*ex vivo* LMPP to DN1) and binding of ICN1-Bio (*Pax5*-deficient pro-B cells) and/or Rbpj-Bio within 50 kb of the gene body (*Rag2*-deficient DN3a cells). (B) Heatmap showing the expression profiles through early lymphocyte development of 71 direct Notch-activated target genes identified by the criteria shown in A. Hierarchical clustering by pairwise average-linkage was performed on log-transformed RPKM expression values of wild-type/control LMPP, ALP, DN1, DN2a, DN2b, DN3a and DN3b cells. The number of columns equals the number of independent RNA-seq samples per cell type. The distance between rows (genes) was measured by Pearson correlation. Expression values are row-normalised. Brackets on the left depict a strongly simplified clustering tree. Max indicates the maximum RPKM expression value of a gene within the DN1 to DN3b stages. Values are averages of all samples in one population, and values below 5 RPKM are red.

2.4.8 Notch signalling induces expression of *Tcrb* germline transcripts

T cell development in the thymus involves sequential, receptor-based selection processes. The first of these check-points for T cells of the $\alpha\beta$ -lineage is the β -selection check-point, which is required for the DN-to-DP cell stage transition. Passage through β -selection depends on the expression of a productively rearranged *Tcrb* gene (Fehling and von Boehmer, 1997; Mathieu et al., 2000). The *Tcrb* gene is assembled by V(D)J recombination from separate V, D, and J gene segments of the ~700 kb-spanning gene locus. V(D)J recombination at the *Tcrb* locus is restricted to cells of the lymphoid lineage and occurs in an ordered fashion with D_β -to- J_β recombination preceding V_β -to- DJ_β assembly. The 'accessibility model' postulates that V(D)J recombination activity is controlled through the modulation of recombinase access to its target substrates (Sleckman et al., 1996). Accessibility of the recombination machinery to the *Tcrb* locus is regulated by *cis*-acting elements, such as promoters and enhancers that control transcription of gene elements within the locus. The expression of sterile germline transcripts originating from promoters just upstream of *Tcrb* D segments ($PD_\beta 1$ and $PD_\beta 2$) was shown to be essential to introduce active chromatin within D_β - J_β - C_β regions of the *Tcrb* locus (Oestreich et al., 2006; Osipovich et al., 2007; Kondilis-Mangum et al., 2010). Germline transcription of these regions correlates with the onset of recombination (Sikes et al., 2010) and critically depends on the activity of the TCR β enhancer (E_β) that is located at the 3' end of the *Tcrb* locus (Mathieu et al., 2000). Consequently, deletion of E_β blocks D_β -to- J_β recombination as well as V_β -to- DJ_β rearrangement, resulting in a block in $\alpha\beta$ T cell development at the pre- β selection DN3a stage (Bories et al., 1996; Bouvier et al., 1996). Interestingly, it was suggested that Notch signalling is required for the recombination of the *Tcrb* locus, as conditional deletion of *Notch1* in DN2 thymocytes leads to a severe impairment of V_β -to- DJ_β rearrangement, resulting in a partial block of thymocyte development at the pre-TCR checkpoint (Wolfer et al., 2002). However, the mechanism explaining this finding remains unclear.

RNA-seq of OP9-DL1 co-cultured LMPPs (Fig. 2.20B,C) and Pax5-deficient pro-B cells (Suppl. Fig. 7.11A) showed that transcription of *Prss2* was rapidly activated by Notch. The *Prss2* gene encodes a trypsinogen, which is a member of the trypsin family of serine proteases. Like many other trypsinogen genes, *Prss2* is located within the *Tcrb* locus and it is the most proximal trypsinogen to E_β , which has a prominent Notch1-dependent Rbpj-binding site in Pax5-mutant pro-B cells (Fig. 2.25A). The region between E_β and *Prss2* contains the duplicated cluster of D_β - J_β - C_β gene segments.

Interestingly, the RNA-seq analysis of LMPPs co-cultured on OP9-GFP or OP9-DL1 cells showed a Notch-dependent expression of PD_β germline transcripts (GLTs; Fig. 2.25A,B). GLTs encompassing $C_\beta 2$ were most abundant, suggesting a higher activity of the $PD_\beta 2$ pro-

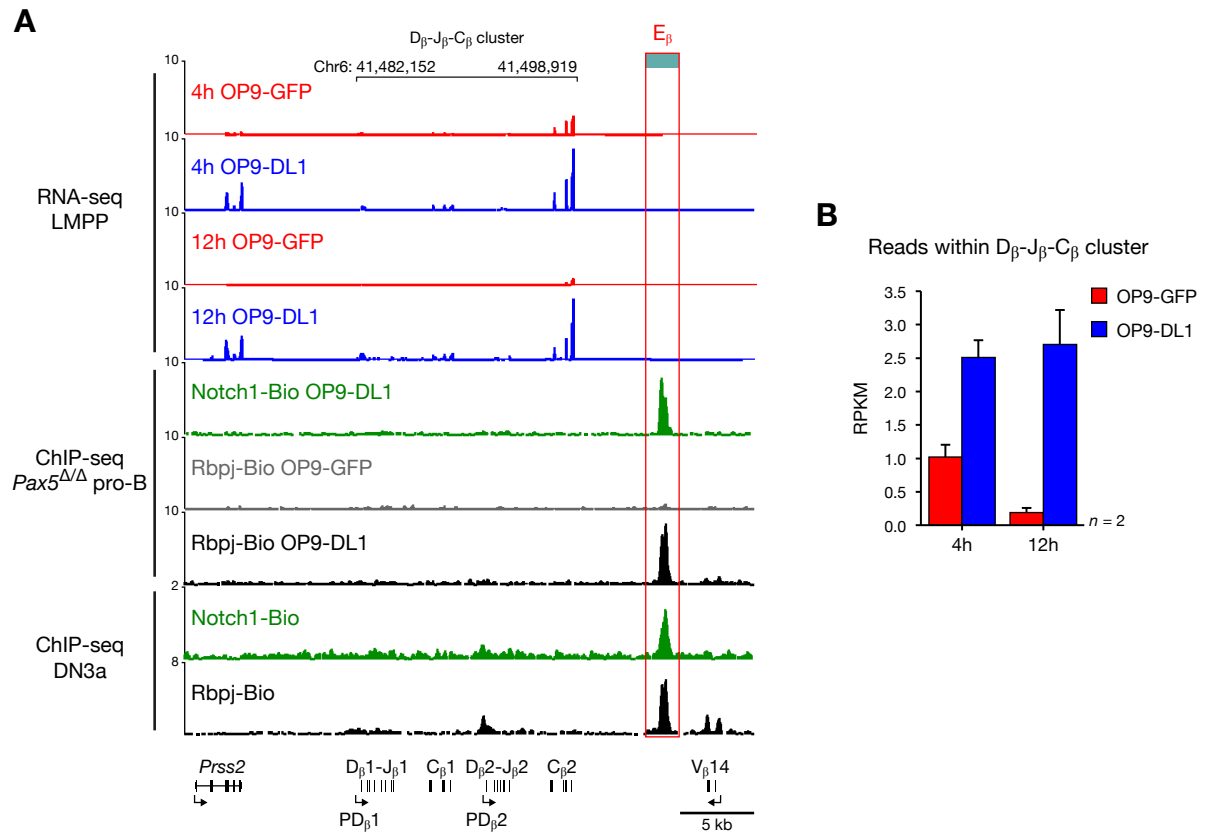


Figure 2.25 Notch-dependent expression of *Tcrb* germline transcripts. (A) RNA-seq read density tracks generated from LMPPs co-cultured for indicated time points on OP9-GFP (red) or OP9-DL1 (blue) feeder cells showing the germline transcripts of the D_β-J_β-C_β clusters 1 and 2 (black bracket and genomic coordinates are shown on the top) together with adjacent regions. Binding of Rbpj/ICN1-Bio to E_β (red box) is shown by read density tracks of Rbpj-Bio (black) and ICN1-Bio (green) Bio-ChIP-seq generated from Notch-stimulated Pax5^{Δ/Δ} pro-B cells and Rag2-mutant DN3a cells. PD_β1 and PD_β2 are the germline transcript promoters. The value on the y-axis represents the RPM-normalised read density of the given track. Arrows mark the TSS and filled black boxes represent exons. The scale in kb is shown at the bottom right of each panel. (B) A bar chart represents the RPKM-normalised RNA-seq signal over the depicted D_β-J_β-C_β cluster (approximately 16.8 kb) at the indicated time points of LMPP co-culture on OP9-GFP (red) or OP9-DL1 (blue).

motor compared to the PD_β1 promoter. PD_β GLTs were also detected in LMPPs after 4 h of OP9-GFP co-culture and were further reduced after prolonged co-culture in the absence of Notch ligand-expressing cells. This may suggest that PD_β activity in the bone-marrow is regulated *in vivo* by an alternative signalling mechanism or that there is a certain level of Notch activity present in bone-marrow LMPPs. A Notch-dependent induction of PD_β GLTs was also seen in OP9-DL1-stimulated Pax5^{-/-} pro-B cells (data not shown). However, under steady-state conditions in OP9-GFP co-cultures, D_β-J_β-C_β transcript levels were approximately 10-times higher in Pax5-mutant pro-B cells compared to 4 h cultured LMPPs (data not shown). Binding of ICN1-Bio and Rbpj-Bio to E_β was also seen in Rag2-deficient DN3a cells (Fig. 2.25A). There were no change in gene expression within the D_β-J_β-C_β cluster upon Notch stimulation

of *Vav-Bcl2* transgenic DN3a cells. Surprisingly, however, when *Notch1* was deleted *in vivo* in Rag2-deficient DN3a cells by *Lck-Cre*, PD β 1 germline transcripts were elevated (data not shown). Rbpj-Bio ChIP-seq identified a small peak at PD β 2 (Fig. 2.25A), which was reported to be regulated by E β (Oestreich et al., 2006). Hence, Notch1 signalling might partially restrict the transcriptional activity of E β to the PD β 2 promoter and diminished Notch1 levels in *Notch1^{fl/fl} Rag2^{-/-} Lck-Cre* thymocytes might reduce this control.

3 General Discussion and Outlook

3.1 Identification of ICN1- and Rbpj-binding sites by ChIP-seq

Genome-wide maps of transcription factor occupancy provide valuable information about potential *cis*-regulatory elements that are used by a transcription factor to control the expression of a gene. Moreover, the type of the bound *cis*-regulatory element (promoter or enhancer) allows mechanistic insights into the control of gene expression through the investigated transcription factor. I mapped ICN1 and Rbpj occupancy in freshly isolated Rag2-deficient DN3a cells, which presents the first genome-wide information about the DNA binding sites of both proteins in primary cells under physiological Notch signalling conditions. The ICN1-Bio ChIP-seq signal had a much lower signal-to-noise ratio compared to the Rbpj-Bio ChIP-seq. Consequently, the Rbpj-Bio ChIP-seq resulted in ~15-times more binding sites than ICN1-Bio ChIP-seq in DN3a cells. Likely reasons for this discrepancy are the indirect binding of ICN1 to DNA and the rapid turnover of ICN1 upon assembly of the transcription-activating complex. Post-translational modifications induce the proteasomal degradation of ICN1, which leads to a short residence time of ICN1 on the DNA. This highly dynamic binding of ICN1 might be difficult to capture through FA-mediated crosslinking followed by ChIP. In contrast to the ICN1-Bio ChIP-seq under steady-state Notch signalling, the controlled induction of Notch signalling *in vitro* is thought to result in a synchronous wave of ICN1-binding to DNA. Consequently, the ICN1-Bio ChIP-seq in *Pax5*-mutant pro-B cells had a better signal-to-noise ratio compared to DN3a cells and detected more ICN1-binding sites. Using the *Pax5*-deficient pro-B cell system, binding sites of Rbpj were determined in the presence and absence of Notch signalling. Activation of Notch signalling increased Rbpj-binding globally, suggesting that the majority of Rbpj sites are Notch1-dependent. A similar result was obtained recently in an *in vivo* system, where Rbpj-Bio ChIP-seq was performed in whole thymocytes (mainly DP T cells), in which ICN1 was artificially expressed from the DP T cell stage onwards (unpublished data from Hiromi Tagoh, Busslinger laboratory). While only few Rbpj-binding sites were detected in the absence of ICN1 (~100 sites), overexpression of ICN1 increased Rbpj-binding and resulted in the detection of ~1000 Rbpj-binding sites. These two ChIP-seq studies indicate that the vast majority of the Rbpj sites are co-occupied by ICN1 when Notch1 signalling is activated, thus suggesting that Rbpj-binding sites can be used as a surrogate for ICN1-binding. In line with this hypothesis, the chromatin at nearly all Rbpj-binding sites in DN3a is in an active state based on DHS site mapping and presence of active histone modifications. This might point towards the presence of ICN1 at those sites.

The inducibility of Rbpj-binding, which was detected in Pax5-deficient pro-B cells after the stimulation of Notch signalling, can be explained by two different mechanisms. One possibility is that, in contrast to the 'classical' textbook view on Notch signalling, Rbpj does not bind to the chromatin in the absence of ICN in the nucleus. This would question the model of the transcriptional switch that is triggered upon the activation of Notch signalling, as Rbpj could not establish transcription-repressing complexes on Notch target genes. Experiments in myogenic cells, partially confirmed this scenario, as the deletion of *Rbpj* did not result in a de-repression of all Notch-regulated genes exhibiting Rbpj-binding sites in their vicinity (Castel et al., 2013). The other possibility is that binding of Rbpj to DNA in the absence of ICN is only transient, yet sufficient to promote the assembly of transcription-repressing complexes. Within these complexes, Rbpj might only weakly interact with DNA, and thus, have a short residence time. The assembly of ICN-dependent transcription-activating complexes increases the residence time of Rbpj and improves its detection by ChIP. In line with the latter possibility, Rbpj binds also weakly in the absence of Notch signalling at most of the ICN1/Rbpj-binding sites that were detected in OP9-DL1 co-cultured Pax5-deficient pro-B cells.

The degree of inducibility of an Rbpj-binding site is the ratio of the Rbpj occupancy in the presence and absence of Notch signalling. The factors that determine the degree of Rbpj occupancy under these two different conditions remain elusive. One factor might be the accessibility of the sites in the absence of Notch signalling. While sites that are located within open/accessible chromatin might allow the binding of Rbpj in the absence of ICN, inaccessible sites could prevent Rbpj-binding. Similar to this, the presence or absence of active histone modifications as well as DNA methylation could play a role here. Regarding the latter case, Bartels et al. (2011) showed that Rbpj binds methylated DNA in the context of a mutated Rbpj consensus motif. Additionally, co-binding of other transcription factors might have an impact on Rbpj occupancy. Such co-occupancy could affect Rbpj-binding in the absence as well as presence of ICN1 and the co-binding transcription factors can promote or prevent the binding of Rbpj. Indeed, the transcription factor Ikaros was shown to restrict the repertoire of Notch target genes in T-ALL cell lines by occupying sites next to Rbpj-binding sites and potentially inducing repressive chromatin, which interferes with Rbpj-binding, and thus, Notch signalling (Geimer Le Lay et al., 2014). Alternatively, cooperative binding between two ICN1-dependent transcription-activating complexes at SPSs could determine the level of Rbpj occupancy in the presence of ICN1. Such dimeric ICN1/Rbpj/MAML complexes interact via the ANK domains of the two ICN1 proteins. This interaction allows the assembly of one complex on a binding site containing a sequence similar to the Rbpj consensus motif TGGGAA, while the secondary site can contain a degenerate Rbpj consensus motif (Bailey and Posakony, 1995; Nellesen et al., 1999; Nam et al., 2007; Arnett et al., 2010; Liu et al., 2010a; Choi et al., 2012).

Accordingly, it would be interesting to investigate: *i*) the chromatin status before and after activation of Notch signalling at the Rbpj-binding sites; *ii*) whether Rbpj-binding sites with low- or high-inducibility are enriched for distinct consensus motifs of other transcription factors; *iii*) whether Rbpj-binding sites with high inducibility are enriched for SPSs.

Dynamic occupancy of Rbpj was also found in other species (Krejčí and Bray, 2007) or mammalian cell types (Fryer et al., 2004; Castel et al., 2013; Wang et al., 2013) and is here described for the first time for T cell progenitors. Castel et al. (2013) reported dynamic and constant Rbpj-binding sites, based on their degree of inducibility in myogenic cell lines. The constant sites described in this study were virtually unaffected by the presence or absence of ICN1. Off note, only very few of such constant sites were found in Pax5-deficient pro-B cells, as Rbpj occupancy increased upon Notch activation at nearly all sites. Whether this observation is a cell type-specific effect or depends on different experimental settings (Bio-ChIP-seq vs. antibody-mediated ChIP-seq) remains to be determined.

3.2 Identification of Notch-regulated genes in DN3 cells

Two different strategies were applied to investigate the Notch-dependent transcriptome in DN3a cells by RNA-seq—the conditional deletion of *Rbpj* or *Notch1* with *Lck-Cre* and the *in vitro* stimulation of Notch signalling. The deletion of *Rbpj* resulted in a partial block of T cell development at the DP T cell stage, while the DN3 stages were less affected. Tanigaki et al. (2004) observed a slightly earlier block at the DN4 stage and consequently a more severe phenotype in the identical experimental setup. This discrepancy could be caused by additional back-crossings of the floxed *Rbpj* allele, that was generated in 129/Sv derived ES cells, into the C57BL/6 background. Deletion of the floxed *Rbpj* allele was only partial at the DN3a stage, and the transcriptome of these cells was more or less identical to the transcriptome of control DN3a cells. A potential explanation for this may be that *Rbpj* deletion occurs too late and that the residual Rbpj protein level is sufficient to maintain the expression of Notch target genes. The same might hold true for DN3b cells, in which *Rbpj* deletion is complete, but the transcript abundance of Notch target genes is not perturbed. As the Notch signalling level is reduced upon β -selection, and as a consequence of this, the amount of ICN1 decreases, even lower levels of Rbpj might be sufficient to mediate the remaining Notch signals in DN3b cells compared to DN3a cells.

The *Lck*-mediated deletion of *Notch1* was more efficient in DN3a cells compared to the deletion of *Rbpj*. The higher deletion efficiency of *Notch1* compared to *Rbpj* could be explained by the highly active chromatin at *Notch1*, which is among the highest transcribed genes in DN3a cells (data not shown). The active chromatin may facilitate Cre-mediated deletion by

granting increased accessibility of the Cre recombinase to the *Notch1* locus compared to the *Rbpj* locus, which is transcribed at a much lower rate (data not shown).

In line with the efficient deletion of *Notch1* at the DN3a stage, T cell development was severely perturbed with a partial block at the β -selection stage. This phenotype is nearly identical to the one observed by Wolfer et al. (2002), who deleted *Notch1* with the same strategy. The reduced surface level of the IL2 receptor α chain (CD25), which is encoded by the Notch target gene *Il2ra*, faithfully identified the DN3a cells, in which the floxed *Notch1* allele was deleted. FACS analysis with an antibody against the N-terminal EGF repeats of Notch1 showed that the deletion results in loss of the surface Notch1 receptor in CD25^{int} DN3a cells. However, several studies reported that 5' deletions of the canonical *Notch1* promoter lead to the activation of cryptic internal promoters (Ashworth et al., 2010; Gómez-Del Arco et al., 2010; Guidos, 2010; Jeannet et al., 2010). The transcripts that are expressed from these promoters start at exons 25-27 of *Notch1* and are translated into Notch1 proteins that lack the ectodomain and are constitutively activated through S2 and S3 cleavages. The RNA-seq read-density profile of the *Notch1* locus in CD25^{int} cells shows a steep increase of the reads over the exons downstream of exon 25 compared to the reads over the exons upstream of exon 25 (see Fig. 2.8E). This could suggest that deletion of the *Notch1* allele in DN3a cells gives rise to truncated Notch1 proteins. These proteins may alleviate the phenotype caused by the loss of the wild-type Notch1 receptor as they could partially maintain the expression of Notch1 target genes at this developmental stage.

Given the strong cellular phenotype observed upon *Notch1* deletion, only relatively mild changes in gene expression were observed in *Notch1*-mutant DN3a cells. This can be explained by the requirement of Notch1 to mediate survival signals at this stage. Accordingly, if the levels of ICN1 are below the minimum required for the survival of the cells at this stage, the cells might die prior to the point, where more severe changes in gene expression could be captured. However, a FACS analysis for the apoptosis markers Annexin V or caspase 9 did not show an increase in apoptotic cells within the DN compartment (data not shown). This might be due to rapid clearance of apoptotic cells in the thymus by macrophages (Surh and Sprent, 1994; Shi and Petrie, 2012).

The induction of Notch signalling leads to the recruitment of several transcription co-activator proteins and finally results in the activation of Notch target gene expression. Hence, Notch is thought to be a positive regulator of transcription. In line with this, the RNA- and ChIP-seq studies, which are presented here, clearly show that Notch-activated genes are enriched for ICN1- and *Rbpj*-binding, compared to Notch-repressed genes. In the *in vivo* deletion experiments, the detection of Notch-activated genes is based on the reduction of their transcripts over time (negative readout). RNA levels are either reduced through proliferation-

dependent dilution of the transcript or through degradation of the RNA molecule over time (stability). As DN3a cells do not cycle, and Notch1 is a positive regulator of proliferation in T cells, the dilution of transcripts through rounds of cell division after *Notch1* deletion is unlikely. Hence, the detection of Notch-activated transcripts largely depends on their degradation. Consequently, the limited life-span of *Notch1*-mutant DN3a cells and the negative-readout (loss of message) might explain the observed relatively mild changes in gene expression.

Notch-dependent regulation of a small set of genes, which are required for proliferation upon β -selection, might also explain the observed phenotype upon deletion of *Notch1*, resulting in strongly reduced cell numbers but only subtle changes in gene expression. However, neither did a 5-Bromodeoxyuridine (BrdU) labelling experiment detect a reduced proliferation rate of Notch1-deficient CD25^{int} DN3a cells (data not shown) nor were any genes with obvious cell cycle functions deregulated in these cells.

The expression of the Notch1 target gene *Notch3* is strongly upregulated in DN3a cells (IMMGEN database and the expression profiling data presented here). As the intracellular domains of ICN1 and ICN3 are partially conserved, a potential rescue of Notch1 target gene expression might happen through activation of the Notch3 receptor. Yet, Notch3 is not thought to be required for early T cell development, since Notch3-deficient thymocytes develop normally (Shi et al., 2011; Suliman et al., 2011). To obtain more insights into potential compensatory effects of truncated Notch1 or ICN3 proteins, nuclear extracts of *Notch1*-deleted CD25^{int} DN3a cells could be probed for the presence of these proteins.

Kreslavsky et al. (2010) reported that a *Bcl2* transgene can rescue the survival of thymocytes in the absence of Notch signalling. I made use of this system to investigate the Notch-regulated transcriptome independently of its role in cell survival. *In vitro* OP9-DL1 stimulation of *Vav-Bcl2* transgenic DN3a cells confirmed several of the target genes that were detected upon *in vivo* deletion. Furthermore, as several genes were activated already after short-term induction of Notch signalling, they are likely to be direct Notch target genes. Upon prolonged Notch stimulation, several genes were activated, which are usually upregulated upon β -selection. Activation of Notch resulted in a fast upregulation of *Ptcra* transcripts, and hence, the potential formation of a functional pre-TCR. Consequently, these genes might be co-regulated by Notch and pre-TCR signalling. In order to discriminate between both signal transduction pathways, a similar experiment could be conducted with *Rag*-deficient DN3a cells, in which the formation of a functional pre-TCR is prevented. Alternatively, the activation of Notch signalling under conditions, where translation is inhibited through compounds such as cycloheximide, might allow discriminating between direct Notch-mediated effects and secondary effects caused by the upregulation of factors, which are downstream of Notch signalling.

3.3 The role of Notch signalling in post- β -selection thymocytes

Notch signalling controls survival, TCR β locus rearrangement and pre-TCR α chain expression of pre- β -selection thymocytes, but it is not required for their proliferation, as these cells are quiescent. In contrast, Notch induces the proliferation of post- β -selection thymocytes, which is required for their developmental progression (Kreslavsky et al., 2012). I compared the Notch-regulated transcriptome as well as the genome-wide occupancy of Rbpj in DN3a and DN3b cells to explain the different outcomes of the same signalling pathway before and after β -selection.

The Notch-regulated transcriptome in post- β -selection cells comprises only a small subset of the Notch-regulated genes identified in pre- β -selection thymocytes. Furthermore, Notch activation did not result in the activation of any novel target genes at the post- β -selection stage. This is in line with the observation that there are hardly any novel Rbpj-binding sites in post- β -selection cells. Instead, Rbpj-binding was globally reduced in these cells compared to DN3a cells. The reduced level of Notch signalling upon β -selection might lead to the genome-wide reduction of Rbpj occupancy, which was shown to be Notch-dependent.

It has to be noted that the DN3b cells were deprived of Notch signalling prior to the OP9-DL1-mediated restimulation of Notch. Perturbation of the Notch pathway results in reduced expression of the pre-TCR α chain, and thus, most likely in reduced pre-TCR signalling. The pre-TCR pathway is the central signal transduction pathway of post- β -selection thymocytes and likely acts in concert with Notch to control the expression of genes in these cells. Thus it is conceivable that reduced pre-TCR levels in Notch signalling-deprived post- β -selection thymocytes limit the detection of some genes, which are co-regulated by both pathways. To circumvent this limitation, a similar experiment could be performed with cells that express a transgenic pre-TCR (Lacorazza et al., 2001). Nevertheless, *Myc* is among the Notch1 target genes in post- β -selection cells. Its role in proliferation likely represents a fundamental function of Notch1 signalling at this cell stage. Clonal expansion upon β -selection is critical in order to generate sufficient T cell progenitors prior to positive and negative selection. Moreover, Kreslavsky et al. (2012) showed that the ectopic activation of proliferation through overexpression of *Myc* is sufficient to rescue DP progression in the absence of Notch1 signalling.

Even though the expression of *Notch1* is reduced upon β -selection, the surface level of the Notch receptor on post- β -selection cells is slightly increased compared to DN3a cells. Furthermore, Kleinmann et al. (2008) showed a strong reduction of ICN1 levels upon β -selection when the lysates of DN3 and DN4 cells were probed by immunoblotting for ICN1. Taken together, this could suggest that activation of the Notch1 receptor might be reduced upon β -selection. It is known that this downregulation of Notch signalling has a tumor-suppressive

function, since prolonged or elevated Notch1 signalling beyond the β -selection checkpoint is oncogenic (reviewed by Grabher et al., 2006). But it is not fully understood how this downregulation of Notch1 signalling is achieved. *Notch1* transcription is repressed through Id3-mediated repression of E2a upon pre-TCR signalling. (Yashiro-Ohtani et al., 2009). Furthermore, the transcription factor Ikaros was suggested to antagonise Notch1 signalling in post- β -selection thymocytes by competing with Rbpj for binding to Notch target genes (Kleinmann et al., 2008; Geimer Le Lay et al., 2014). The data presented here might suggest an alternative mechanism, in which the DLL-mediated activation of the Notch1 receptor is reduced upon β -selection. The glycosyltransferase Lunatic fringe catalyses the addition of N-acetylglucosamines onto specific EGF-like repeats of the Notch1 receptor during early T cell development and thereby enhances Delta-to-Notch signalling (Visan et al., 2006; Yuan et al., 2011). Interestingly, the expression of *Lfng*—the gene encoding the glycosyltransferase—is reduced upon β -selection (expression profiling data presented here and Mingueneau et al., 2013). As a consequence, a reduced generation of DLL signalling-competent Notch1 receptors upon β -selection might result in lower levels of Notch1 signalling, and ultimately, may have a tumour-suppressive function. In order to investigate this, the Notch1 receptor levels could be analysed by FACS in transgenic mice that overexpress *Lfng* in thymocytes (Koch et al., 2001). According to the model suggested here, prolonged *Lfng* expression could cause a reduced Notch1 surface level on post- β -selection cells compared to control cells. However, it has to be noted that Notch1 is known to regulate its own expression (Yashiro-Ohtani et al., 2009). Thus, prolonged Notch1 activation in post- β -selection thymocytes might also partially increase Notch1 surface levels.

3.4 The role of Notch signalling in lymphocytes prior to β -selection

The investigation of the Notch-regulated transcriptomes in combination with the genome-wide binding profiles of Rbpj and ICN1 in uncommitted lymphoid progenitors as well as in DN3a cells presented here, identified several previously described as well as novel potential Notch target genes. Among the previously described Notch target genes are *Hes1*, *Dtx1*, *Nrarp*, *Ptcra*, *Notch1*, *Myc*, *Il2ra*, *Gata3* and several other genes. The identification of these canonical Notch targets validates the overall applicability of the approach presented here.

The directly Notch1-regulated transcriptome in uncommitted lymphoid progenitors is distinct to the one in DN3a cells. While some canonical Notch target genes, such as *Notch1*, *Notch3*, *Hes1* or *Dtx1*, are activated by Notch in both cell types, the majority is regulated in only one cell type. It is conceivable that this is achieved through the cell stage-specific binding of ICN1/Rbpj. As shown by Bio-ChIP-seq, binding of ICN1/Rbpj is dynamic in the course of T cell differentiation. While there are genes, such as *FasI* or *Gata3*, which are only Notch-

regulated and bound by ICN1/Rbpj in uncommitted progenitors, other genes, such as *Ptcra* and *Il2ra*, are regulated by Notch in a DN3a cell-specific manner. Several factors could determine such a cell type-specific control of gene expression. As the binding of Rbpj seems to be Notch-dependent, the level of Notch signalling, and hence, the amount of nuclear ICN1 might dictate, which genes are bound by ICN1/Rbpj. Notch signalling is at the top of the hierarchy of the transcription factor network that controls T cell differentiation (Kueh and Rothenberg, 2012; Rothenberg, 2014). Expression levels of several transcription factors change within the differentiation of multipotent progenitors to T lineage committed thymocytes (David-Fung et al., 2009; Mingueneau et al., 2013). On one hand, other transcription factors might regulate the binding of ICN1/Rbpj to *cis*-regulatory elements through mechanisms, such as cooperative binding or through the regulation of chromatin accessibility. On the other hand, the ability of ICN1/Rbpj to activate transcription might be affected through other transcription factors that co-regulate the expression of the gene. Accordingly, it could be of interest to investigate the local chromatin changes at ICN1/Rbpj-binding sites as well as changes in transcription factor occupancy within the vicinity of Notch target genes during the course of thymocyte differentiation. Unfortunately, there is hardly any genome-wide occupancy data available for other transcription factors in early T cell progenitors, except for E2a (Miyazaki et al., 2011), Gata3 and PU.1 (Zhang et al., 2012b). Two recent studies aimed to address the changes of the chromatin status during thymopoiesis by profiling of active and repressive histone modifications at distinct stages of T cell development (Zhang et al., 2012b; Vigano et al., 2013). Exploration of these datasets might also provide insights into, why some previously reported direct Notch target genes, such as *Tcf7*, respond to Notch signalling only after long-term stimulation of the pathway. For instance, it was shown that a distal enhancer of the T lineage-specific transcription factor *Bcl11b* is initially decorated with repressive chromatin in DN1 cells, and activation of this enhancer coincides with maturation of the cells (Li et al., 2013).

The functions of the identified Notch-regulated target genes in uncommitted T cell progenitors as well as in DN3a thymocytes are of a pleiotropic nature. Pathway analysis tools such as DAVID (<http://david.abcc.ncifcrf.gov/>) or Ingenuity (IPA, QIAGEN Redwood City, www.qiagen.com/ingenuity) did not point towards a Notch-driven regulation of genes of a specific pathway or process, except for the Notch pathway (*Notch1*, *Notch3*, *Dtx1*, *Hes1*, *Nrarp*). This finding is not surprising, since a similar result was obtained for another lymphocyte lineage commitment factor, namely Pax5, that controls the function, identity and development of B cells (Revilla-I-Domingo et al., 2012). This study, together with a complementary microarray expression profiling approach by Schebesta et al. (2007), showed that Pax5 directly regulates the expression of genes that encode signalling and adhesion receptors, intracellular signal transducers and transcriptional regulators. Interestingly, there was little overlap between

Pax5-regulated genes in pro-B and mature B cells, similar to what I observed for the Notch-regulated genes in uncommitted T cell progenitors and DN3a thymocytes. Revilla-I-Domingo et al. (2012) suggested that global changes in the expression profile upon B cell maturation, which are accompanied by changes in the chromatin landscape of the cells, result in a different spectrum of Pax5 target genes. Similar mechanisms are conceivable for Notch target genes during T cell differentiation.

During the earliest stages of T cell development, Notch1 is required to suppress non-T lineage potentials and promote T cell lineage development of multipotent progenitors. Repression of lineage-inappropriate genes during B cell development is directly mediated by a Pax5-dependent recruitment of co-repressor complexes to these genes (McManus et al., 2011; Medvedovic et al., 2011). However, activation of Notch signalling leads to the recruitment of several transcriptional co-activators (see Section 1.3.3.3). The Notch pathway thus functions as positive regulator of transcription, which is supported by the gene expression profiling and Bio-ChIP-seq results presented in this study. This rises the question, how Notch signalling represses lineage-inappropriate genes. Among the directly Notch-regulated target genes in LMPPs are *Gata3* and *Hes1*, which both encode transcription factors that directly repress genes of the B and myeloid lineage, respectively (De Obaldia et al., 2013b; García-Ojeda et al., 2013). Consequently, Notch might indirectly control T lineage commitment through the direct activation of transcriptional repressors. OP9-DL1 co-cultured LMPPs repress *Cepba*, which encodes the myeloid lineage-specific transcription factor C/EBP α . This repression is likely to be the result of *Hes1* activation by Notch. *Gfi1b*, which encodes the transcription factor Gfi1b (growth factor independent 1B) was also repressed upon Notch activation of LMPPs. Schulz et al. (2012) showed that Gfi1b negatively regulates *Rag1* and *Rag2* expression in the B cell lineage. As Rag activity is required for *Tcr* locus rearrangement during T cell development, an indirectly Notch-mediated downregulation of *Gfi1b*, and thus, a derepression of *Rag* genes, might be important for thymocyte development. In accordance with this hypothesis, *Gfi1b* expression is downregulated *in vivo* upon transition to the DN3a stage (IMMGEN database and the expression profiling data presented here)

Gata3 as well as *Lck* belong to a group of genes that is Notch1-dependent in LMPPs but not in DN3 cells, where the expression levels of both genes peak. *Lck* is of particular interest, as it encodes the tyrosine kinase Lck that integrates pre-TCR and TCR signals (Papatriantafyllou, 2013). Signalling through the (pre-)TCR is not required prior to the DN3a stage. Accordingly, *Lck* expression is not required at the LMPP stage, where it is Notch1-dependent, but from the DN3a stage onwards, where it is Notch1-independent. This suggests that Notch1 initially induces the expression of genes with critical roles for the T cell lineage. At later stages of

development, however, their expression is maintained by other members of the transcription factor network that controls T cell differentiation.

Notch signalling directly activates many genes in LMPPs, which are only transiently up-regulated during the ETP or DN2 stages. Many of these genes are specifically expressed in non-T cell lineages. It is conceivable that activation of these genes is a non-specific byproduct of Notch signalling and that continued expression of these genes is incompatible with T cell differentiation. Therefore, these genes must be repressed by other factors. A likely scenario could be that this is mediated by a Notch1-induced transcriptional repressor. Potential candidates for this function are Hes1, Gata3, Bcl11b and/or TCF-1. As the latter three exhibit a delayed expression profile compared to Hes1, and since the transiently expressed genes are mostly repressed at the DN2 stage, Hes1 seems to be the most promising candidate. Accordingly, it would be of interest to investigate if the transiently expressed genes are bound by Hes1, and if their expression is prolonged to later DN2 or DN3 stages in the absence of Hes1.

The Bray lab studied the genome-wide response to Notch activation in *Drosophila* muscle progenitor cells through transcriptome profiling and Su(h) ChIP (Krejci et al., 2009). The authors observed several cases of so called 'incoherent network logic', where Notch directly induces the expression of both a gene and the repressor of this gene. The result of such incoherent feed-forward loops in transcription factor networks is a transient window of competence, in this case, after Notch activation. If such Notch-activated network loops are conserved, and whether they have an effect on haematopoiesis, remains to be determined. Of note, the microarray analysis by Krejci et al. (2009) as well as the RNA-seq analyses in LMPPs and ETPs presented here, were performed on a population level. Therefore, it is possible that the transcripts of the transiently expressed genes originate from cells, that received low levels of Notch signalling or no Notch signals at all. A single cell gene expression analysis of LMPPs after Notch stimulation could answer this question.

Among the Notch-regulated target genes in DN3a cells are several genes, such as *Myc* (Dose et al., 2006) or *Ptcra* (Fehling et al., 1995), that were previously reported to be Notch target genes with important roles during T cell development. In addition, Notch was reported to regulate the PI3K/Akt pathway in DN3 cells, which is required for pre-TCR-induced survival (Ciofani and Zúñiga-Pflücker, 2005; Shah and Zúñiga-Pflücker, 2014). However, none of the directly Notch-regulated genes in DN3a cells has a direct function in the PI3K/Akt pathway. This observation could be explained by an indirect regulation of the PI3K/Akt pathway by Notch, such as the previously reported Hes1-dependent repression of the PI3K/Akt pathway inhibitor Pten (Wong et al., 2012a). The directly Notch-activated target gene in DN3a cells *Mpzl2* (myelin protein zero-like 2), also known as *Eva1* (epithelial V-like antigen 1), was previously not connected to Notch signalling. The encoded protein is a homotypic ad-

hesion molecule, which is also expressed by cells of the thymic stroma (Guttinger et al., 1998). *Eva1* was thought to mediate homotypic adhesion interactions between thymocytes and thymic stroma (Tydell et al., 2007). The depletion of *Mpzl2* resulted in delayed DN1-DN3 transition of progenitor cells *in vitro* as well as in a defect in thymocyte maturation *in vivo*, which showed its requirement for thymocyte development (Iacovelli et al., 2009). The gene is located adjacent to the *Cd3* cluster. The neighbouring gene *Cd3e* contains two prominent ICN1/Rbpj-binding sites, which represent the closest binding sites relative to *Mpzl2*. As the expression of *Cd3e* is Notch1-independent, these sites might actually control the expression of *Mpzl2* (and of *Cd3d*) at the DN3a stage.

Several other directly Notch-regulated genes in DN3a cells seem to be dispensable at this stage, such as *Hes1* (Wendorff et al., 2010) and *Dtx1* (Storck et al., 2005; Lehar and Bevan, 2006). Accordingly, it is important to determine which of the identified Notch target genes in DN3a cells (as well as in uncommitted progenitors or post- β -selection cells) are functionally relevant for thymopoiesis. A multiplexed negative-selection RNA interference (RNAi) screen, similar to screens that aimed to identify essential genes in acute myeloid leukaemia (Fellmann et al., 2011; Zuber et al., 2011a,b), could be performed to answer this question. To this end, haematopoietic progenitor cells are transduced with a library of small hairpin RNAs (shRNAs) against the identified Notch-regulated target genes, and the changes in the composition of the shRNA pool are monitored over the course of T cell differentiation. High-throughput sequencing technology allows to analyse the composition of the shRNA pool within the cell population over time. Knockdown of a gene with an essential function in thymopoiesis should result in the loss of the cells that express the respective shRNA within the cell pool. Such a screen can be performed in the *in vitro* OP9-DL1 co-culture system in order to prevent skewing of the shRNA representation, which is likely to occur *in vivo* at the point of thymus seeding. This approach could ultimately reveal which Notch-regulated genes affect thymocyte development, and thus, the function of Notch at different stages of T cell development.

3.5 Insights into the Notch-mediated regulation of gene expression

Information on the genome-wide Rbpj and ICN1 occupancy has provided insights about, how Notch signalling regulates the expression of its target genes. Interestingly, some of the findings presented here, are contradictory to previously published results.

For instance, Maillard et al. (2006) suggested that Notch signalling regulates *Il2ra* (CD25) expression in DN thymocytes through Rbpj-binding sites in the promoter and/or an enhancer that is located within intron 3. As determined by Bio-ChIP-seq in DN3a cells, neither ICN1 nor ICN1 bind to these elements at this stage. However, there are two sites within the *Il2ra*

locus with prominent Rbpj occupancy. The site, that is ~20 kb upstream of the *Il2ra* promoter, is also co-occupied by ICN1. The second prominent Rbpj-binding site is located in intron 1 and is not bound by ICN1. Interestingly, Rbpj-binding to the site ~20 kb upstream of the promoter is lost in CD25⁺ post- β -selection thymocytes, while Rbpj-binding to the intronic binding site is less affected. This could suggest that the upstream element acts as the enhancer, which controls *Il2ra* expression in response to Notch1 signalling.

Deregulation of *Myc* expression is a critical event in the development of Notch-dependent T-ALL. Several studies in murine T-ALL cell lines as well as in DN3 cells suggest a Notch-dependent activation of *Myc* through an ICN1/Rbpj-bound element, which is located ~1.5 kb upstream of the *Myc* promoter (Palomero et al., 2006; Sharma et al., 2006; Weng et al., 2006). Despite rapid upregulation of *Myc* upon *in vitro* Notch activation in DN3a cells, Bio-ChIP-seq did not detect prominent ICN1 or Rbpj occupancy anywhere close to the *Myc* locus. *Myc* expression was shown to be regulated in acute myeloid leukaemia cells through a super-enhancer, which is located 1.7 megabases downstream of the *Myc* locus (Shi et al., 2013). This *cis*-regulatory element is occupied by Rbpj and ICN1 in *Pax5*-mutant progenitors, yet, *Myc* is repressed by Notch in these cells. Binding of Rbpj and ICN1 to the super-enhancer is stage-specific, since this element is not bound in DN3a cells. In DN3a cells, ICN1/Rbpj co-occupy two sites that are located in between the super-enhancer and the *Myc* gene. Hence, it is intriguing to speculate that one of these sites is involved in the regulation of *Myc* in DN3a cells.

In general, even though ICN1- and Rbpj-binding was predominantly detected at the promoters of genes, many of the Notch targets, did not exhibit ICN1 and Rbpj occupancy at their promoters. This suggests that Notch signalling regulates the expression of several genes via enhancers. For some of the Notch1-regulated genes, potential ICN1/Rbpj occupied *cis*-regulatory elements were found several hundred kb away from the promoter of the genes. This suggests that Notch signalling controls the expression of some genes through distal *cis*-regulatory elements that interact with the promoter of the Notch-regulated gene. In accordance with this hypothesis, Wang et al. (2013) reported a Notch-mediated regulation of target gene expression through interactions with super-enhancers. These *cis*-regulatory elements control the expression of genes with crucial roles in lineage specification during development (Whyte et al., 2013) as well as of oncogenes in transformed cells (Hnisz et al., 2013; Wang et al., 2013). Since genome-wide information on these elements is available for several (leukaemia) cell lines, it is on one hand interesting to determine which of the ICN1/Rbpj occupied elements qualify as super-enhancers. On the other hand, it might be informative to define super-enhancers in uncommitted T cell progenitors or DN3a cells. In the case of DN3a cells, ChIP-seq data for factors that are enriched in super-enhancers, such as BRD4, mediator (MED1) and H3K27ac, could be generated and integrated into the *cis*-regulatory landscape presented here.

Besides the previously mentioned ICN1/Rbpj-occupied *cis*-regulatory elements, which might mediate the Notch-dependent expression of *Myc*, ICN1/Rbpj-occupied enhancers or promoters of other genes with crucial roles in thymopoiesis could be analysed for their Notch-dependency. Interesting candidates are the downstream enhancer of *Gata3* (Hosoya-Ohmura et al., 2011), *Bcl11b* (Li et al., 2013) as well as ICN1/Rbpj-occupied sites upstream of *Tcf7* (Germar et al., 2011). To test these elements for their Notch-dependency, classical *in vitro* reporter assays could be performed. However, their *in vivo* Notch-dependency can ultimately only be proven through mutation of the Rbpj motif at the endogenous enhancer locus. The CRISPR/Cas9 genome editing technology could be used to introduce the required mutations of the Rbpj motifs within potential Notch-dependent *cis*-regulatory elements (Gilbert et al., 2013; Jinek et al., 2012). On one hand, these mutations could be introduced into *in vitro* cultured *Pax5*-mutant progenitors that after transfer onto OP9-DL1 feeders, should fail to up-regulate the corresponding potential Notch target gene of the mutated *cis*-regulatory element. On the other hand, these mutations could be introduced into ES cells, which can be used to generate transgenic mice to test the functionality of the ICN1/Rbpj-binding site *in vivo* during thymopoiesis.

3.6 The role of Notch signalling in the regulation of the *Tcrb* locus

Notch signalling was implicated in *Tcrb* rearrangement (Wolfer et al., 2002); yet the molecular mechanism remains unexplored. It is conceivable that Notch controls the expression of genes that encode proteins of the recombination machinery. The aforementioned Notch-dependent repression of *Gfi1b* and its potential impact of *Rag* gene expression might point towards a role of Notch in expression of genes associated with the V(D)J recombination machinery. However, neither *Rag* genes themselves were found to be Notch-regulated, nor genes encoding other members of the recombination machinery were among the Notch-regulated targets in my RNA-seq analysis. Instead, RNA-seq of LMPPs revealed a Notch-dependent expression of *Tcrb* GLTs, which is likely to be regulated directly via ICN1/Rbpj-binding sites in the *Tcrb* locus enhancer E_β . The promoters $PD_\beta 1$ and $PD_\beta 2$ were shown to initiate the expression of sterile D_β - J_β - C_β transcripts (Sikes et al., 1998, 1999; McMillan and Sikes, 2008; Abarrategui and Krangel, 2009). Expression of these GLTs induces active chromatin within the D_β - J_β - C_β region and results in repositioning of nucleosomes downstream of the PD_β promoters. This together allows the recombination machinery to access the D_β - J_β - C_β region and thereby facilitates D_β -to- J_β rearrangements (Abarrategui and Krangel, 2006, 2007; Liu et al., 2007; Matthews et al., 2007; Osipovich et al., 2007; Kondilis-Mangum et al., 2010).

Like most promoters, PD $_{\beta}1$ and PD $_{\beta}2$ themselves do not confer a T cell-specific expression of their transcripts (Sikes et al., 1999). Instead their activity was shown to depend on the E $_{\beta}$ enhancer, which physically interacts with both germline promoters (Oestreich et al., 2006). Interestingly, the promoter-enhancer interaction generates a holocomplex containing two histone acetylases (p300/CBP and PCAF) that are known to be recruited by ICN1 (Spicuglia et al., 2002; Oestreich et al., 2006). Deletion of E $_{\beta}$ (Bories et al., 1996; Bouvier et al., 1996; Leduc et al., 2000; Ryu et al., 2003; Bonnet et al., 2009b) or of PD $_{\beta}1$ (Whitehurst et al., 1999, 2000) partially prevent the formation of active chromatin within the D $_{\beta}$ -J $_{\beta}$ -C $_{\beta}$ region, and consequently impairs D $_{\beta}$ -to-J $_{\beta}$ rearrangements. Wolfer et al. (2002) reported a defect in V $_{\beta}$ -to-DJ $_{\beta}$ recombination upon *Lck*-Cre-mediated deletion of *Notch1*, while D $_{\beta}$ -to-J $_{\beta}$ rearrangements were not affected. This is surprising, since my results point towards a role of Notch in D $_{\beta}$ -to-J $_{\beta}$ recombination. However, D $_{\beta}$ -to-J $_{\beta}$ rearrangements mainly occur at the DN2 stage (Godfrey et al., 1994), where *Lck*-Cre-mediated deletion is incomplete. This could explain why Wolfer and colleagues did not observe a defect in D $_{\beta}$ -to-J $_{\beta}$ rearrangements.

The E $_{\beta}$ -mediated regulation reaches across an ~25 kb large region 5' of the enhancer, encompassing both D $_{\beta}$ -J $_{\beta}$ -C $_{\beta}$ clusters but none of the 5' V $_{\beta}$ genes (Mathieu et al., 2000). This rises the question why the loss of ICN1 might affect in V $_{\beta}$ -to-DJ $_{\beta}$ rearrangements, in particular since there is basically no prominent ICN1/Rbpj occupancy within the entire *Tcrb* locus except for E $_{\beta}$. A possible explanation is the requirement of active chromatin within DJ $_{\beta}$ region to facilitate V $_{\beta}$ -to-DJ $_{\beta}$ rearrangements. This hypothesis is supported by the 'recombination centre model', which was put forward by the Schatz laboratory. This model defines the recombination centre as a region of an antigen receptor locus that is characterised by strong binding of Rag, high levels of RNA polymerase II-mediated germline transcription, histone acetylation and H3K4me3 (Schatz and Ji, 2011). The two D $_{\beta}$ -J $_{\beta}$ -C $_{\beta}$ clusters qualify as such recombination centres, since they meet all of these criteria (Schatz and Ji, 2011). Mechanistically, this model proposes that the V(D)J recombination machinery pre-assembles at these centres due to the presence of the previously mentioned features. This is followed by large-scale chromatin reorganisation that, in the case of the *Tcrb* locus, positions V $_{\beta}$ gene segments proximal to the recombination centre, which facilitates V $_{\beta}$ -to-DJ $_{\beta}$ recombination. Taken together, Notch might play two different roles in the regulation of the *Tcrb* locus. On one hand, Notch signalling could induce the expression of GLTs to introduce active chromatin and to allow D $_{\beta}$ -to-J $_{\beta}$ rearrangements. On the other hand, Notch signalling might be required to maintain active chromatin at D $_{\beta}$ -J $_{\beta}$ -C $_{\beta}$ recombination centres to allow V $_{\beta}$ -to-DJ $_{\beta}$ rearrangements. The loss of active chromatin upon *Notch1* deletion within recombination centres at the *Tcrb* locus could be investigated by ChIP for active histone modifications in FACS-purified Rag2-deficient CD25^{int} DN3a cells. As described for the analysis of other interesting ICN1/Rbpj-binding sites (see above), only

the mutation of the endogenous Rbpj motifs in the E_β enhancer can reveal the potential Notch-dependent regulation of E_β enhancer activity. Consequently, ES cells with specific mutations of the Rbpj motifs in the E_β enhancer can be generated and used for blastocyst injections to generate transgenic mice. The mutations should result in the loss of ICN1/Rbpj-binding to the E_β enhancer, which can be analysed by ChIP. If the function of E_β is Notch-dependent, the mice should have a defect in *Tcrb* rearrangement, and thus, in $\alpha\beta$ T cell development. Of note, ICN1/Rbpj binding was also observed within *cis*-regulatory elements of the *Tcra/Tcrd* and *Tcrd* locus. Assessing the functionality of the binding sites would require similar strategies to those described above for the *Tcrb* locus.

4 Material and Methods

4.1 Mice

PCR genotyping primer pairs

Locus	MBU ID	fw/rev	5' – 3'
<i>Lck</i> -Cre	1784A	fw	CAATTTACTGACCGTACAC
	1784B	rev	TAATCGCCATCTTCCAGCAG
<i>Mox2</i> -Cre	9599	fw	GGGACCACCTTCTTTTGGCTTC
	9600	rev wt	AAGATGTGGAGAGTTCGGGGTAG
	9598	rev Cre	CCAGATCCTCCTCAGAAATCAGC
<i>Notch1</i> ^{Bio}	11807	fw	TCTTCTGCGAGTCGAGGATT
	11808	rev wt	ATTCTGCCCCAGGAAAGC
	11809	rev Bio	CTGGAAAGGACTCCCAACAG
<i>Notch1</i> ^{fl}	11932	fw	GTCCTGGAATGTGCTTTCACACTG
	11933	rev wt	CAGATCACCACCCTCGGAATCC
	11934	rev Δ	CCGCCTACTCCGACACCCAATAC
<i>Rag2</i> ⁻	1824A	fw	GCAACATGTTATCCAGTAGCCGGT
	1824B	rev wt	TTGGGAGGACACTCACTTGCCAGT
	1824C	rev Neo	GTATGCAGCCGCCGCATTGCATCA
<i>Rbpj</i> ^{Bio}	8578	fw	TTCTGTCACCCAGGGGTTGG
	9613	rev wt	AAGCTTATGCGAGTCCCTTG
	9612	rev Bio	TCTTCTGCGAGTCGAGGATT
<i>Rbpj</i> ^{fl}	7626	fw	GATGAGATGCTGACATCTGAG
	7627	rev wt	GGTACCTGGTACTAACTGTCT
	11765	rev Δ	GCTGAGAGACAAGCCTAGAA
<i>Rosa26</i> ^{BirA}	7884	fw	GTGTAAGTGTGGACAGAGGAG
	7885	rev wt	GAACCTTGATGTGTAGACCAGG
	10755	rev BirA	GCCAGCCAGAATTTATATGCAG
<i>Vav-Bcl2</i>	5307	fw	ACGGTGTTGGAGGAGCTCTTC
	5231	rev	AAAACCTCCCACACCTCCCCCTGAA

These mouse strains were maintained on the C57BL/6 background: transgenic *Lck*-Cre (Lee et al., 2001); *Meox2*^{Cre} also known as More-Cre (Tallquist and Soriano, 2000); *Notch1*^{Bio} (Dorninger, 2008); *Notch1*^{fl} (Radtke et al., 1999); *Pax5*^Δ (Horcher et al., 2001); *Pax5*⁻ (Urbánek et al., 1994); *Rag2*⁻ (Shinkai et al., 1992); *Rbpj*^{Bio} (unpublished); *Rbpj*^{fl} (Han et al., 2002); *Rosa26*^{BirA} (Driegen et al., 2005); transgenic *Vav-Bcl2* (Ogilvy et al., 1999). Genotyping PCRs were performed with DNA isolated from mouse tail clips. All animal experiments

were done according to valid project licenses, which were approved and regularly controlled by the Austrian Veterinary Authorities.

4.2 Bone marrow transplantation

Bone marrow cells from *Vav-Bcl2* mice (Ly5.2) were depleted of T cells by MACS depletion with TCR β , CD4 and CD8a antibodies. A minimum of 2×10^5 cells was retro-orbitally injected (Yardeni et al., 2011; see) into C57BL/6-Ly5.1 and C57BL/6-Ly5.2 mice, which were γ -irradiated with 12 Gy 24 h and 6 h before injection. Chimerism was analyzed by FACS 8 weeks after reconstitution in thymocyte suspensions of C57BL/6-Ly5.1 mice and was $> 95\%$ donor contribution.

4.3 Intraperitoneal injection of mice with CD3 ϵ antibody

Rag2^{-/-} or *Rbpj*^{Bio/Bio} *Rosa26*^{BirA/BirA} *Rag2*^{-/-} mice were injected intraperitoneally with 300 μ g of purified CD3 ϵ antibody (clone: 145-2C11). Thymus suspensions were analyzed by FACS 24 h, 48 h or 72 h post-injection. For Rbpj-Bio-ChIP-seq, thymi were harvested 48 h post-injection and single cell suspensions were immediately crosslinked with FA (see Section 4.13 for further details).

4.4 Flow cytometry and definition of haematopoietic cell types

Mice between 6-12 weeks of age were used for FACS analysis of lymphoid progenitors and mature T cell types, respectively. Single cell suspensions of respective organs or tissue culture cells were incubated with γ Fc receptor block (BD Biosciences) to block unspecific binding of antibodies to CD16 (Fc γ III) and CD32 (Fc γ II), which are expressed on B cells, monocyte/macrophages, NK cells and neutrophils. Cells were incubated for 20 min at 4°C with the respective antibody cocktail at a density of 4×10^7 cells per ml in FACS staining buffer (1 $\times$ PBS; 2% FCS; 1 mM EDTA). After washing with FACS staining buffer, cells were acquired on a FACS Canto (BD Biosciences) or LSRFortessa cell analyzer (BD Biosciences). Data analysis was done in FACSDiva (BD Biosciences) and FlowJo (TreeStar).

The following antibodies were used for FACS: anti-B220/CD45R (RA3-6B2), CD4 (GK1.5), CD8a (53-6.7), CD11b/Mac1 (M1/70), CD11c (HL3), CD19 (1D3), CD25/IL-2Ra (PC61), CD27 (LG.7F9), CD28 (37.51 or E18), CD34 (RAM34), CD44 (IM781), CD71/Transferrin receptor (RI7217), CD117/c-Kit (ACK4), CD127/IL-7Ra (A7R34), CD135/Flt3

(A2F10.1), Gr1 (RB6- 8C5), NK1.1 (PK136), Notch1 (22E5.5), Ly5.1 (A20), Ly5.2 (104), Sca1/Ly6A (D7), TCR β (H57-597), TCR $\gamma\delta$ (GL3), Ter119 (TER119), Thy1.2 (53-2.1).

The haematopoietic cell types were identified as follows: **LMPP**: Lin⁻ Flt3⁺ Sca1^{hi} c-Kit^{hi}; **DN1/ETP**: CD44⁺ c-Kit^{hi} CD25⁻ CD4⁻ CD8⁻; **DN2**: CD44⁺ c-Kit⁺ CD25⁺ CD4⁻ CD8⁻; **DN2a**: CD44⁺ c-Kit^{hi} CD25⁺ CD4⁻ CD8⁻; **DN2b**: CD44⁺ c-Kit^{int} CD25⁺ CD4⁻ CD8⁻; **DN3**: CD44⁻ CD25⁺ Thy-1.2⁺ CD4⁻ CD8⁻; **DN3a**: CD44⁻ CD25⁺ Thy-1.2⁺ CD28^{lo} CD71^{lo} CD27^{lo} CD4⁻ CD8⁻; **DN3b**: CD44⁻ CD25⁺ Thy-1.2⁺ CD28^{hi} CD71^{hi} CD27^{hi} CD4⁻ CD8⁻; **DN3b-DN4**: CD44⁻ CD25^{int} Thy-1.2⁺ CD28^{hi} CD71^{hi} CD27^{hi} CD4⁻ CD8⁻; **DN4**: CD44⁻ CD25⁻ Thy-1.2⁺ CD4⁻ CD8⁻; **DP**: CD4⁺ CD8⁺; **CD4-SP**: CD4⁺ CD8⁻; **CD8-SP**: CD4⁻ CD8⁺; *in vitro*-cultured **pro-B** cells: CD19⁺. Lin mix: Gr1, CD11c, CD11b, CD19, TCR β , TCR $\gamma\delta$, CD4, CD8a, Ter119, NK1.1.

4.5 FACS sorting for RNA-seq

For cells that were subjected to RNA-seq after cell-sorting, EDTA was omitted in FACS staining buffer as it elicits Notch activation (Rand et al., 2000). FACS sorting was performed on FACS Aria I or FACS Aria III machines (BD Biosciences). For the *Lck*-Cre-mediated deletion of *Notch1* and *Rbpj* in DN3a and DN3b thymocytes, a minimum of two to six male and female littermates were sacrificed. To analyse the deletion of *Notch1* in Rag2-sufficient cells, experiential *Notch1*^{fl/fl} *Lck*-Cre CD25^{hi} or CD25^{int} DN3a cells as well as control *Notch1*^{fl/fl} DN3a were sorted. To analyse the deletion of *Rbpj* in Rag2-sufficient cells, experiential *Rbpj*^{fl/fl} *Lck*-Cre DN3a and DN3b cells as well as control *Rbpj*^{fl/fl} DN3a and DN3b cells were sorted. To analyse the deletion of *Notch1* in Rag2-deficient cells, experiential *Notch1*^{fl/fl} *Rag2*^{-/-} *Lck*-Cre CD25^{hi} or CD25^{int} DN3a cells as well as pooled *Notch1*^{fl/fl} *Rag2*^{-/-} and *Notch1*^{fl/fl} *Rag2*^{-/-} DN3a were sorted. Genotyping PCRs were performed on DNA isolated from sorted cells. For *in vitro* OP9-DL1 stimulation experiments with committed thymocytes, DN3a and DN3b-DN4 cells from approximately 50 *Vav-Bcl2* bone marrow chimeric mice were sorted. Cell fixation and permeabilization for intracellular staining of TCR β were done with the Cytofix/Cytoperm™ kit (BD Biosciences). Approximately 50 male wild-type mice were sacrificed to obtain LMPPs, ETPs/DN1, DN2a and DN2b cells for *in vivo* expression profiles as well as for Notch-stimulation experiments with LMPPs. To reduce cell aggregates during the preparation of single-cell suspensions, 1 μ g/ml DNaseI (Sigma) was added to the FACS buffer. For Notch stimulation of *Pax5*-deficient pro-B cells, a *Pax5*^{-/-} *Vav-Bcl2* pro-B cell line was expanded on OP9-GFP cells, sorted and replated on either OP9-GFP or OP9-DL1 cells. All *in vitro* Notch-stimulation experiments were performed at a cell density of 5×10^5 progenitor cells per one well of a 24-well cell culture plate.

4.6 OP9-GFP and OP9-DL1 feeder cell culture

Stromal OP9-GFP and OP9-DL1 feeder cell lines (Schmitt and Zúñiga-Pflücker, 2002) were cultured in IMDM supplemented with 10% FCS (Gibco), 100 U/ml penicillin, 0.1 mg/ml streptomycin and 2 mM glutamine (all Sigma). Cells were seeded at a density of approximately 10^6 cells per 15 ml in a 20 cm cell culture dish and passaged every 2-3 days in a ratio of 1:3.

4.7 *In vitro* expansion of T cell progenitors

DN1 and DN2 progenitors were enriched from thymus suspension of 3 week-old *Notch1*^{Bio/Bio} *Rosa26*^{BirA/BirA} *Rag2*^{-/-}, *Rbpj*^{Bio/Bio} *Rosa26*^{BirA/BirA} *Rag2*^{-/-} and *Rosa26*^{BirA/BirA} *Rag2*^{-/-} mice by immunomagnetic (c-Kit) selection (Miltenyi Biotec). Cells were plated on 6 cm cell culture dishes that were seeded 24 h earlier sub-confluent with stromal OP9-DL1 cells. Thymocytes were co-cultured in IMDM supplemented with 1% supernatant of rIL-7 producing J558L cells (Karasuyama and Melchers, 1988), 10% FCS (Gibco), 100 U/ml penicillin, 0.1 mg/ml streptomycin, 50 μ M 2-Mercaptoethanol (2-ME) and 2 mM glutamine (all Sigma), for up to 4 weeks, while the cells were transferred every 2-4 days onto fresh feeder monolayers.

4.8 Pro-B cell culture for Bio-ChIP-seq and RNA-seq

Notch1^{Bio/Bio} *Rosa26*^{BirA/BirA} *Pax5* ^{Δ/Δ} *Vav-Bcl2* or *Rbpj*^{Bio/Bio} *Rosa26*^{BirA/BirA} *Pax5* ^{Δ/Δ} pro-B cells for Bio-ChIP-seq and *Pax5*^{-/-} *Vav-Bcl2* pro-B cells for RNA-seq were obtained from bone marrow suspensions of 2-3 week-old mice by immunomagnetic (B220) selection (Miltenyi Biotec). Cells were plated on 6 cm cell culture dishes that were seeded 24 h earlier sub-confluent with stromal OP9-GFP cells. Pro-B cells were co-cultured in IMDM supplemented with 1% supernatant of rIL-7 producing J558L cells (Karasuyama and Melchers, 1988), 10% FCS (Gibco), 100 U/ml penicillin, 0.1 mg/ml streptomycin, 50 μ M 2-ME and 2 mM glutamine (all Sigma), as previously described by Nutt et al. (1997). Approximately 2×10^7 pro-B cells were plated on two 20 cm confluent OP9-DL1 feeder cells dishes in 10 ml pro-B cell culture medium for *Notch1*^{Bio/Bio} *Rosa26*^{BirA/BirA} *Pax5* ^{Δ/Δ} *Vav-Bcl2* Bio-ChIP-qPCR time courses, while for *Rbpj*^{Bio/Bio} *Rosa26*^{BirA/BirA} *Pax5* ^{Δ/Δ} the volume was reduced to 5 ml. Cells were subsequently crosslinked at room temperature for 10 min on the plates by addition of 1 volume of pro-B cell medium with 2% FA (Sigma). The pro-B cells were harvested from the plates during the glycine quenching step without transfer of feeder cells. $\sim 3 \times 10^8$ cells were used for Bio-ChIP-seq experiments (see Section 4.13 for further details).

4.9 Antibodies

Rabbit polyclonal antibodies recognising the following histone tail modifications were used for ChIP-seq analysis: H3K4me3 (Abcam; ab8580), H3K4me2 (Millipore; 07-030), H3K9ac (Millipore; 07-352) and H3K27me3 from T Jenuwein (MPI Freiburg). A rabbit monoclonal antibody against a synthetic peptide corresponding to the sequence at the Val1754 cleavage site in human Notch1, which is equivalent to Val-1744 in mouse Notch, was used for ICN1 ChIP-qPCR (Cell Signaling Technology; clone D3B8; #4147). Rbpj-Bio and ICN1-Bio proteins were detected in immunoblots with a V5 antibody (produced in-house; clone 5C5).

4.10 CAGE analysis

RNA was isolated from whole thymocyte suspensions of *Rag2*^{-/-} mice. Total RNA (~50 mg) was sent to ImaGenes (Berlin) for preparation of CAGE libraries, which were analysed by Solexa deep sequencing.

4.11 DHS site mapping

Single cell suspensions of thymi from 7 week-old *Rag2*^{-/-} mice were prepared and purified twice using density gradient purification with Lympholyte M (Cedarlane Laboratories). Approximately 4×10^7 permeabilised cells were treated with DNase I followed by cell lysis and isolation of DNA fragments between 150 and 250 bp by sucrose gradient centrifugation as previously described by Sabo et al. (2006). Fractions were analysed by qPCR for enrichment of DHS sequences. The fractions with the highest enrichments were pooled and analysed by Solexa deep sequencing.

4.12 ChIP-seq analysis of histone modifications

Single cell suspensions of thymi from 4-8 week-old *Rag2*^{-/-} mice were prepared and purified once using density gradient purification with Lympholyte M (Cedarlane Laboratories). ChIP with histone modification-specific antibodies was performed as described by Schebesta et al. (2007). The precipitated DNA was analysed by qPCR (Decker et al., 2009) for enrichment of genomic regions that are known to contain the analysed active or repressive histone modifications prior to Solexa deep sequencing.

4.13 Transcription factor ChIP analysis

For ICN1-Bio and Rbpj-Bio ChIP-seq from Rag2-deficient mice, whole thymocyte single cell suspensions of five mice were prepared simultaneously and fixed immediately in 5 ml IMDM containing 1% FA for 10 min at RT. The reaction was quenched with 0.25 M glycine for 5 min at RT. For whole cell lysis ChIP, cells were washed twice with ice-cold PBS supplemented with 2% FCS. The cell pellet was resuspended in ice-cold ChIP lysis buffer (25 mM Tris-HCl pH 8.0, 2 mM EDTA, 1% SDS, Complete® protease inhibitors (Roche)) and incubated overnight at 4°C at a concentration of 5×10^7 cells per ml. The crosslinked chromatin was mechanically sheared by sonication to an average fragment size of ~500 bp by circa seven 30 s pulses using a Bioruptor™ (Diagenode). Cell debris were removed by centrifugation and the chromatin content was quantified with a NanoDrop™ 1000 spectrophotometer (PEQLAB Biotechnologie GmbH) by A260 nm. The lysate was diluted 10-fold using ChIP dilution buffer (25 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, Complete® protease inhibitors). Preclearing was performed with 40 µl magnetic protein A beads (Dynabeads® protein A, Invitrogen) per 400 µg of chromatin for 2 h at 4 °C. Precleared chromatin was incubated for 2 h at RT with 40 µl of SA-coated magnetic beads (Dynabeads® M-280 SA, Invitrogen) for affinityprecipitation. The ICN1-Bio and Rbpj-Bio-containing chromatin complexes were sequestered by magnetic separation and unspecific chromatin was removed by a stringent washing protocol: 3× with stringent washing buffer 1 (0.5 M LiCl, 1 mM EDTA, 1% Nonidet NP-40, 1% Na-deoxycholate), 4× with stringent washing buffer 2 (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 3% SDS) and 2× with TE. Transfer of the beads to fresh tubes during the washing steps and prior to elution helped to remove nonspecific chromatin. Protein-DNA complexes were enzymatically released from the beads by proteinase K digest (500 µg/ml) in 500 µl elution buffer (10 mM Tris-HCl pH7.5, 1 mM EDTA, 1% SDS, 100 mM NaHCO₃, 200 mM NaCl) at 55°C overnight, paralleled by temperature-induced reversal of FA-crosslinks. Purification of ChIP and input DNA (1/100 of IP material) was performed by Phenol:Chloroform:Isoamyl Alcohol (25:24:1) extraction and isopropanol precipitation. The precipitated DNA was quantified by qPCR analysis (Decker et al., 2009) prior to Solexa sequencing. The results of the ChIP efficiency assessment by qPCR was plotted either as ChIP-signal relative to the input or relative enrichment over the average of two negative control (ctrl) regions. The two negative control regions were a gene-desert region on chromosome 1 (Chr1) or a region downstream of the *Cd19* gene (see table below). For Bio-ChIP-seq, 25-50 mice were used for one ChIP and 2-4 experiments were pooled to obtain enough material for library preparation.

Alternatively to the whole cell lysis for ChIP, nuclei were prepared by disruption of the cell membrane in ice-cold buffer A (10 mM HEPES ph 8.0, 10 mM EDTA, 0.5 mM EGTA,

0.25% Triton X-100, Complete[®] protease inhibitors). The nuclei were pelleted after 10 min incubation at 4°C and resuspended in ice-cold buffer B (10 mM HEPES pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.01% Triton X-100, Complete[®] protease inhibitors). After 10 min incubation at 4°C followed by centrifugation, pelleted nuclei were lysed overnight in ice-cold ChIP lysis buffer (25 mM Tris-HCl pH 8.0, 2 mM EDTA, 0.25% SDS, Complete[®] protease inhibitors) at a concentration of 2.5×10^7 nuclei per ml. Sonication and debris removal were performed as described above. The lysate was diluted 3-fold in dilution buffer (25 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, Complete[®] protease inhibitors). The remaining steps were performed as described above.

For the antibody-mediated ICN1 ChIP, single cell suspensions of thymi from 4-8 week-old *Rag2*^{-/-} mice were prepared and immediately crosslinked with FA as described above. Remaining steps were performed as described in the previous section. The chromatin was precleared with 50 µl protein A sepharose beads (pre-adsorbed with BSA) for 1 h at 4°C followed by incubation with 5 µg of antibody per 400 µg of chromatin overnight at 4°C. Antibody complexes were harvested by incubation with 40 µl protein A sepharose for 2 h at 4°C. The sequestered complexes were washed with the following buffers for 10 min at 4°C each: low salt buffer (20 mM Tris-HCl pH 8.1, 2 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, Complete[®] protease inhibitors), high salt buffer (20 mM Tris-HCl pH 8.1, 2 mM EDTA, 500 mM NaCl, 1% Triton X-100, 0.1% SDS, Complete[®] protease inhibitors), LiCl buffer (10 mM Tris-HCl pH 8.1, 1 mM EDTA, 250 mM LiCl, 1% Nonidet NP-40, 1% Na-deoxycholate, Complete[®] protease inhibitors) and twice with 1× TE. Transfer of the beads to fresh tubes during the washing steps and prior to elution helped to remove nonspecific chromatin. The protein-DNA complexes were eluted from the beads twice with 250 µl elution buffer (100 mM NaHCO₃, 1% SDS) for 15 min at RT. For crosslink reversal and protein digest, NaCl (0.2 M), EDTA (10 mM), Tris-HCl pH 8.0 (20 mM) and Proteinase K (500 µg/ml) were added to the pooled eluates prior overnight incubation at 55°C. The remaining steps were performed as described in the previous section.

The V5 antibody mediated ICN1-Bio ChIPs were performed on standard FA-crosslinked material from prepared nuclei of *Notch1*^{Bio/Bio} *Rosa26*^{BirA/BirA} *Rag2*^{-/-} whole thymocyte suspensions. Sheared chromatin was pre cleared with 50 µl protein A sepharose beads per 400 µg chromatin as described above. The IP was performed with 40 µl V5 antibody-coupled agarose beads overnight at 4°C. The remaining steps were performed as described in the previous sections.

In contrast to conventional dual crosslinking approaches, FA crosslinking was performed prior to DGS crosslinking in order to inhibit turnover of ICN1 containing complexes by proteasomal decay. FA crosslinking was performed as described above. Since DSG activity is

inhibited by trace concentrations of glycine, we tested standard glycine-mediated quenching of FA as well as wash out of FA without prior quenching. To this end, the cells were washed 3× with ice-cold PBS supplemented with 2% FCS. The second crosslinking reaction was performed in PBS with 2 mM DSG for 45 min at RT followed by quenching with 0.25 M glycine for 5 min. The remaining steps were performed as described above.

Primers used for qPCR to test ChIP efficiency

Locus	MBU ID	fw/rev	5' – 3'
Chr1	10558	fw	CATAGATGAAGCTGCCACATAGGT
	10559	rev	GTGGGCAAGGACAAAGCATT
<i>Cd19</i>	10818	fw	CCCTCTTCTCATTCGTTTTCCA
	10819	rev	CCAGGAAAGAATTTGAGAAAAATCA
<i>Hes1</i>	10734	fw	TGGGAAAGAAAGTTTGGGAAG
	10735	rev	ACCAGCTCCAGATCCTGTGT
<i>Notch1</i>	11333	fw	CCTCAATCCAACCAGCTGTA
	11334	rev	CAACATGTGGGAGAGTGCTG
<i>Notch3</i>	11150	fw	AGCTCAAGCCCTGGGAAC
	11151	rev	GTCAAGGCAACTTCCTGAGC
<i>Rbpj</i>	11250	fw	TCTCGCGAGGCTTAGGAAAT
	11251	rev	CGGAGTCTCACGCTACACCT

4.14 cDNA preparation for RNA-sequencing

Total RNA was isolated with the RNeasy Plus Mini Kit (Qiagen) and processed for deep sequencing as described by Mortazavi et al. (2008). Briefly, RNA was purified by one or two rounds of poly(A)-selection using the Dynabeads® mRNA purification kit (Invitrogen). RNA was fragmented in the presence of divalent cations to a size of 200-500 nucleotides at 94°C for 3 min. The fragmented RNA was used for the first-strand cDNA synthesis with the SuperScript VILO™ cDNA Synthesis Kit (Invitrogen) primed by random hexamers. The second-strand synthesis was performed with 25 mM dATP, dCTP, dGTP and dUTP in the presence of RNase H, *E. coli* DNA polymerase I and DNA ligase (Invitrogen). The cDNA was purified using the MinElute Reaction Cleanup kit (Qiagen) or AMPure® XP beads (Beckman Coulter).

4.15 Library preparation for Solexa sequencing

Between 1-5 ng of ChIP-precipitated DNA, DNA excised from DHS sites or cDNA prepared from RNA was used as starting material for library preparation based on Zhong et al. (2011). End repair, dA-tailing and ligation of the pre-annealed adaptors (TruSeq adaptors 1-12) were performed with the NEB Next™ DNA Sample Prep Reagent Set 1 (NEB). DNA products of the enzymatic reactions were purified with AMPure® XP beads (Beckman Coulter). To this end, DNA was mixed with 1.8 volumes of AMPure® XP beads and incubated for 15 min at RT. The beads were magnetically sequestered and washed twice with 75% EtOH. DNA was eluted from the beads with 1× TE by incubation for 1 min at 37°C. Because the ligation buffer contained 7.5% PEG, which increased the binding capacity of the beads, 1.0 volume of AMPure® XP beads was used after the adaptor ligation reaction to purify adaptor-ligated DNA, to remove non-ligated adaptors as well as adaptor dimers. A second round of DNA purification was performed with 1.4 volumes of AMPure® XP beads. For selection of DNA fragments with a range of 200-700 bp, DNA was incubated with 0.525 volumes of AMPure® XP beads for 15 min at RT. Larger DNA fragments were removed by magnetic separation of the beads, while the supernatant was transferred to a fresh tube. Finally, the size selected DNA was purified once more with 1.0 volume of AMPure® XP beads. For strand-specific RNA-seq, cDNA samples with uracil-labelled second-strands were incubated with *E. coli* uracil-DNA glycosylase (NEB), followed by AMPure® XP bead DNA purification. Sequencing libraries were amplified using the KAPA Real-Time Library Amplification Kit (Kapa Biosystems). To avoid over-amplification of the libraries, each sample was individually amplified until it reached a standardised fluorescent signal (between 8-13 cycles). Next, 1.4 volumes of AMPure® XP beads were used to remove unused PCR primers and clean up amplified libraries. Molarity of the libraries was determined by DNA measurements with the PicoGreen™ assay for dsDNA (Thermo scientific), Bioanalyzer dsDNA 1000 assay kit (Agilent) and qPCR NGS library quantification kit (Agilent). Cluster generation and sequencing were carried out by using the Genome Analyzer II or HiSeq 2000 system (Illumina) according to the manufacturer's guidelines.

Primers/adaptors used for library amplification

Primer name	Sequence 5' – 3'
Multiplexing 1.0	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGA
Multiplexing 2.0	CAAGCAGAAGACGGCATACGAGAT
TruSeq universal	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACAC GACGCTCTTCCGATC*T
TruSeq TS1	P-GATCGGAAGAGCACACGTCTGAACTCCAGTCACATCACG ATCTCGTATGCCGTCTTCTGCTTG
TruSeq TS2	P-GATCGGAAGAGCACACGTCTGAACTCCAGTCACCGATGT ATCTCGTATGCCGTCTTCTGCTTG
TruSeq TS3	P-GATCGGAAGAGCACACGTCTGAACTCCAGTCACTTAGGC ATCTCGTATGCCGTCTTCTGCTTG
TruSeq TS4	P-GATCGGAAGAGCACACGTCTGAACTCCAGTCACTGACCA ATCTCGTATGCCGTCTTCTGCTTG
TruSeq TS5	P-GATCGGAAGAGCACACGTCTGAACTCCAGTCACACAGTG ATCTCGTATGCCGTCTTCTGCTTG
TruSeq TS6	P-GATCGGAAGAGCACACGTCTGAACTCCAGTCACGCCAAT ATCTCGTATGCCGTCTTCTGCTTG
TruSeq TS7	P-GATCGGAAGAGCACACGTCTGAACTCCAGTCACCAGATC ATCTCGTATGCCGTCTTCTGCTTG
TruSeq TS8	P-GATCGGAAGAGCACACGTCTGAACTCCAGTCACACTTGA ATCTCGTATGCCGTCTTCTGCTTG
TruSeq TS9	P-GATCGGAAGAGCACACGTCTGAACTCCAGTCACGATCAG ATCTCGTATGCCGTCTTCTGCTTG
TruSeq TS10	P-GATCGGAAGAGCACACGTCTGAACTCCAGTCACTAGCTT ATCTCGTATGCCGTCTTCTGCTTG
TruSeq TS11	P-GATCGGAAGAGCACACGTCTGAACTCCAGTCACGGCTAC ATCTCGTATGCCGTCTTCTGCTTG
TruSeq TS12	P-GATCGGAAGAGCACACGTCTGAACTCCAGTCACCTTGTA ATCTCGTATGCCGTCTTCTGCTTG

4.16 Sequence alignment

Sequence reads that passed the Illumina quality filtering were considered for alignment. For RNA-seq, samples were trimmed at the 5' end of the read to a read length of 44 bp fol-

lowed by alignment to the mouse transcriptome with the TopHat splice junction mapper for RNA-seq reads, (version 1.4.1; Trapnell et al., 2009). Reads corresponding to mouse ribosomal RNA (BK000964.1) were removed. The mouse genome assembly version of July 2007 (NCBI37/mm9) with a genome size of 2,654,911,517 bp was used for all bioinformatic calculations. The NCBI reference sequence database (RefSeq) from 29 April 2010 was used to obtain genomic, transcript and protein features. Reads from ChIP-seq were aligned using the BOWTIE program, (version 12.5; Langmead et al., 2009) allowing up to two mismatches and ignoring any read that would match more than once.

4.17 Peak calling of ChIP-seq data

Peaks were called using the MACS software (version 1.3.6.1; Zhang et al., 2008), embedded in the Galaxy platform (<http://www.galaxyproject.org>), with default parameters: read length of 36 bp or 50 bp single-end for ChIP-seq and 76 bp paired-end for DHS data; mm9 genome size (see Section 4.16) and a ChIP input control track from *Notch1*^{Bio/Bio} *Rosa26*^{BirA/BirA} *Rag2*^{-/-} whole thymocytes (DN3a). The default mfold parameter for selecting the peaks to generate the read shifting model was 32. In cases where not sufficient (< 100) high quality peaks could be found, the mfold was lowered until enough peaks were found. For analysis of DHS and chromatin data, the generation of a *de novo* read shifting model was omitted. If not stated differently, peaks were filtered for a *P*-value < 10⁻¹⁰.

4.18 Peak-overlap analysis

Overlaps between the identified genomic peak regions were analysed with the MULTOVL application suite (Aszódi, 2012), which is embedded in the Galaxy platform. The minimal overlap length was set to one. Results were parsed and then converted into tables with custom-made bash, Perl and R scripts as described earlier (Schwickert et al., 2014). Further analysis and visualisation were performed with the TIBCO Spotfire® program (version 5.0.1).

4.19 Peak-to-gene assignment

All peak assignments were performed with scripts, which are embedded in the Galaxy platform. For peak-to-gene assignments used to determine direct Notch target genes, Rbpj-Bio and/or ICN1-Bio peaks were assigned to genes using a script described earlier (Revilla-I-Domingo et al., 2012). Briefly, the assignment was performed in a stepwise manner by prioritising genes containing peaks in their promoter and/or gene body. Firstly, peaks within the

promoter region (−2.5 kb to +2.5 kb relative to TSS) or gene body (+2.5 kb to TTS) were assigned to the corresponding gene. Secondly, remaining peaks within 50 kb upstream of the TSS or 50 kb downstream of the TTS were assigned to the prioritised peak-containing gene. Other peaks within 50 kb upstream of a TSS or 50 kb downstream of a TTS of a gene without peaks in the promoter region or gene body were assigned to the nearest gene.

The CEAS tool (version 0.0.5; Ji et al., 2006; Shin et al., 2009) was used to visualise the distribution of peaks across the mouse genome with respect to different features of the annotated genes. The program was run with default settings. To improve the display, promoter-proximal regions were combined. Distal intergenic sites refer to all peaks that are not within any of the other described categories.

4.20 ChIP-seq heatmaps and composite density plots

Read density heatmaps were generated with scripts described earlier by Tedeschi et al. (2013), which are embedded in the Galaxy platform. The jnomics program (Tamir, unpublished) was used to calculate normalised read densities. To this end, 150 bp extended reads were counted in 100 bins across 2.5 kb upstream and downstream of the peak centre. The counted reads of each track were individually normalised per million aligned reads of the sample. Finally, heatmaps of the read densities of all peak regions were produced and sorting of the regions was determined by the bin with the highest/lowest read density. Three partitions were generated by calculating the median absolute deviation (MAD) cutoffs of the track that was used for sorting. Composite median read density profiles were calculated across all regions within a given partition and plotted as line plots.

4.21 Scatterplots of read densities within peak regions

The read densities were calculated with the simple RPKM tool (version 0.4; Tamir, unpublished), which is embedded in the Galaxy platform. To this end, the reads of a given ChIP-seq track were extended for 100 bp and their occurrence within the peak regions was counted. The counts were RPKM-normalised to the length of the peak region (kb) and to the number of aligned reads (million) of each individual sample. The data was plotted with the TIBCO Spotfire® software (version 5.0.1). Alignments with identical parameters (trimmed read length, single-end sequenced) were used in all cases where the compared samples have been sequenced with different read lengths or sequencing modes.

The histone mark peak density correlations between the two replicas were calculated as $\log_2$ of the sum over all read count-normalised binned read densities from a peak-centred 5 kb region (see Section 4.21).

4.22 Analysis of RNA-seq data

The Galaxy platform was used for analysis of differential gene expression. The number of reads per gene was counted using the HTseq Python package (version 0.5.3) for processing data from high-throughput sequencing assays with the overlap resolution mode option set to 'union' (<http://www-huber.embl.de/users/anders/HTSeq>). Two independent RNA-seq experiments were performed for each cell type except for CD25^{int} *Notch1*^{fl/fl} *Lck*-Cre and *Rbpj*^{fl/fl} DN3a cells (both 1) and wild-type DN1, DN2a, DN2b (3 each). Replicates were compared by using the R package DESeq (version 1.6.1; Anders and Huber, 2010) to calculate the significance level of differential expression. To this end, the samples were normalised and the dispersions were estimated using the default DESeq settings. Genes with an adjusted *P*-value of < 0.1 were called as differentially expressed. For *in vitro* stimulation experiments with LMPPs, DN3a and DN3b cells, I applied an additional dispersion cutoff of < 0.1 between the replicates. This step helped to remove false positive genes, which were the result of OP9-GFP and OP9-DL1 feeder cell contamination during cell sorting. OP9 cells contain large amounts of RNA. Hence, already small differences in the degree of feeder cell contamination between OP9-GFP and OP9-DL1 co-cultured samples led to false positive genes (feeder cell transcriptome signature). However, since the degree of feeder cell contamination and consequently also the amounts of feeder-derived RNA varied between replicates, the gene expression values of these false positive genes had an increased variation. The dispersion filter was omitted for *Pax5*^{-/-} *Vav-Bcl2* pro-B cell stimulation experiments, since the cells were sorted for two sequential rounds after stimulation, resulting in a negligible feeder cell contamination. Scatterplots of the normalised counts or moderated $\log_2$ fold-changes in gene expression were created in the TIBCO Spotfire® program (version 5.0.1).

4.23 Principal component analysis – PCA

The Population PCA tool (<http://cbdm.hms.harvard.edu/LabMembersPges/SD.html>) was used for principal component analysis. The analysis was performed as described earlier by Mingueneau et al. (2013) and adapted for RPKM based gene expression values determined by RNA-seq. For each track, the reads were counted with HTseq (see Section 4.22) and the RPKM value of a gene was calculated with the RPKM tool embedded in the Galaxy platform (Axelsson, un-

published). Next, the RPKM-normalised transcript abundance was $\log_2$ -transformed. All data sets were filtered for expression values greater than 10 RPKM in any subset and included only 15% of the genes with the highest variation across analysed populations.

RNA-seq tracks of different cell types used for PCA

Cell type	MBU ID	Genotype	reference
ALP	8485 8558	wild-type	Revilla-I-Domingo et al. (2012)
BLP	8486 8559	wild-type	Revilla-I-Domingo et al. (2012)
DN1	14588 14591 14593	wild-type	this study
DN2a	14589 14594 14596	wild-type	this study
DN2b	14590 14595 14597	wild-type	this study
DN3a	12521 12522 13326 13034 13038	<i>Notch1</i> ^{fl/fl} <i>Rbpj</i> ^{fl/fl} <i>Gata3</i> ^{fl/fl}	this study Busslinger lab, unpublished
DN3b	13324 13330 13035 13039	<i>Rbpj</i> ^{fl/fl} <i>Gata3</i> ^{fl/fl}	this study Busslinger lab, unpublished
LMPP	14587 14592	wild-type	this study
pro-B	12073 12806 12119 13020	<i>Ikzf1</i> ^{fl/+} <i>Cd79</i> ^{Cre/+} <i>Eed</i> ^{fl/fl}	Schwickert et al. (2014) Busslinger lab, unpublished
pre-B	12078 12079	wild-type	this study

4.24 Hierarchical clustering of genes

The *ex vivo* expression profiles of the identified Notch-regulated genes through early lymphocyte development were based on the LMPP, ALP, DN1, DN2a, DN2b, DN3a and DN3b populations listed in section 4.23. Clustering was performed with the HierarchicalClustering module from the GenePattern genomic analysis platform (<http://genepattern.broadinstitute.org>) on $\log_2$ -transformed RPKM values. Gene/row distances were measured by Pearson correlation and hierarchical clustering was performed by pairwise average-linkage (see <http://genepattern.broadinstitute.org> for details). The gene expression changes were row-normalised and the resulting data table was visualised as heat map with the HierarchicalClusteringViewer module of GenePattern.

4.25 *De novo* motif finding and PWM scans

Motif analyses were performed with scripts embedded in the Galaxy platform. The Gimme-Motifs program (van Heeringen and Veenstra, 2011) with default settings was used for *de novo* Rbpj motif prediction from the common Rbpj-Bio peaks identified in Rag2-deficient DN3a cells. Occurrences of the motif within peak regions were determined by PWM scans with the scan.py script (<https://github.com/simonvh/gimmemotifs>) with a cutoff of 0.85. For background detection of the Rbpj motif within a given set of peaks, the sequence of the peak region was randomised by shuffling of the nucleotide positions once using the shuffleseq tool (Rice et al., 2000; Blankenberg et al., 2007). By doing this, the relative contribution of each nucleotide remained unchanged.

4.26 Determination of active TSSs based on a minimal CAGE signal

For the determination of active TSSs for the *cis*-regulatory landscape of Rag2-deficient DN3a cells, a minimum CAGE signal was determined as described earlier by Revilla-I-Domingo et al. (2012). Briefly, full length RNA-seq was performed on the same material from which the CAGE library was generated and the RPKM of each gene was determined. Next, a three-wise overlap was performed with: 249,070 CAGE islands; 38,975 DHS peaks and 26,812 TSSs (RefSeq as of 29 April 2010), whereby only TSSs were taken which are associated with genes that have an RPKM greater than one. As in many cases multiple CAGE islands overlap one DHS site, we filtered on the CAGE island with the highest RPKM value. Finally, the RPKM cutoff of the CAGE signal was set in a way that 95% of those CAGE islands (DHS⁺ and RPKM of the associated gene > 1) have an higher RPKM value than the cutoff. This resulted

in a minimal CAGE island signal of 2.62 RPKM for active TSSs of genes encoded on the Watson (+) stand and a minimal CAGE island signal of 2.03 RPKM for active TSSs of genes encoded on the Crick (–) stand. In total, from all identified 249,070 CAGE islands, 18,314 CAGE islands on the Watson stand and 26,094 CAGE islands on the Crick strand matched the described criteria.

4.27 RT-PCR and qPCR for gene expression analysis

Total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen). RNA was reverse-transcribed into cDNA with Oligo(dT)12-18 Primers and the SuperScript II™ kit as described in the manual (Invitrogen). Each primer combination spanned an exon-exon splice junction region of the respective gene. qPCR was performed for 40 cycles with the iQ5 real-time PCR detection system (Bio-Rad). Reactions were performed as technical triplicates using reagents described earlier (Decker et al., 2009). Relative concentrations of *Hprt* and *Dtx1* cDNA were determined on basis of standard curves of cDNA, followed by normalisation for *Hprt* contents with the software provided by the manufacturer. Melt curves were run to ensure amplification of a single product.

qPCR primer pairs to test gene expression

Gene	MBU ID	fw/rev	5' – 3'	Reference
<i>Dtx1</i>	12709	fw	GAGGATGTGGTTCGGAGGTA	David-Fung et al. (2006)
	12710	rev	CCCTCATAGCCAGATGCTGT	
<i>Hprt</i>	8980	fw	AGTGTTGGATACAGGCCAGAC	RT primer database http://tinyurl.com/o5c4v3d
	8981	rev	CGTGATTCAAATCCCTGAAGT	

4.28 Nuclear extract preparation

Approximately 10^8 *in vitro* cultured pro-T cells (see Section 4.7) were harvested from OP9-DL1 co-cultures and washed twice with ice-cold PBS. Pelleted cells were first equilibrated with sucrose buffer (10 mM Tris-HCl pH 8.0, 0.32 M sucrose, 50 mM KCL, 20 mM NaCl, 3 mM CaCl₂, 2 mM MgCl₂, 1 mM dithiothreitol (DTT), 2 mM 6-aminocaproic acid (6-AA), 0.15 mM spermine, 0.5 mM spermidine, 0.5 M Phenylmethylsulfonylfluorid (PMSF), Complete® protease inhibitor) before the cell membrane was lysed by addition of one volume of sucrose buffer containing 0.2% NP-40. Next, the pelleted nuclei were resuspended in low

salt buffer (10 mM HEPES pH 7.9, 20% glycerol, 20 mM KCL, 2.0 mM MgCl₂, 2 mM 6-AA, 0.5 mM DTT, Complete[®] protease inhibitor) before one volume of high salt buffer (10 mM HEPES pH 7.9, 20% glycerol, 700 mM KCL, 2.0 mM MgCl₂, 2 mM 6-AA, 0.5 mM DTT, 0.2% NP-40, Complete[®] protease inhibitor) was added in a stepwise manner to extract the proteins. The nuclear extract was centrifuged and the protein content in the supernatant was measured by Bradford assay (Bio-Rad) prior to snap freezing in liquid nitrogen and storage at -80°C.

4.29 Streptavidin pulldown of biotinylated proteins

1:2 diluted (with low salt buffer from Section 4.28) nuclear extracts from $\sim 10^8$ *in vitro* cultured pro-T cells (see Section 4.7) were incubated with streptavidin-coated magnetic beads (Dynabeads[®] M-280 SA, Invitrogen) at 4°C overnight, while 10% of the nuclear extract were saved as input controls. The beads were sequestered on a magnet and the supernatant was removed. 10% of the supernatant and the input control were denatured by addition of one volume of 2× Laemmli buffer (5% SDS, 150 mM Tris-HCl pH 6.7, 30% glycerol, 720 mM 2-ME and 0.0025% bromophenol blue) followed by boiling for 5 min at 94°C. The beads were washed 3× with washing buffer (20 mM Tris-HCl pH 8, 250 mM KCl, 2 mM MgCl₂, 10% glycerol, 1 mM DTT, 2 mM 6-AA, Complete[®] protease inhibitor) and the bound protein was eluted from the beads by boiling in 2× Laemmli buffer for 5 min. Input, supernatant and eluate samples were separated by SDS-PAGE and subjected to western blotting.

4.30 Streptavidin supershift assay of biotinylated proteins

One volume of 2× Laemmli buffer was added to 10 µg of 1:2 diluted (with low salt buffer from Section 4.28) nuclear extract from *in vitro* cultured *Notch1*^{Bio/Bio} *Rosa26*^{BirA/BirA} *Rag2*^{-/-} pro-T cells (see Section 4.7). Proteins were denatured by boiling for 5 min at 95°C. The denatured nuclear extract was preincubated overnight at 4°C with 2.75 µg SA resuspended in PBS or with PBS alone before proteins were separated by SDS-PAGE and subjected to immunoblot analysis.

4.31 SDS PAGE and Western Blot analysis

Approximately 20 µg of denatured protein extract were separated on 7.5% or 10% acrylamide gels with the NEXT GEL[®] system (Amresco). Proteins were transferred to a Polyvinylidene fluoride (PVDF) membrane by wet transfer, followed by incubation of the membrane in block-

ing buffer (1× TBS, 5% non-fat dry milk powder, 0.3% Tween 20). The membrane was incubated with the primary V5 antibody (1:2000 in blocking buffer) overnight at 4°C. Unbound antibodies were removed from the membrane by three washing steps with TBST (1× TBS, 0.3% Tween 20) followed by incubation of the membrane with a secondary horseradish peroxidase (HRP) conjugated secondary anti mouse antibody (1:5000 in blocking buffer) for 1 h at RT. Unbound antibodies were removed from the membrane by three washing steps with TBST. Finally, protein bands were visualised by incubation of the membrane with ECL plus reagent (GE Healthcare) followed by exposure of the membrane to X-omat AR film (Kodak).

5 Credits for Contributions to this Work

Several people have contributed to the results presented in this thesis:

Elin Axelson provided scripts for all RNA-seq data related analysis and assisted with the analysis for the determination of the CAGE signal threshold and RNA-seq data analysis.

Meinrad Busslinger performed the functional annotation of Notch-regulated target genes.

Ivan Bilic and Roger Revilla-i-Domingo generated the CAGE dataset of Rag2-deficient DN3a cells and the corresponding full length RNA-seq dataset.

The CSF NGS unit performed Solexa Deep Sequencing as well as library preparation of the following samples: All histone modification ChIP-seq experiments, one of the two DHS site mapping experiments in Rag2-deficient DN3a cells, all Bio-ChIP-seq experiments with Pax5-deficient pro-B cells, one of the three ICN1-Bio ChIP-seq experiments from *Notch1*^{Bio/Bio} *Rosa26*^{BirA/BirA} *Rag2*^{-/-} DN3a cells and one of the three Rbpj-Bio ChIP-seq experiments from *Rbpj*^{Bio/Bio} *Rosa26*^{BirA/BirA} *Rag2*^{-/-} DN3a cells.

Heike Dorninger and Qiong Sun generated the *Notch1*^{Bio} and *Rbpj*^{Bio} mice.

Markus Jaritz provided scripts which were used for ChIP-seq peak calling, peak overlap analysis, peak-to-gene assignment, motif finding and ChIP-seq density heatmaps. He also performed analysis for ChIP-seq peak finding, determination of the CAGE signal threshold and peak overlaps to generate the *cis*-regulatory landscape of Rag2-deficient DN3a cells, which was visualised in TIBCO Spotfire® layouts generated by him.

Taras Kreslavsky performed the following experiments: *i*) reverse transcription of mRNA followed by real-time PCR to determine the relative *Dtx1* transcript levels in Rag2-sufficient DN3 cells. *ii*) cell culture, CellTrace™ Violet labelling and FACS sorting for RNA-seq of Notch-stimulated LMPPs, Pax5-deficient pro-B cells, DN3a cells and post-β-selection cells. *iii*) RNA-seq library preparation for Notch-stimulated Pax5-deficient pro-B cells and post-β-selection cells. *iv*) The majority of retro-orbital and intraperitoneal injections. *v*) FACS analysis of thymocytes after CD3ε antibody injection. *vi*) FACS sorting of LMPPs, DN1/ETPs, DN2a and DN2b cells.

Hiromi Tagoh performed the DHS site mapping for one replicate from Rag2-deficient DN3a cells and all Bio-ChIP-seq experiments with ICN1-Bio and Rbpj-Bio in Pax5-deficient pro-B cells.

All remaining experiments and analysis was performed by Markus Schäfer.

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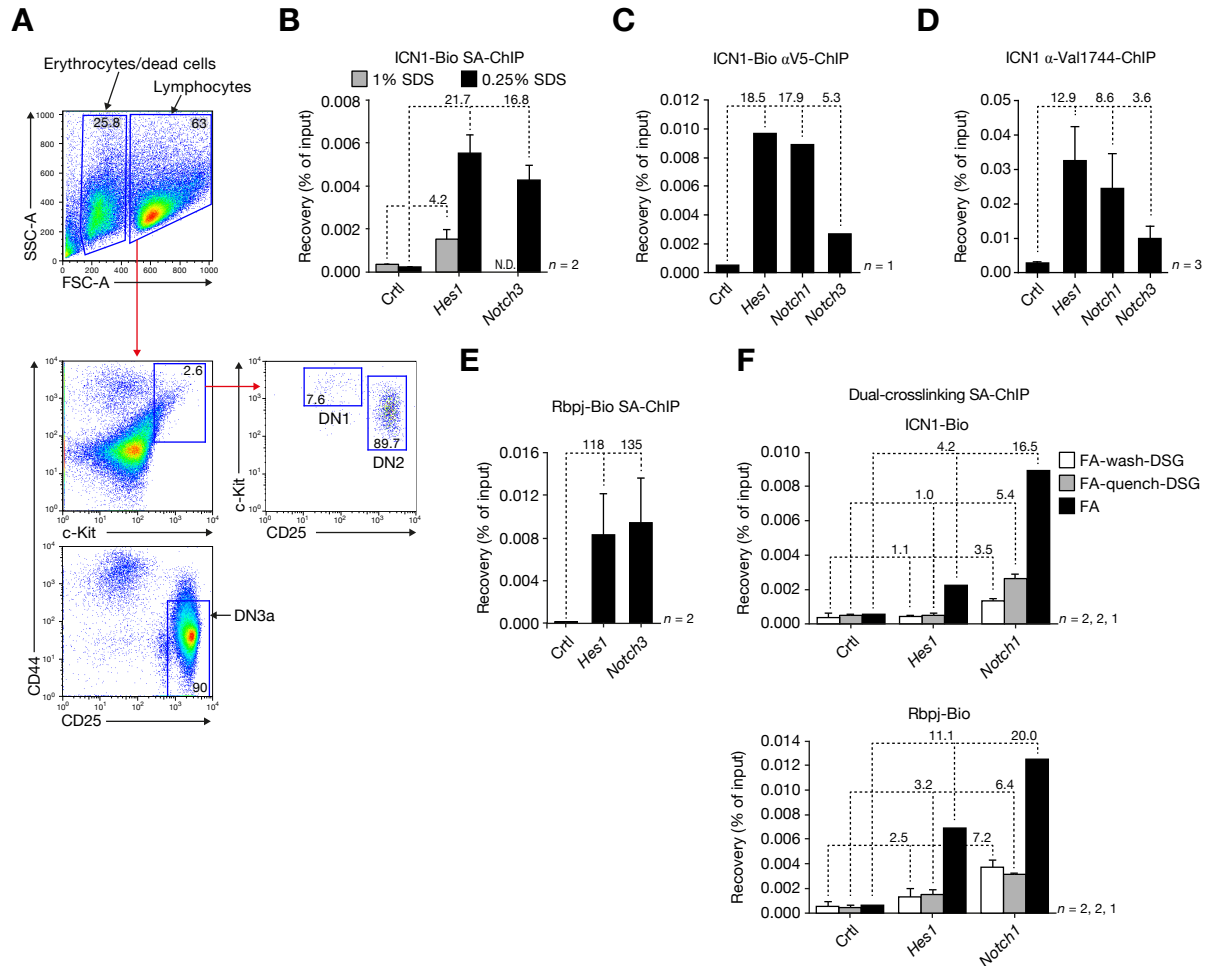
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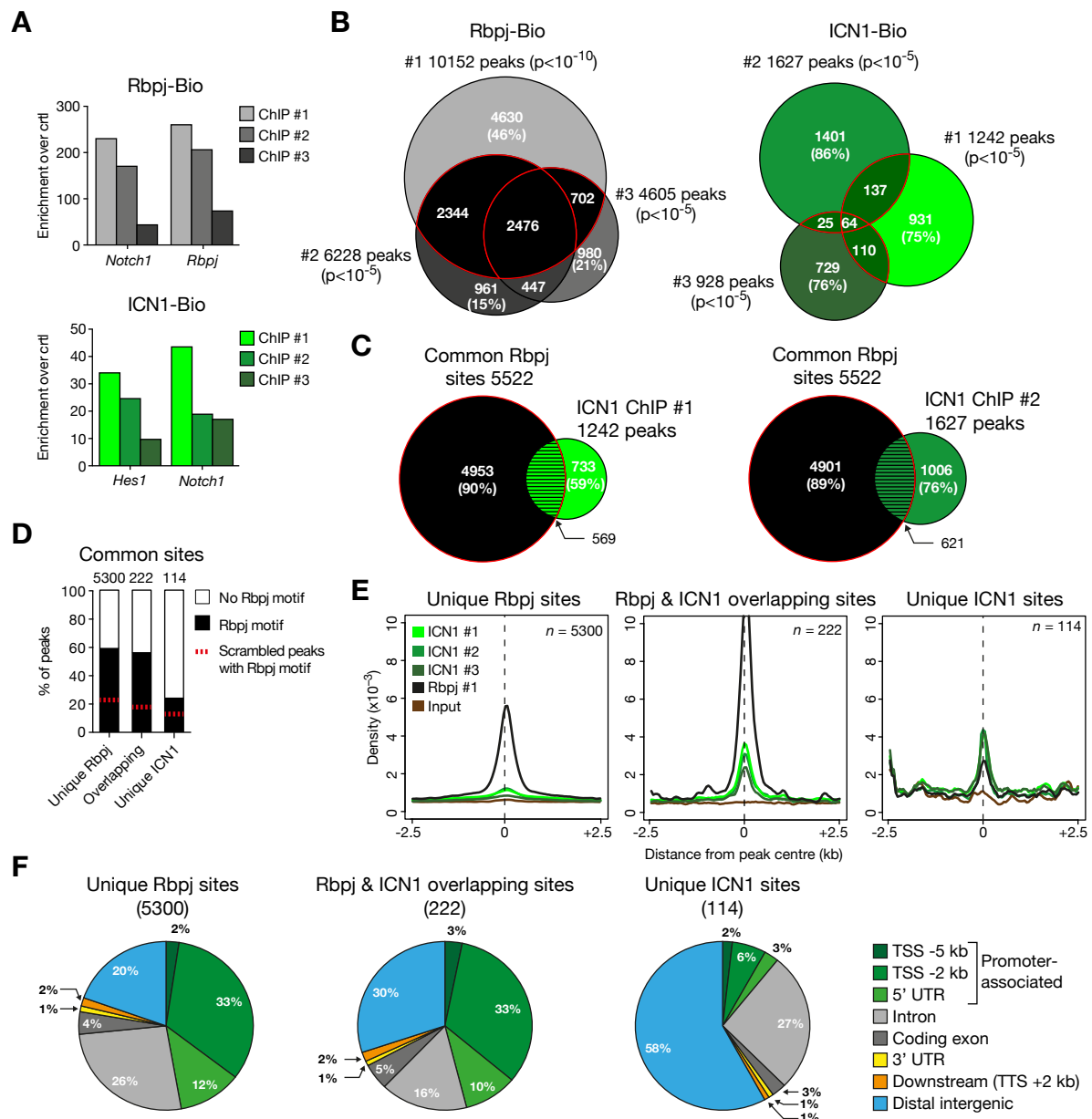
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7 Supplementary Materials

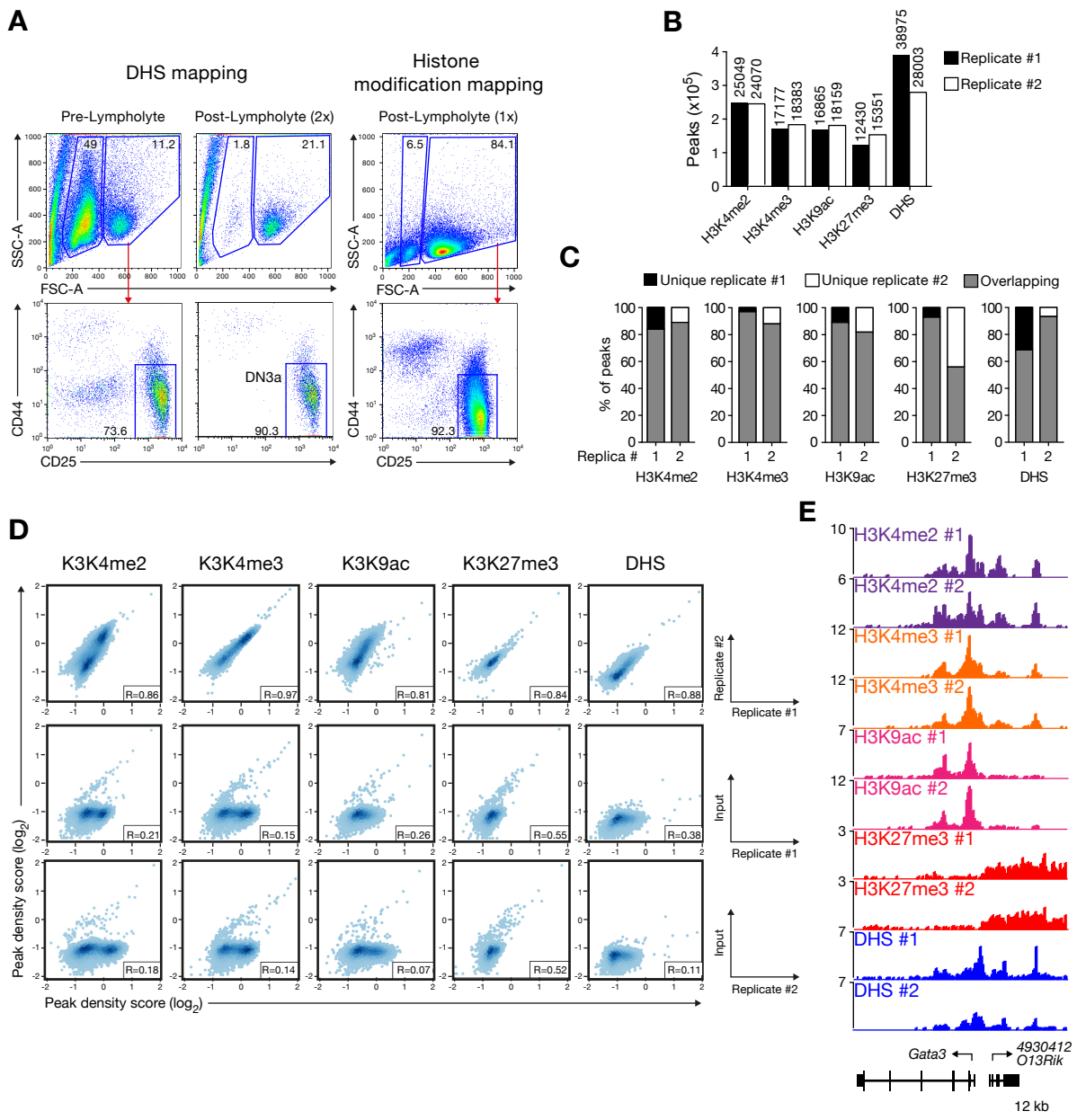
7.1 Supplemental figures



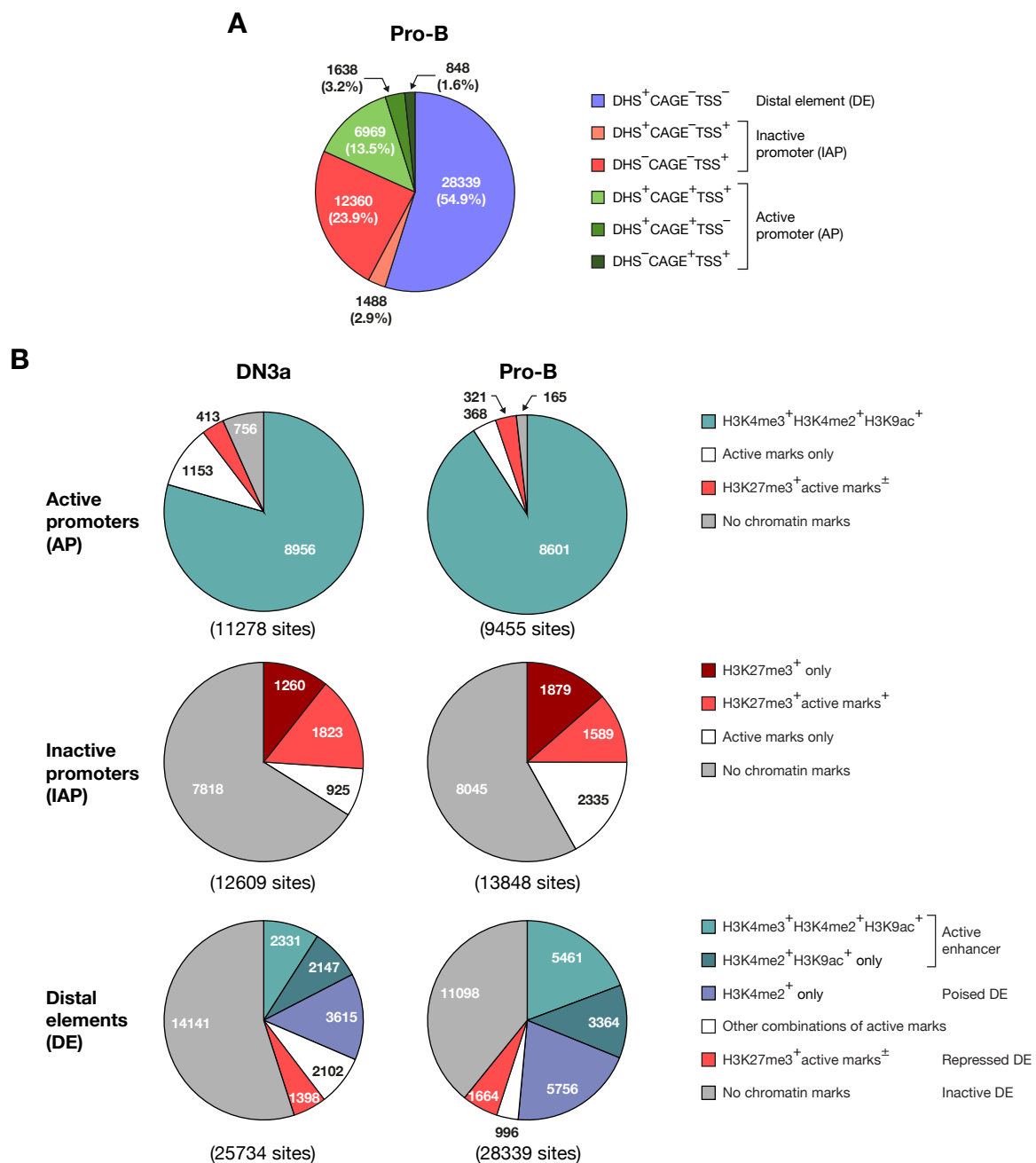
Supplemental Figure 7.1 Testing of different protocols for ICN1 and Rbpj ChIPs. (A) A representative FACS analysis of a thymus from a 4 week-old *Notch1^{Bio} Rag2^{-/-}* mouse shows a nearly homogenous DN3a population. (B - G) ChIP-qPCR analysis showing the recovery of DNA elements relative to the total input DNA of the indicated genes with known ICN1/Rbpj-binding sites and the average of the recovery of two control regions (Ctrl). Values above the corresponding dashed lines represent enrichment relative to the negative control regions. Error bars indicate the SD and *n* indicates the number of experiments. ChIPs were performed after preparation of nuclei that were lysed in a buffer containing 0.25% SDS if not indicated differently. (B) SA-mediated ICN1-Bio-ChIP from thymocytes of *Notch1^{Bio} Rag2^{-/-}* mice using whole-cell lysis in the presence of 1% SDS (grey bars) or preparation of nuclei followed by lysis in the presence 0.25% SDS (black bars). (C) V5 antibody-mediated ICN1-Bio ChIP from thymocytes of *Notch1^{Bio} Rag2^{-/-}* mice. (D) α -Notch1 Val-1744 antibody-mediated ICN1 ChIP from thymocytes of *Rag2^{-/-}* mice. (E) SA-mediated Rbpj-Bio-ChIP from thymocytes of *Rbpj^{Bio} Rag2^{-/-}* mice. (F) SA-mediated Bio-ChIPs from *in vitro* expanded *Notch1^{Bio} Rag2^{-/-}* (top) or *Rbpj^{Bio} Rag2^{-/-}* (bottom) pro-T cells (Fig. 2.2A) using standard FA-mediated crosslinking (black bars) or dual crosslinking with DSG after quenching of FA with glycine followed by washing steps (grey bars) or washing out of FA without prior quenching (white bars).



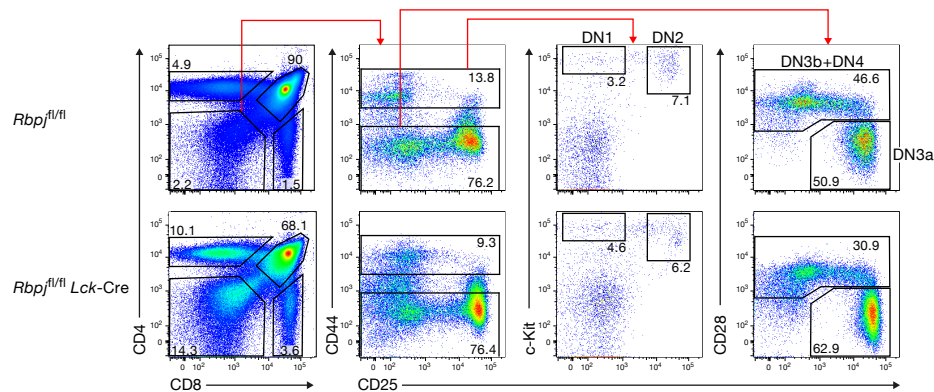
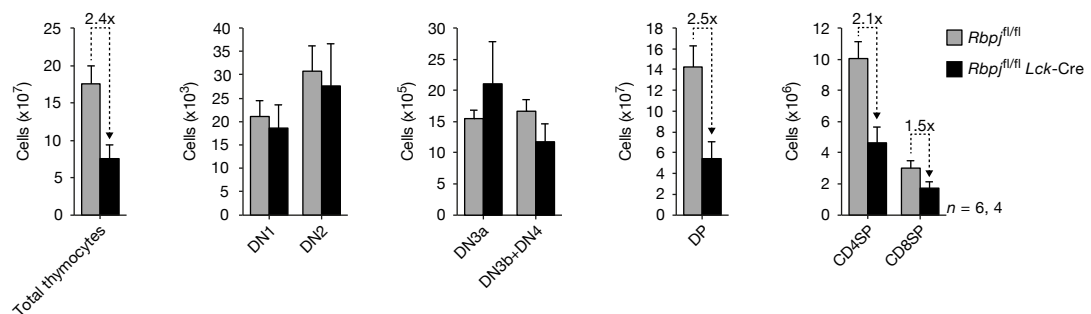
Supplemental Figure 7.2 Quality of generated ICN1 and Rbpj Bio-ChIP-seq datasets in Rag2-deficient DN3a cells. (A) qPCRs analysis of precipitated and input Bio-ChIP DNA from total thymocytes of *Rbpj*^{Bio} *Rag2*^{-/-} (top) and *Notch1*^{Bio} *Rag2*^{-/-} (bottom) mice prior to high-throughput sequencing library generation. Relative enrichments of indicated positive control regions with known ICN1/Rbpj-binding sites over the average of two negative control regions are shown. (B) Definition of the common peaks by overlap analysis of the three Bio-ChIP-seq replicas for Rbpj (left) and ICN1 (right) displayed as Venn diagram. Peak numbers of each experiment identified by MACS peak calling with the indicated *P*-value threshold are indicated. Percentages refer to the ratio of unique peaks of each experiment. Common Rbpj-Bio-binding sites (red border) are defined by a minimum 2-fold overlap of a peak detected in experiment #2 or #3 with a peak of experiment #1. Common ICN1-Bio-binding sites (red border) are defined by a minimum 2-fold overlap of any peak region with another region of any of the three replicas. (C) Overlap analysis of ICN1-binding sites from Bio-ChIP-seq experiment #1 (left) and #2 (right) with common Rbpj-Bio-binding sites. (D) Bar chart representing the ratio of the unique and overlapping ICN1-Bio and Rbpj-Bio sites containing the *de novo* identified Rbpj motif (Fig. 2.3B). The same motif was found in random DNA sequences at the frequency indicated by a dashed red line. (E) Read density plots of ICN1 (green) and Rbpj (black) Bio-ChIP-seq tracks together with a sequencing track from sonicated ChIP-input control DNA (brown) at unique and overlapping ICN1-Bio and Rbpj-Bio-binding sites. (F) Pie charts representing the frequencies of unique and overlapping ICN1-Bio and Rbpj-Bio-binding sites based on their distance to different features of annotated genes. Promoter-associated regions are defined by their proximity to the TSS. The '-' and '+' signs indicate upstream or downstream sequences, respectively.



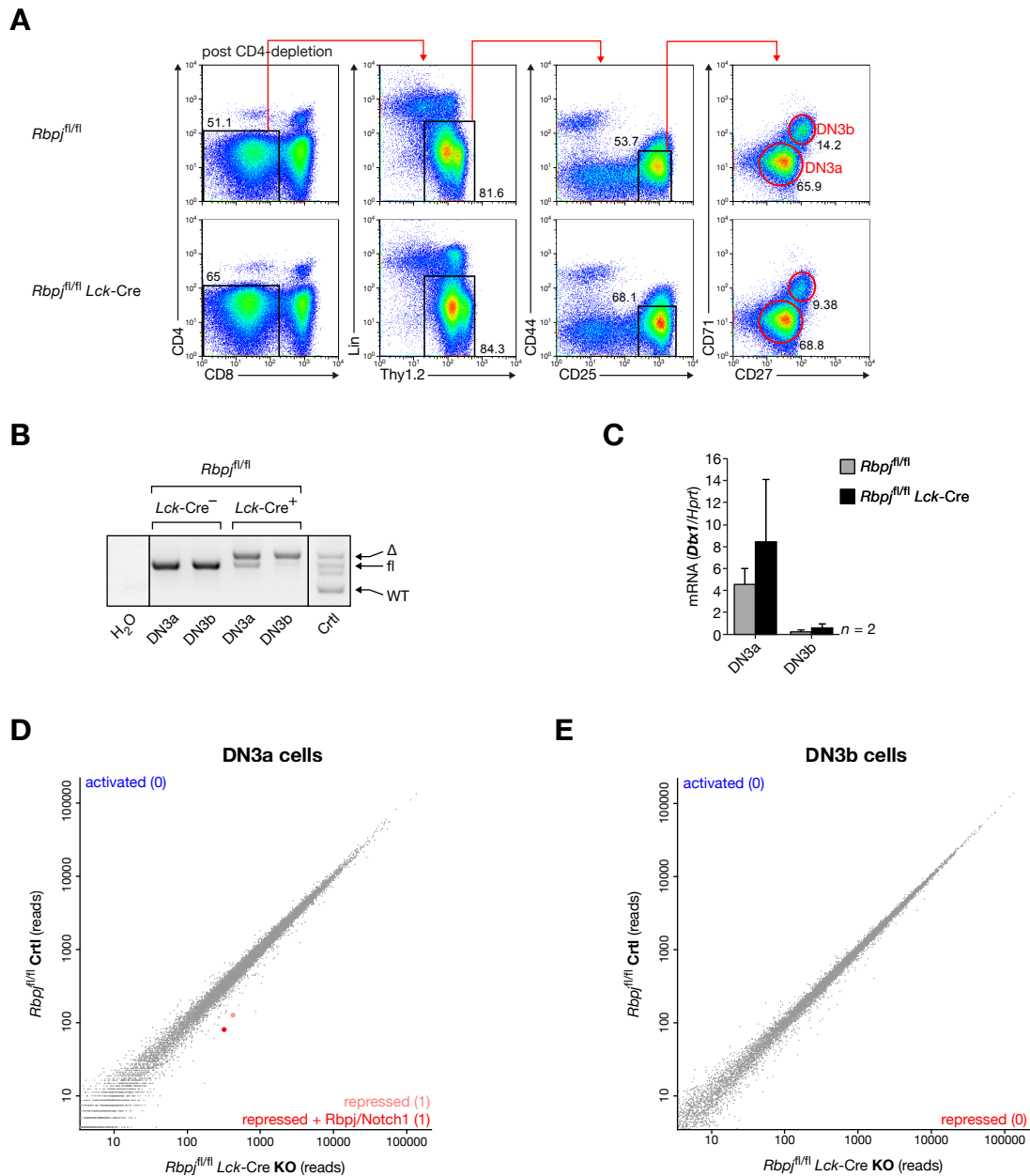
Supplemental Figure 7.3 Quality of the samples used for the generation of the *cis*-regulatory landscape of *Rag2*-deficient DN3a cells. (A) FACS analysis of whole-thymocyte suspensions of *Rag2*^{-/-} mice used for DHS mapping (left) and histone modification ChIP-seq (right) before and after Lympholyte cell purification. Numbers refer to percent cells in the indicated gate. (B) MACS peak calling results (P -value $< 10^{-10}$) of DHS site mapping and histone modification ChIP-seq (H3K4me2, H3K4me3, H3K9ac, H3K27me3). (C) Overlap analysis of the two replicas for DHS and histone modification ChIP-seq. (D) Scatterplots visualising the peak density correlation (R) of all identified peaks (P -value $< 10^{-10}$) between the two replicas of each profiled chromatin feature, and input. Individual peak densities are expressed as $\log_2$ of the sum over all read count-normalised binned read densities from a peak-centred 5 kb region. (E) An IGB screenshot of the *Gata3* locus showing individual RPM-normalised DHS and histone modification ChIP-seq read density profiles generated from *Rag2*^{-/-} DN3a cells. Values on the y-axis represent the read density. Arrows mark TSSs and filled black boxes represent exons. The scale in kb is shown at the bottom right.



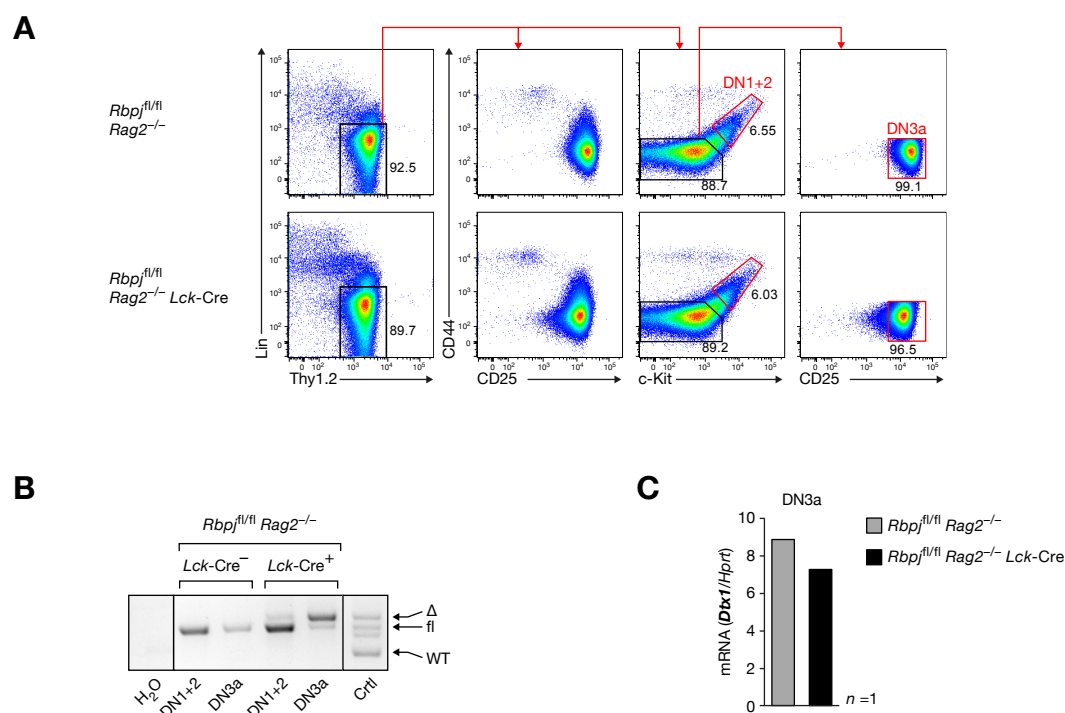
Supplemental Figure 7.4 Comparison of the number of *cis*-regulatory elements of Rag2-deficient DN3a and pro-B cells. (A) Pie chart showing the total numbers and percentages of all *cis*-regulatory elements (shown by different colours) identified in *Rag2*^{-/-} pro-B cells (Revilla-I-Domingo et al., 2012; Schwickert et al., 2014). (B) Pie charts indicating the absolute numbers of each combination of histone modifications (shown by different colours) at AP (top), IAP (middle) and DE (bottom), which were defined by the strategy shown in Fig. 2.4A.

A**B**

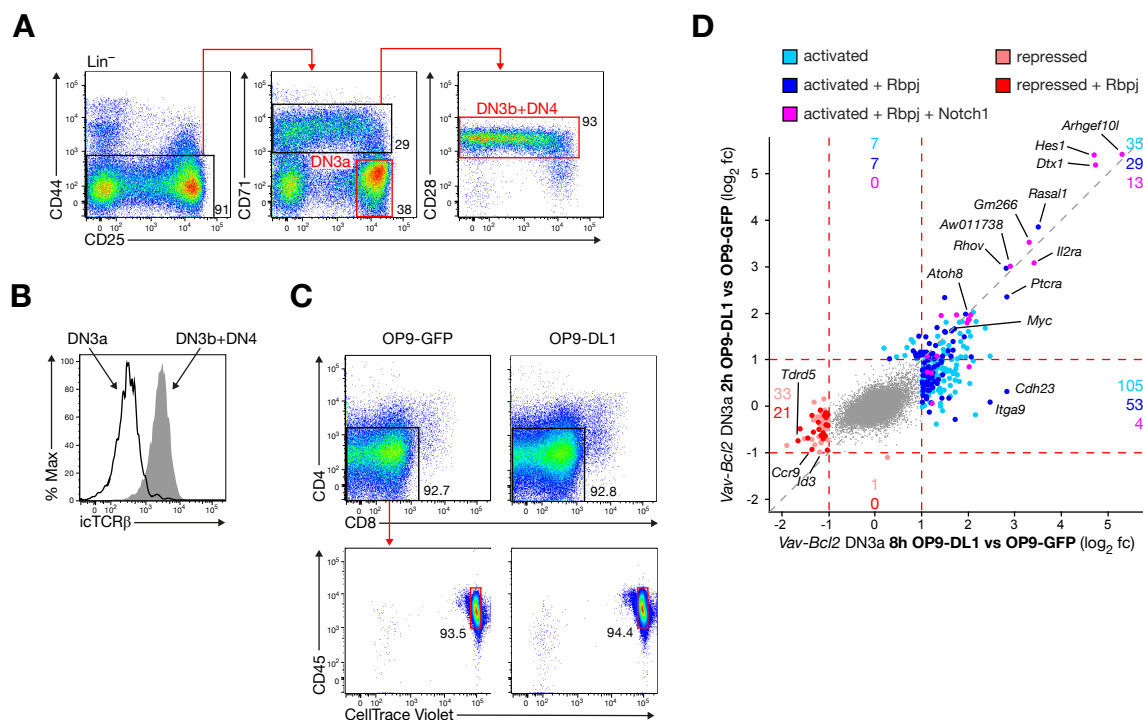
Supplemental Figure 7.5 Intrathymic deletion of *Rbpj*. (A,B) Flow cytometry (A) and absolute numbers (B) of the indicated thymocyte subsets from 6-8 week-old *Rbpj^{fl/fl}* (grey bars) and *Rbpj^{fl/fl} Lck-Cre* littermates (black bars). Numbers refer to percent cells in the indicated gate (A), *n* indicates the number of mice analyzed and error bars represent SEM (B).



Supplemental Figure 7.6 Transcriptome analysis of *Rbpj*^{fl/fl} Lck-Cre DN3 cells. (A) FACS analysis showing the gating strategy for the indicated thymocyte subsets from 6-8 week-old *Rbpj*^{fl/fl} and *Rbpj*^{fl/fl} Lck-Cre littermates. Numbers refer to percent cells in the indicated gate. Red gates depict final sorting gates. (B) Deletion of the floxed *Rbpj* allele in DN3a and DN3b cells was analyzed by PCR. PCR fragments corresponding to the wild-type (WT), intact (fl) and deleted (Δ) floxed alleles are indicated. A 1:1 mix of tail DNA from *Rbpj*^{fl/+} and *Rbpj* ^{Δ /+} mice was used as template in a control PCR. (C) *Dtx1* mRNA abundance relative to *Hprt* mRNA in *Rbpj*^{fl/fl} (grey bars) and *Rbpj*^{fl/fl} Lck-Cre (black bars) DN3a and DN3b cells was analyzed by RT-qPCR. *n* indicates the number of experiments. Error bars represent the SD. (D,E) Scatter plot visualising gene expression differences identified by RNA-seq of *Rbpj*^{fl/fl} and *Rbpj*^{fl/fl} Lck-Cre DN3a (D) or DN3b (E) cells. Genes, which are more than 2-fold differentially expressed and have an adjusted *P*-value of < 0.1, are shown in blue (*Rbpj*-activated; 0 genes) or red (*Rbpj*-repressed; 2 genes). Darker colours indicate binding of *Rbpj*-Bio within 50 kb of the gene, identified by Bio-ChIP-seq in DN3a cells (see chapter 2.1.3). Numbers in brackets correspond to the number of genes in a given group.

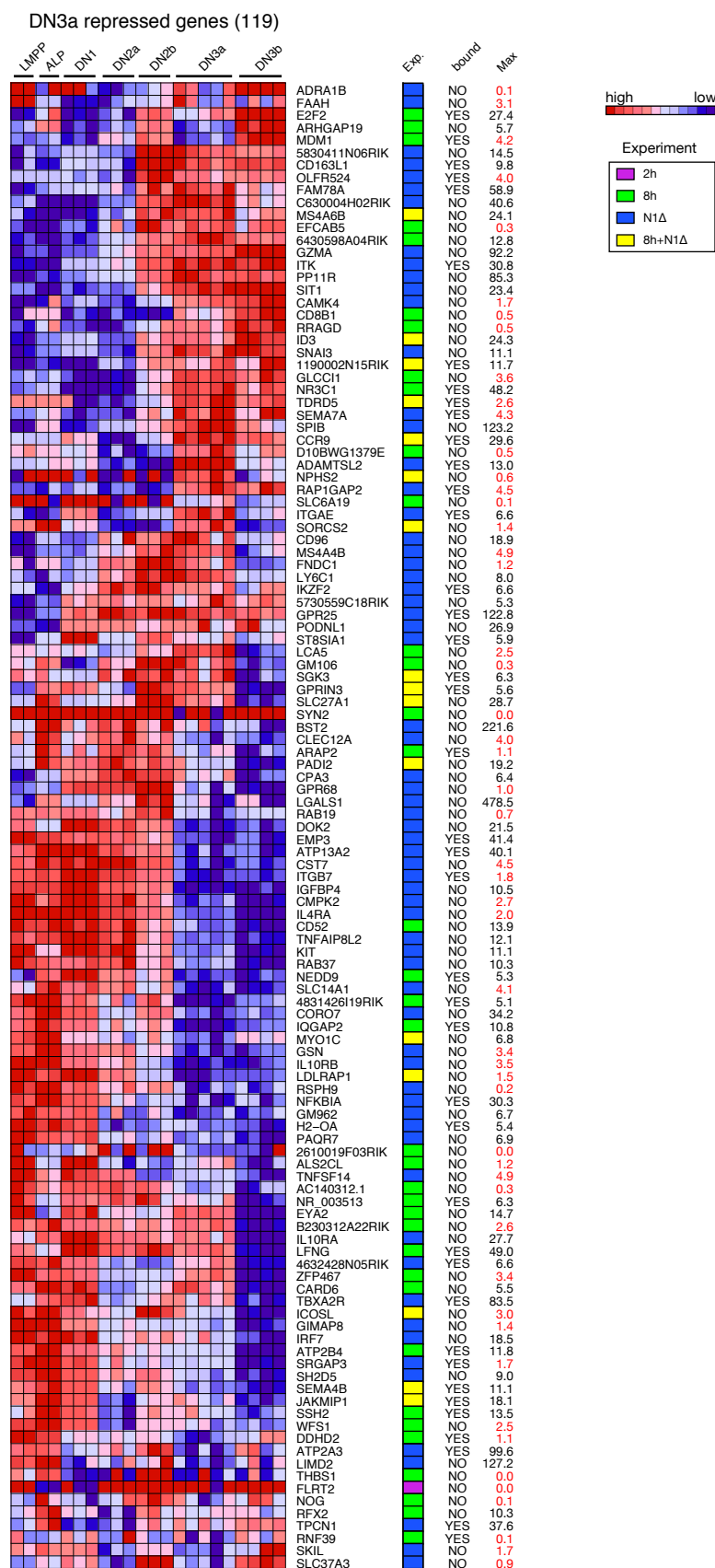


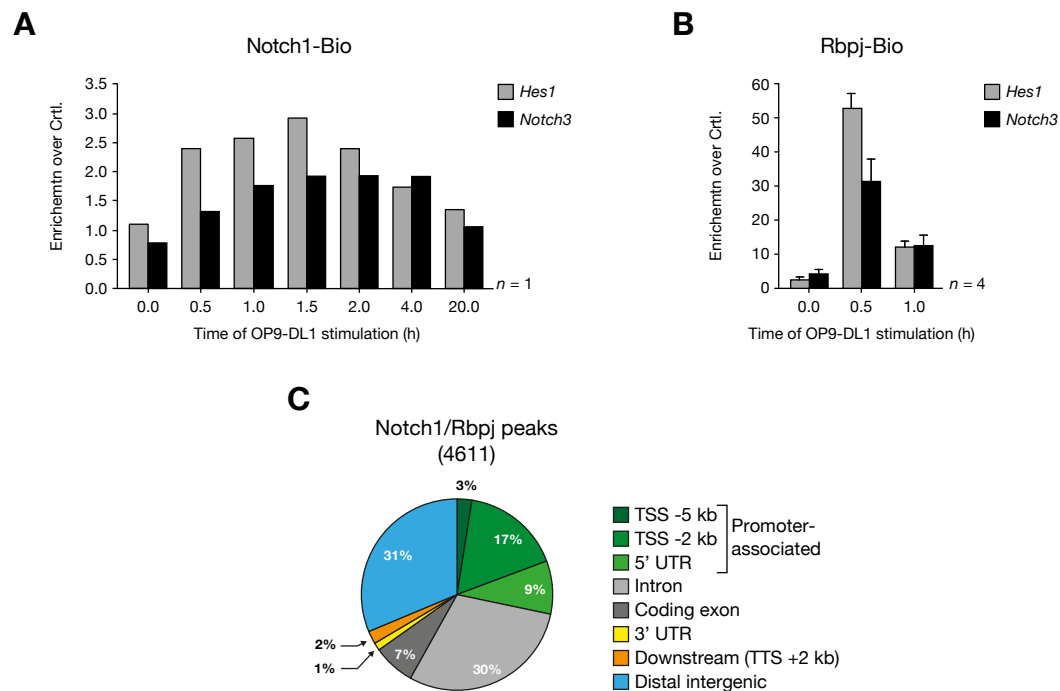
Supplemental Figure 7.7 Intrathymic deletion of *Rbpj* in *Rag2*-deficient mice. (A) FACS analysis showing the gating strategy for the indicated thymocyte subsets from 6-8 week-old *Rbpj*^{fl/fl} *Rag2*^{-/-} and *Rbpj*^{fl/fl} *Rag2*^{-/-} *Lck-Cre* littermates. Numbers refer to percent cells in the indicated gate. Red gates depict final sorting gates. (B) Deletion of the floxed *Rbpj* allele in *Rag2*^{-/-} DN3a cells was analyzed by PCR. PCR fragments corresponding to the wild-type (WT), intact (fl) and deleted (Δ) floxed alleles are indicated. A 1:1 mix of tail DNA from *Rbpj*^{fl/+} and *Rbpj* ^{Δ /+} mice was used as template in a control PCR. (C) *Dtx1* mRNA abundance relative to *Hprt* mRNA in *Rbpj*^{fl/fl} *Rag2*^{-/-} (grey bars) and *Rbpj*^{fl/fl} *Rag2*^{-/-} *Lck-Cre* (black bars) DN3a and DN3b cells was analyzed by RT-qPCR. *n* indicates the number of experiments. Error bars represent the SD.



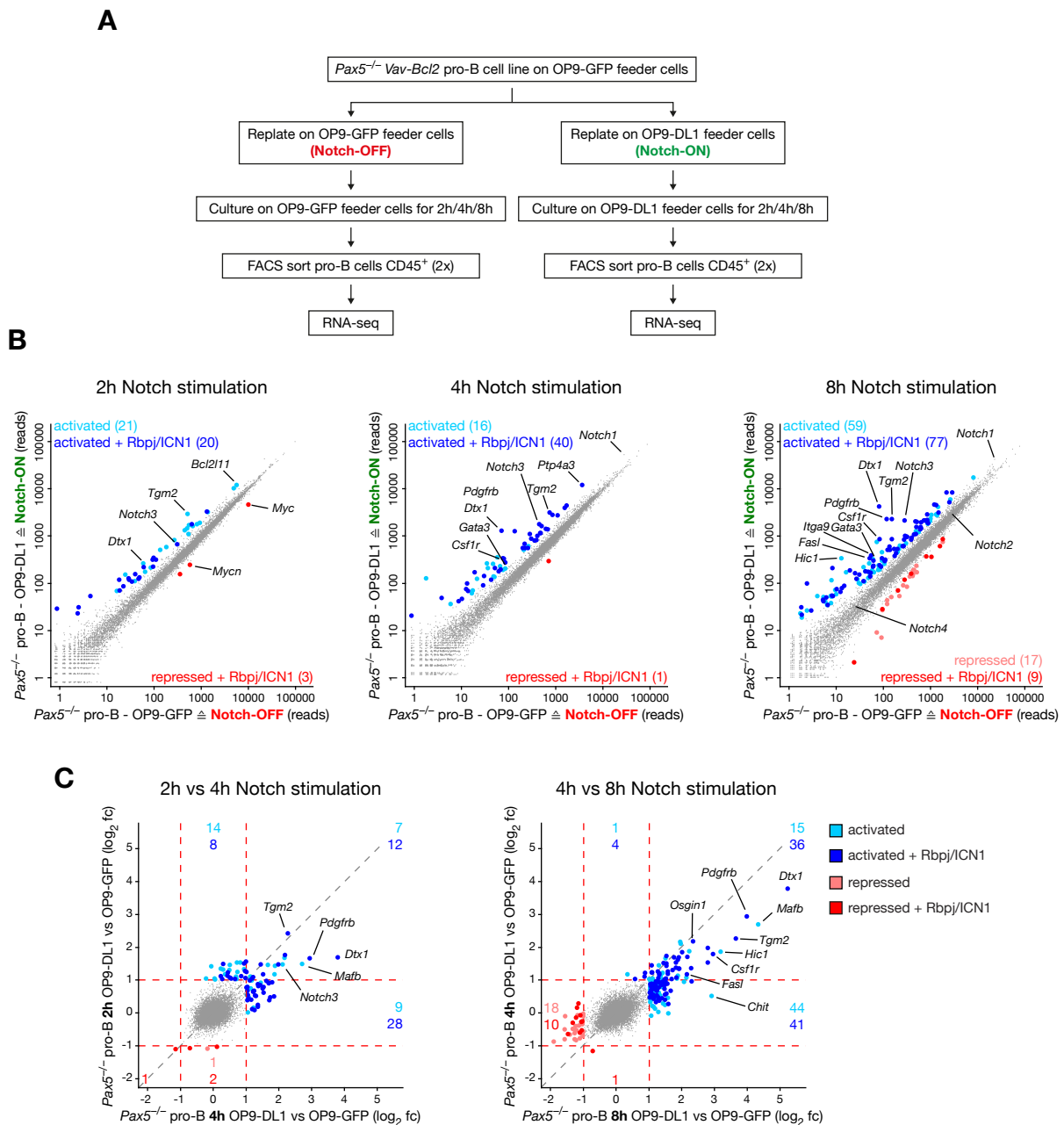
Supplemental Figure 7.8 Sorting and RNA-seq of Notch-stimulated *Vav-Bcl2* DN3a and *Vav-Bcl2* DN3b+DN4 cells. (A,C) FACS analysis showing the gating strategy for DN3a and DN3b+DN4 subsets of *Vav-Bcl2* bone marrow chimeric mice (A) and the resort of CD45⁺ CellTrace^{hi} CD4⁻ CD8⁻ GFP⁻ DN3a cells after co-culture on OP9-DL1 or OP9-GFP (both GFP⁺) cells. Numbers in (A,C) refer to percent cells in the indicated gate, and red gates depict final sorting gates. (B) Flow cytometry with intracellular TCRβ (icTCRβ) in sorted subsets depicted in (A), which demonstrated that, as expected, sorted DN3a cells were icTCRβ⁻, while DN3b+DN4 cells were icTCRβ⁺. (D) Scatter plot visualising moderated fold expression differences identified by RNA-seq of *Vav-Bcl2* DN3a cells after 2 h (y-axis) and 8 h (x-axis) of Notch stimulation on OP9-DL1 feeder cells. Genes, which were more than 2-fold differentially expressed (dashed red lines) are shown in blue (Notch-activated) or red (Notch-repressed). Darker colours indicate binding of Rbpj-Bio and magenta co-occupancy of ICN1-Bio and Rbpj-Bio within 50 kb of the gene, identified by Bio-ChIP-seq in DN3a cells (see chapter 2.1.3). Numbers correspond to the number of genes in a given group. The *P*-value and dispersion threshold was omitted for this correlation analysis. The dashed grey line depicts a correlation of 1.

Supplemental Figure 7.9 (following page) Expression profiles through early lymphocyte development of genes repressed by Notch signalling in DN3a cells A heatmap showing the expression profiles of genes that were repressed by Notch signalling in DN3 cells. Hierarchical clustering by pairwise average-linkage was performed on log-transformed RPKM expression values of LMPP, ALP, DN1, DN2a, DN2b, DN3a and DN3b cells. The number of columns equals the number of independent RNA-seq samples per cell type. The distance between rows (genes) was measured by Pearson correlation. Expression values are row-normalised. The colour code on the right side depicts, in which experiments the gene was identified (2/8 h = *in vitro* Notch stimulation on OP9-DL1 feeders; N1Δ = *in vivo* Notch1 deletion by *Lck-Cre* in Rag2-deficient mice). Binding of Rbpj-Bio in DN3a cells within 50 kb of a gene (see chapter 2.1.3) is indicated in the bound column. Max RPKM indicates the maximum RPKM expression value of a gene within thymic DN1 to DN3b stages. Values are averages from all samples of one population, and values below 5 RPKM are red.



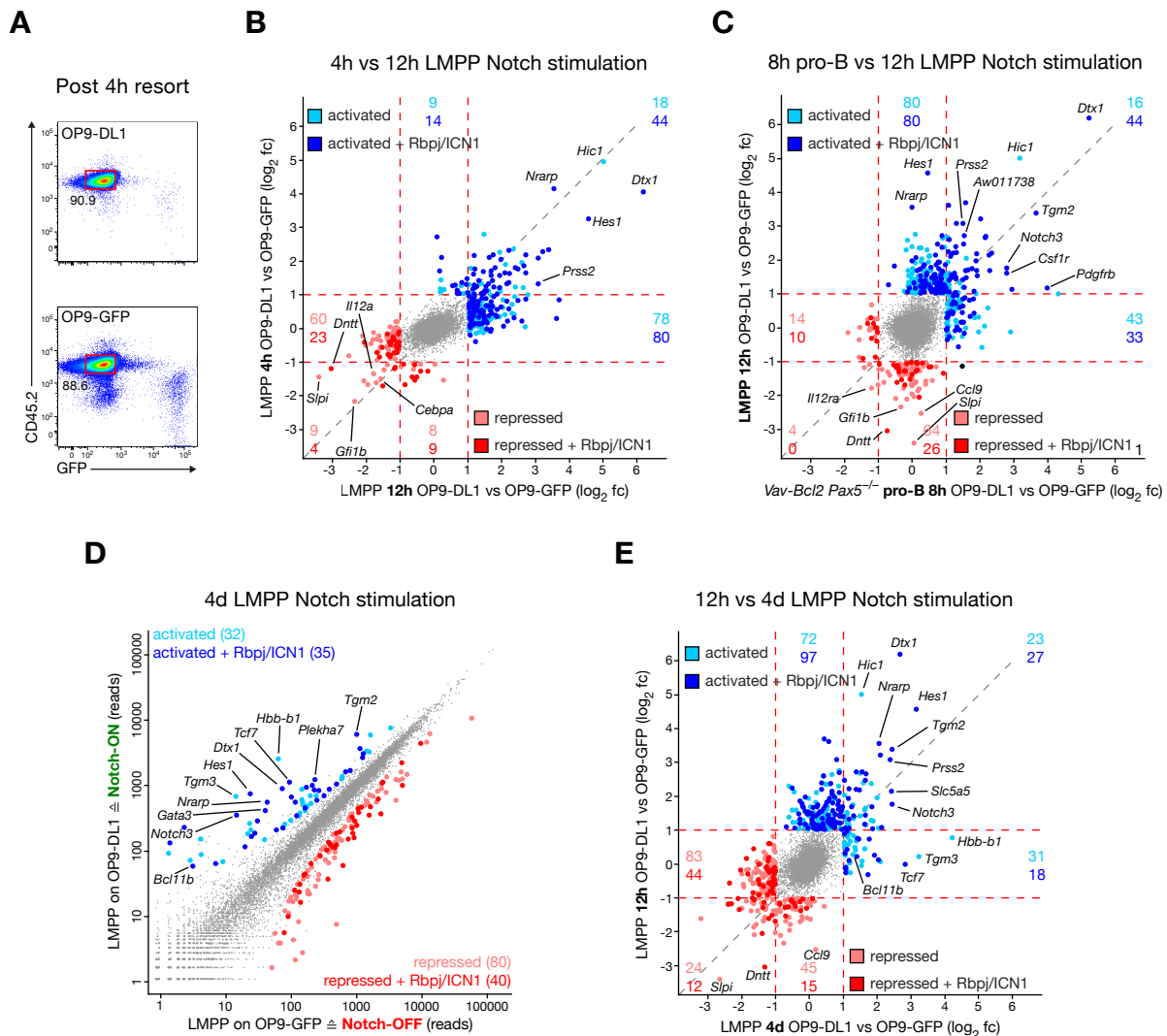


Supplemental Figure 7.10 Kinetics of ICN1-Bio and Rbpj-Bio recruitment to chromatin in Pax5 deficient pro-B cells. (A,B) Bio-ChIP-qPCR with ICN1 from *Notch1*^{Bio} *Pax5*^{Δ/Δ} (A) and Rbpj *Rbpj*^{Bio} *Pax5*^{Δ/Δ} (B) pro-B nuclear lysates. Notch signalling in pro-B cells was induced by transfer from OP9-GFP co-cultures onto OP9-DL1 co-cultures. The cells were harvested and crosslinked with FA after the indicated time points. Bio-ChIP-seq mediated enrichment of *Hes1* (grey) and *Notch3* (black) promoter elements with known ICN1/Rbpj-binding sites over control regions was tested by qPCR. (C) Pie charts representing the ratios of all 4611 ICN1-Bio and Rbpj-Bio-binding sites identified by Bio-ChIP-seq based on their distance to different features (shown by different colours) of mm9-annotated genes. Promoter-associated regions are defined by their proximity to the TSS. The '–' and '+' signs indicate upstream or downstream sequences, respectively.



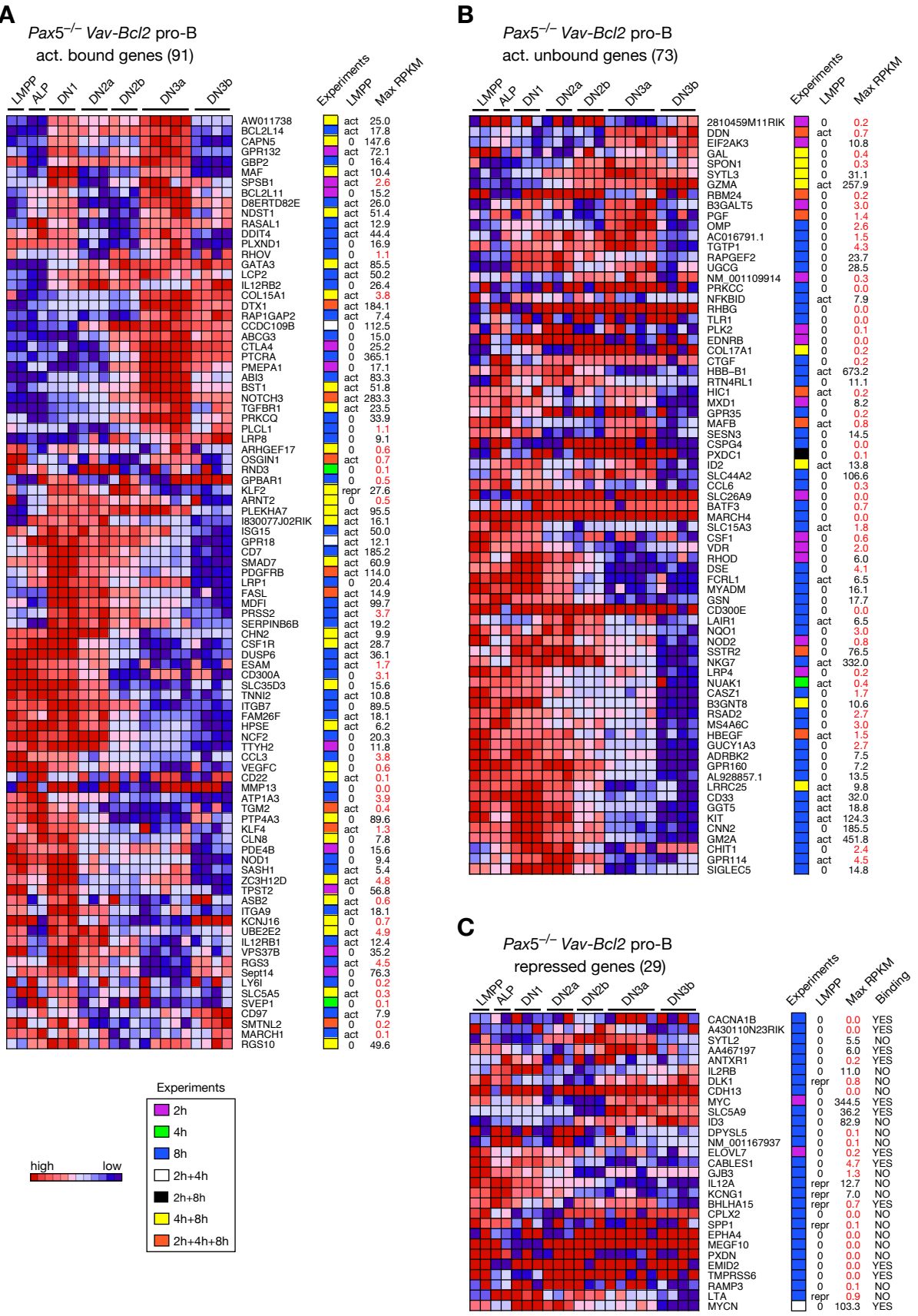
Supplemental Figure 7.11 RNA-seq of Notch-stimulated Pax5-deficient Vav-Bcl2 pro-B cells.

(A) Flowchart describing the experimental design. (B) Scatter plot visualising gene expression differences identified by RNA-seq of Pax5^{-/-} Vav-Bcl2 pro-B cells after 2 h (left), 4 h (middle) and 8 h (right) of Notch-stimulation on OP9-DL1 feeder cells. Genes, which are more than 2-fold differentially expressed and have an adjusted *P*-value of < 0.1, are shown in blue (Notch-activated) or red (Notch-repressed). Darker colours indicate binding of Rbpj-Bio in Rag2-deficient DN3a cells (see chapter 2.1.3) and/or ICN1-Bio in Pax5-deficient pro-B cells (see chapter 2.4.1) within 50 kb of the gene, identified by Bio-ChIP-seq. Numbers in brackets correspond to the number of genes in a given group. (C) Scatter plots visualising the correlation of gene expression differences identified by RNA-seq shown in (B) after 2 h and 4 h (left) and 4 h and 8 h (right) of Notch stimulation. Genes, that are more than 2-fold differentially expressed (dashed red lines), have the same colour code as described in (B). Numbers correspond to the number of genes in a given group. The *P*-value and dispersion threshold was omitted for this correlation analysis. The dashed grey line depicts a correlation of 1.

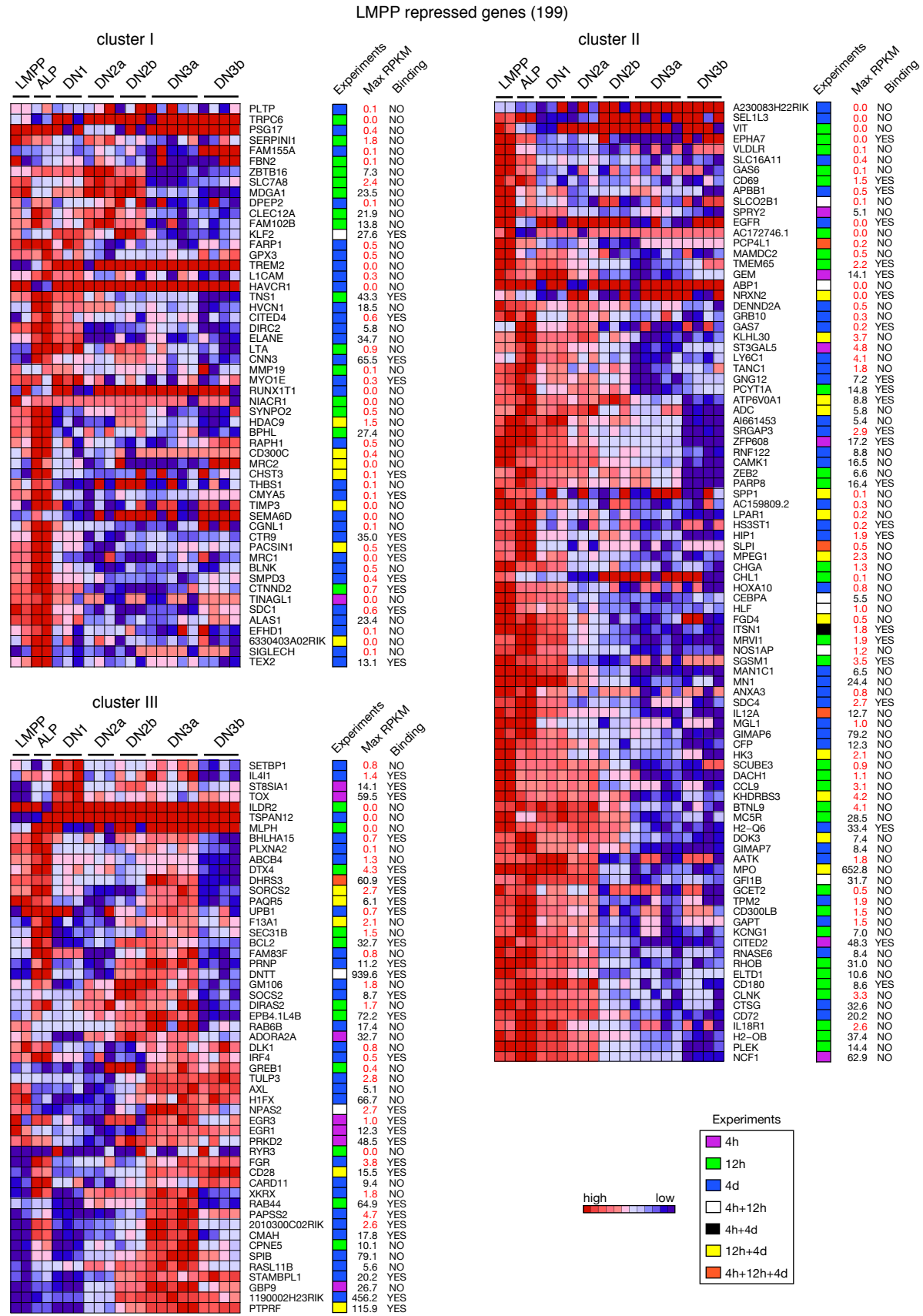


Supplemental Figure 7.12 Correlation of RNA-seq of Notch-stimulated LMPPs. (A) FACS analysis showing the resorting strategy of LMPPs after 4 h *in vitro* culture on OP9-DL1 (top) or OP9-GFP (bottom). Final sorting gates are depicted in red. (B,C) Scatter plots visualising the correlation of gene expression differences identified by RNA-seq of LMPPs (see Figure 2.20B) after 4 h and 12 h of Notch stimulation (B) and between 8 h Notch-stimulated *Pax5*^{-/-} pro-B cells (see Figure 7.11) and 12 h Notch-stimulated LMPPs (C). Genes, that are more than 2-fold differentially expressed (dashed red lines), are shown in blue (Notch-activated) or red (Notch-repressed). Darker colours indicate binding of Rbpj-Bio in Rag2-deficient DN3a cells (see chapter 2.1.3) and/or ICN1-Bio in Pax5-deficient pro-B cells (see chapter 2.4.1) within 50 kb of the gene, identified by Bio-ChIP-seq. Genes, which are inversely regulated by Notch signalling in different cell types are shown in black. Numbers correspond to the number of genes in a given group. The *P*-value and dispersion threshold was omitted for this correlation analysis. The dashed grey line depicts a correlation of 1. (D) Scatter plot visualising gene expression differences identified by RNA-seq of LMPPs after 4 d of Notch stimulation on OP9-DL1 feeder cells. Genes, which are more than 2-fold differentially expressed and have an adjusted *P*-value of < 0.1, are shown in blue (Notch-activated) or red (Notch-repressed). Direct target genes are shown in darker colours as described above. Numbers in brackets correspond to the number of genes in a given group. (E) Scatter plots visualising the correlation of gene expression differences identified by RNA-seq of LMPPs after Notch stimulation for 12 h (see Figure 2.20B) or 4 d as shown in D. Colour code and threshold values of depicted lines are explained above.

Supplemental Figure 7.13 (following page) Expression profiles through early lymphocyte development of genes regulated by Notch signalling in Pax5-deficient pro-B cells. (A-C) Heatmaps showing the expression profiles of Notch-activated genes that are bound by Rbpj-Bio in *Rag2*^{-/-} DN3a cells and/or ICN1-Bio in *Pax5* mutant pro-B cells (A), Notch-activated genes to which no Rbpj-Bio or ICN1-Bio-binding site was assigned to (B) and Notch-repressed genes (C). Hierarchical clustering by pairwise average-linkage was performed on log-transformed RPKM expression values of wild-type/control LMPP, ALP, DN1, DN2a, DN2b, DN3a and DN3b cells. The number of columns equals the number of independent RNA-seq samples per cell type. The distance between rows (genes) was measured by Pearson correlation. Expression values are row-normalised. The colour code on the right side depicts, in which experiments (duration of Notch stimulation) the gene was identified. The LMPP column indicates if the gene was activated (act), repressed (repr) or less than 2-fold differentially expressed (0) in LMPPs upon Notch stimulation (see Figure 2.21 & 7.14). Max refers to the maximum RPKM expression value of a gene within thymic DN1 to DN3b stages. Values are averages from all samples of one population, and values below 5 RPKM are red.



Supplemental Figure 7.14 (following page) Expression profiles through early lymphocyte development of genes repressed by Notch signalling in LMPPs. Heatmaps showing the expression profiles of 199 Notch-repressed genes identified in LMPPs. Hierarchical clustering by pairwise average-linkage was performed on log-transformed RPKM expression values of wild-type/control LMPP, ALP, DN1, DN2a, DN2b, DN3a and DN3b cells. The number of columns equals the number of independent RNA-seq samples per cell type. The distance between rows (genes) was measured by Pearson correlation. Expression values are row-normalised. The analysis resulted in three main clusters which were separated in order to improve their display. The colour code on the right side depicts, in which experiments (duration of Notch stimulation) the gene was identified. Max refers to the maximum RPKM expression value of a gene within thymic DN1 to DN3b stages. Values are averages from all samples of one population, and values below 5 RPKM are red.



7.2 Supplemental tables

Supplemental Table 7.1: Directly Notch-activated genes identified in DN3a cells (135)

Gene	Code	2 h stimulation			8 h stimulation			Notch1 ^{fl/m} Rag2 ^{-/-}			In vivo expression (RPKM)								LMPP		Pax5 ^{-/-} pro-B		B _{DN3,B}
		fc	GFP	DL1	fc	GFP	DL1	fc	Cre ⁺	Cre ⁻	LMPP	ALP	DN1	DN2a	DN2b	DN3a	DN3b	Max	↑	↓	↑	↓	
3110082117RIK	010	1.3	2.43	3.11	2.8	2.06	6.03	0.9	14.62	12.76	36.5	29.3	27.0	31.5	21.3	14.2	44.1	44.1	-	-	-	-	B
ABCB1A	010	2.2	0.03	0.06	9.2	0.04	0.40	0.9	0.01	0.01	0.2	0.1	0.2	0	0	0	0	0.2	010	-	-	-	B
ABCE1	010	1.4	11.60	16.31	2.3	5.98	13.86	1.1	17.95	19.29	11.7	14.6	12.7	20.3	13.3	32.4	55.2	55.2	-	-	-	-	B
ABHD15	110	2.9	26.85	76.46	2.2	25.03	56.13	1.5	63.65	94.73	23.2	22.9	35.1	25.2	23.2	39.3	6.2	39.3	-	-	-	-	B
ADAMTS2	010	3.0	0.04	0.12	22.7	0.02	0.43	-	0.00	0.01	0	0.1	0.1	0	0	0	0	0.1	-	-	-	-	B
ADK	010	0.9	2.81	2.45	2.9	2.59	7.64	1.3	9.30	12.24	16.3	18.2	17.2	23.5	19.8	19.6	46.7	46.7	-	-	-	-	B
ADRB2	110	2.3	13.77	31.59	2.6	11.77	30.76	1.6	4.18	6.73	5.6	2.5	0.7	1.6	3.2	5.4	2.6	5.4	-	-	-	-	B
AGFG1	011	1.7	18.16	31.00	2.3	17.81	41.38	3.2	27.69	89.30	15.6	13.2	16.4	14.0	14.4	41.3	40.2	41.3	-	-	-	-	B
AKAP6	010	1.3	0.02	0.02	4.4	0.12	0.53	1.0	1.20	1.20	0	0.1	0.2	1.0	1.1	1.4	0.5	1.4	-	-	-	-	B
ALDH1B1	111	2.4	19.26	45.53	3.4	19.98	68.38	3.4	65.82	225.57	14.2	26.2	32.8	117.4	96.0	81.8	48.8	117.4	-	-	-	-	B
ARHGEF10L	111	58.1	1.43	82.96	49.5	2.15	107.60	5.3	19.59	105.79	2.3	1.5	2.3	4.3	2.0	59.3	9.2	59.3	-	-	-	-	B
ARNT2	001	0	0.00	0.00	0	0.02	0.00	14.2	0.09	1.36	0	0.1	0.2	0.5	0.2	0.2	0	0.5	-	-	011	-	B
ATIC	010	1.6	19.45	30.64	2.3	16.37	39.01	1.1	42.33	48.95	74.0	90.0	61.0	56.5	55.7	70.8	151.2	151.2	-	-	-	-	B
ATOH8	111	4.1	17.81	72.91	4.2	10.56	44.65	2.8	13.73	38.94	0.2	0.2	0	0	0	15.2	0.3	15.2	-	-	-	-	B
AW011738	111	9.3	5.30	49.06	8.8	6.57	58.64	2.6	16.16	42.67	3.2	1.8	13.6	10.1	10.7	25.0	5.9	25.0	110	-	011	-	B
B4GALNT2	001	1.0	0.68	0.68	2.2	0.75	1.70	9.5	0.51	4.98	0.5	0.2	0.5	1.0	1.0	1.2	1.1	1.2	-	-	-	-	B
BID	110	2.4	6.49	15.47	2.2	6.94	15.65	0.7	12.27	8.59	25.9	42.2	27.4	28.8	16.9	17.8	62.5	62.5	-	-	-	-	B
BST1	111	2.2	23.82	51.40	2.7	13.67	36.88	2.7	37.21	103.13	0.1	0.9	1.5	4.5	6.6	51.8	5.2	51.8	010	-	011	-	B
BTG1	100	2.9	37.69	107.98	1.9	41.74	82.49	1.0	89.44	93.95	7.6	8.3	14.1	9.8	11.1	30.3	4.1	30.3	-	-	-	-	B
BZW2	010	1.9	36.34	68.52	2.6	26.38	70.53	1.1	72.11	77.59	62.1	78.1	88.8	113.8	87.4	100.9	152.1	152.1	-	-	-	-	B
C1QBP	010	2.0	38.39	75.95	2.8	37.06	105.95	0.9	98.99	95.09	167.7	81.5	166.7	203.9	156.2	143.6	333.3	333.3	-	-	-	-	B
CAPN5	111	3.7	33.54	122.87	4.3	26.92	117.17	3.3	81.72	271.55	26.1	30.9	70.1	42.7	34.5	147.6	23.2	147.6	-	-	011	-	B
CBLB	001	1.3	30.00	40.09	1.1	25.87	28.67	2.2	50.54	112.75	7.0	12.8	12.8	10.9	11.4	57.9	10.1	57.9	-	-	-	-	B
CCDC109B	001	1.0	3.92	3.94	1.0	3.01	2.98	2.8	62.04	177.82	0.2	0.4	0.6	17.4	112.5	88.2	31.4	112.5	-	-	110	-	B
CD3D	001	1.0	117.44	115.33	1.4	85.60	123.06	2.6	370.94	967.13	0.1	0.1	3.1	0.7	25.3	397.1	142.5	397.1	-	-	-	-	B
CD82	011	1.9	22.82	42.40	4.7	11.78	55.92	3.3	105.77	352.28	64.5	85.3	126.0	37.0	64.8	155.9	48.4	155.9	010	-	-	-	B
CDH23	011	1.3	1.06	1.35	8.5	1.15	9.95	9.1	1.04	9.58	0.1	0	0	0.1	0.3	11.8	0.8	11.8	010	-	-	-	B
CDK5RAP2	001	1.4	5.69	7.71	2.0	5.31	11.02	3.4	4.79	16.40	9.3	12.7	8.9	7.9	10.2	14.3	14.7	14.7	-	-	-	-	B
CDV3	100	2.3	22.88	53.02	1.8	23.71	43.15	1.2	47.36	58.82	17.3	13.1	36.2	36.2	48.8	42.5	59.0	59.0	-	-	-	-	B
CHSY1	001	1.1	9.03	9.74	1.2	10.05	12.37	2.6	19.30	51.18	18.7	28.1	30.8	20.5	24.4	33.8	19.7	33.8	-	-	-	-	B
CLUH	010	1.6	13.69	21.44	2.4	13.37	32.72	1.1	17.77	19.20	40.5	62.7	41.7	30.8	27.4	30.8	67.5	67.5	-	-	-	-	B
CMSS1	010	1.2	5.27	6.14	2.7	3.96	10.85	1.1	14.17	16.20	18.6	13.1	15.6	20.1	17.3	13.6	31.2	31.2	-	-	-	-	B
CNTNAP2	001	1.8	0.08	0.15	8.6	0.02	0.14	4.7	0.35	1.66	0	0	0	0	0	0.6	0.1	0.6	-	-	-	-	B
CORO2A	110	2.3	7.16	16.31	2.1	9.27	20.06	1.5	26.86	39.69	88.9	129.5	159.1	113.5	81.4	46.2	30.5	159.1	-	-	-	-	B
CSDA	010	1.4	17.75	23.94	2.5	13.51	34.58	1.6	42.37	70.81	176.0	155.1	169.2	133.7	118.2	128.9	318.7	318.7	-	-	-	-	B
CTAGE5	010	1.6	23.01	37.61	2.2	18.53	42.36	1.3	13.45	17.84	15.1	14.2	15.1	14.1	13.5	31.4	99.6	99.6	-	-	-	-	B
DCTPP1	010	1.8	24.76	44.87	2.5	26.77	67.98	1.1	84.83	95.86	310.3	174.9	229.6	411.4	323.0	107.0	357.2	411.4	-	-	-	-	B
DEPDC6	001	1.2	1.20	1.43	1.3	1.73	2.29	3.7	1.19	4.51	1.9	1.0	20.0	21.0	6.0	4.2	1.2	21.0	-	-	-	-	B
DHX33	010	2.1	6.07	12.52	2.3	5.78	13.54	1.0	9.73	10.07	12.8	13.5	15.2	11.9	8.7	14.6	18.6	18.6	-	-	-	-	B
DTX1	111	37.6	20.81	779.42	28.0	21.98	625.31	4.6	114.66	529.96	0.2	1.5	9.6	7.0	2.5	184.1	41.8	184.1	111	-	111	-	B

Supplemental Table 7.1 Continued: Directly Notch-activated genes identified in DN3a cells (135)

Gene	Code	2 h stimulation			8 h stimulation			<i>Notch1^{flM} Rag2^{-/-}</i>			<i>In vivo</i> expression (RPKM)								LMPP		<i>Pax5^{-/-}</i> pro-B		B _{DN3B}
		fc	GFP	DL1	fc	GFP	DL1	fc	Cre ⁺	Cre ⁻	LMPP	ALP	DN1	DN2a	DN2b	DN3a	DN3b	Max	↑	↓	↑	↓	
EAF1	010	1.8	15.95	29.28	2.3	14.56	33.85	0.8	22.72	19.52	11.2	14.4	12.5	17.2	21.0	30.5	48.4	48.4	–	–	–	–	B
ECE2	010	1.9	1.59	3.05	2.6	1.84	4.82	0.8	6.66	5.71	8.5	5.0	8.2	11.3	6.6	5.1	6.6	11.3	–	–	–	–	B
EGFR	010	1.3	0.10	0.13	3.8	0.16	0.64	–	0.00	0.09	0	0.1	0	0	0	0	0	0	–	001	–	–	B
ELOVL6	010	1.7	1.90	3.31	2.3	2.36	5.52	0.6	7.11	3.98	6.6	7.0	5.9	7.3	8.4	9.0	8.1	9.0	–	–	–	–	B
ERG	001	0.9	6.36	5.63	1.0	4.23	4.43	2.6	9.34	24.63	71.0	56.4	63.2	36.6	35.3	21.5	0.5	63.2	–	–	–	–	B
ETS2	001	1.4	190.48	271.44	1.5	156.44	236.07	2.1	43.22	93.32	10.2	6.9	4.8	3.0	6.7	52.7	33.5	52.7	–	–	–	–	B
FAM163B	001	1.2	222.42	269.14	1.2	153.10	189.97	4.1	26.98	111.34	0	0	0	0	0	73.3	21.8	73.3	–	–	–	–	B
FAM43A	001	1.3	16.86	21.30	1.4	10.43	14.51	3.4	6.85	23.71	20.8	15.3	17.0	5.6	2.9	13.6	4.3	17.0	–	–	–	–	B
FRMD4A	001	1.9	23.35	43.25	1.8	21.33	38.36	2.8	23.25	66.91	15.9	18.1	27.1	16.0	17.3	46.6	9.6	46.6	–	–	–	–	B
FSTL4	001	1.0	1.18	1.14	0.6	1.00	0.60	17.0	0.07	1.29	0	0	0	0	0	0.2	0.1	0.2	–	–	–	–	B
GADD45G	100	3.7	1.10	4.03	1.3	2.53	3.36	4.3	0.46	2.04	0.7	1.1	0.1	0.1	0.3	1.0	0.6	1.0	–	–	–	–	B
GART	010	1.7	25.95	43.20	2.4	23.31	56.45	1.0	55.88	58.64	80.3	100.9	92.8	93.8	87.3	76.4	177.7	177.7	–	–	–	–	B
GFRA1	111	3.8	14.72	55.49	4.1	17.64	74.44	2.5	69.45	178.13	1.3	16.2	25.1	3.9	11.5	89.3	68.7	89.3	–	–	–	–	B
GLS	100	2.3	16.29	36.69	2.0	15.38	31.45	1.1	26.05	29.48	7.2	3.9	7.8	8.9	8.9	27.4	18.1	27.4	–	–	–	–	B
GM266	001	21.3	1.97	41.79	40.5	0.63	25.29	31.3	0.11	3.50	0	0	0	0	0	0.3	0.1	0.3	–	–	–	–	B
GNL3	110	2.4	11.37	27.60	3.3	8.10	27.22	1.0	11.03	11.44	25.6	27.9	22.1	31.5	24.0	37.1	76.3	76.3	–	–	–	–	B
GPR56	100	2.2	60.60	132.86	1.8	55.80	104.95	1.5	256.77	396.84	918.3	411.6	978.0	879.4	489.3	263.9	73.9	978.0	–	–	–	–	B
GRM4	001	1.1	3.91	4.20	0.6	6.29	4.11	2.7	2.22	6.01	0.1	0	0.2	0.8	1.5	12.4	2.5	12.4	–	–	–	–	B
GSR	001	1.7	27.40	46.48	2.0	26.07	51.91	2.1	54.11	117.34	18.5	13.6	12.9	15.1	23.9	57.4	96.7	96.7	–	–	–	–	B
HDGFRP3	110	4.0	14.51	57.89	4.4	8.59	38.48	1.3	7.67	9.83	3.9	0.6	0.7	0.5	0.7	10.2	7.0	10.2	–	–	–	–	B
HES1	110	72.8	2.19	159.43	41.0	2.76	114.38	1.4	174.84	253.88	4.5	1.8	94.3	80.8	78.7	159.8	27.7	159.8	111	–	–	–	B
HEY1	010	7.3	0.05	0.34	5.9	0.18	1.13	0.8	0.21	0.18	0.1	0	0.1	0.1	0	0.1	0	0.1	001	–	–	–	B
HEYL	011	2.5	1.30	3.22	3.7	1.03	3.81	2.5	3.30	8.52	0	0	0.1	0.3	0.3	2.9	0.9	2.9	110	–	–	–	B
HIVEP3	010	1.4	13.49	18.52	2.3	13.58	32.28	2.1	15.27	33.17	1.6	1.1	0.4	0.3	3.0	53.4	48.7	53.4	–	–	–	–	B
HSPA9	010	1.4	30.39	43.55	2.0	25.29	53.06	1.2	42.23	50.12	66.3	59.2	67.3	65.7	57.5	83.7	177.7	177.7	–	–	–	–	B
HSPD1	010	1.5	7.67	11.14	2.4	6.76	16.72	1.4	11.95	16.59	30.9	20.2	38.1	44.9	30.8	31.9	108.6	108.6	–	–	–	–	B
IL10	001	0	0.00	0.19	0	0.00	0.36	–	0.00	1.83	0.2	0	0	0	0	0.5	0.1	0.5	–	–	–	–	B
IL2RA	111	8.6	52.10	446.10	10.8	39.50	433.58	4.1	240.94	1005.97	0.3	0	13.6	267.5	232.6	516.0	93.7	516.0	–	–	–	–	B
JAG1	010	1.6	0.33	0.51	2.8	0.54	1.55	1.7	0.03	0.06	0.1	0.2	0	0	0	0	0	0	–	–	–	–	B
LDLRAD3	001	1.3	17.26	22.34	1.1	14.19	15.39	5.0	0.62	3.13	6.6	5.8	5.6	3.8	1.4	2.7	1.6	5.6	–	–	–	–	B
LRIG1	001	1.6	33.04	51.11	1.3	21.04	28.04	2.6	1.92	5.08	0.5	1.1	1.0	0.3	0.5	4.5	4.3	4.5	–	–	–	–	B
LRP1	001	1.1	6.10	6.38	1.1	9.39	10.72	2.2	4.39	9.72	3.1	11.6	20.4	14.0	5.7	13.1	0.9	20.4	–	–	001	–	B
MDN1	010	1.1	7.01	7.91	3.0	6.85	21.24	1.2	4.44	5.42	6.2	6.9	6.6	5.4	5.9	16.1	16.8	16.8	–	–	–	–	B
METTL13	110	2.2	8.41	18.23	2.3	8.20	19.71	1.0	15.57	15.68	14.1	17.3	18.5	18.9	16.9	19.1	27.0	27.0	–	–	–	–	B
METTL6	010	1.5	12.58	18.44	2.3	11.73	27.49	1.2	22.24	26.66	12.6	12.0	10.3	26.2	23.5	27.5	30.0	30.0	–	–	–	–	B
MFHAS1	110	3.2	5.68	17.99	2.9	4.88	14.59	2.1	1.12	2.38	2.4	6.4	0.7	0.5	0.9	1.7	2.6	2.6	–	–	–	–	B
MRPS18B	010	1.4	8.47	12.06	2.4	6.34	15.64	1.1	10.77	11.82	14.2	16.4	13.9	20.3	16.5	12.1	24.9	24.9	–	–	–	–	B
MRT04	010	1.9	10.13	18.68	2.6	8.92	23.37	1.1	20.87	23.58	32.8	19.3	28.2	46.2	36.1	28.1	64.8	64.8	–	–	–	–	B
MYC	110	3.1	90.04	278.06	3.2	69.41	225.10	1.0	205.61	214.85	278.7	169.7	245.8	132.9	109.4	276.6	344.5	344.5	–	–	–	100	B
MYCN	110	3.4	8.61	29.56	2.9	6.29	18.23	2.0	14.77	30.70	39.6	7.8	103.3	83.3	17.9	16.3	0.7	103.3	110	–	–	110	B
MYL4	001	1.7	0.61	1.05	2.0	0.96	1.95	3.9	5.84	23.30	3.8	26.2	28.5	28.2	29.7	8.7	2.4	29.7	010	–	–	–	B
MYOF	001	1.1	2.26	2.46	1.2	3.57	4.48	3.1	2.55	8.02	6.3	3.6	1.5	0.3	1.2	4.0	0.4	4.0	–	–	–	–	B
NCKAP5	011	0.8	1.27	0.98	4.3	0.72	3.17	3.6	0.97	3.58	0	0	0	0.1	0.3	2.1	0.2	2.1	–	–	–	–	B
NLN	011	1.9	1.03	1.98	3.4	1.57	5.45	4.6	4.95	23.21	5.4	5.5	6.4	5.3	2.9	7.8	6.2	7.8	–	–	–	–	B
NME1	010	1.5	24.22	37.03	2.7	23.48	64.87	1.0	88.74	91.11	107.6	135.7	104.7	216.8	154.2	98.1	238.3	238.3	–	–	–	–	B

Supplemental Table 7.1 Continued: Directly Notch-activated genes identified in DN3a cells (135)

Gene	Code	2 h stimulation			8 h stimulation			<i>Notch1^{ΔM} Rag2^{-/-}</i>			<i>In vivo</i> expression (RPKM)								LMPP		<i>Pax5^{-/-}</i> pro-B		B _{DN3,B}
		fc	GFP	DL1	fc	GFP	DL1	fc	Cre ⁺	Cre ⁻	LMPP	ALP	DN1	DN2a	DN2b	DN3a	DN3b	Max	↑	↓	↑	↓	
NOC3L	010	1.6	2.81	4.36	2.8	2.21	6.34	0.9	4.38	4.04	6.3	6.7	5.4	5.1	4.3	6.5	9.9	9.9	-	-	-	-	B
NOP16	010	1.8	12.73	23.31	2.2	10.92	24.96	1.0	29.65	29.29	44.6	34.0	39.2	49.8	36.2	32.1	55.6	55.6	-	-	-	-	B
NOTCH1	111	3.9	219.94	847.96	2.7	270.76	740.52	2.6	205.86	536.54	68.6	236.3	228.5	163.7	162.1	526.1	230.2	526.1	110	-	-	-	B
NOTCH3	110	3.9	58.88	230.62	3.4	66.70	231.89	1.7	217.38	369.88	0.5	0.3	2.7	12.6	29.8	283.3	33.7	283.3	011	-	111	-	B
NRP1	110	3.0	1.15	3.40	2.8	1.95	5.53	1.0	14.75	15.14	0.6	7.6	1.7	0.5	1.8	16.2	22.9	22.9	-	-	-	-	B
ODZ1	111	2.4	1.26	3.06	2.6	1.32	3.45	2.5	2.46	6.26	0	0	0	0	0	3.3	2.8	3.3	-	-	-	-	B
OSGIN1	110	3.3	4.33	14.33	4.7	3.89	18.41	1.5	0.65	0.99	8.1	0.2	0.2	0.1	0	0.7	0.1	0.7	110	-	111	-	B
PA2G4	010	1.6	48.82	76.53	2.3	45.14	107.35	1.2	102.61	128.78	129.5	124.4	137.8	171.5	160.7	186.4	370.0	370.0	-	-	-	-	B
PDZD2	001	1.1	0.22	0.24	1.0	0.23	0.23	3.1	0.30	0.95	0.1	0.1	0.1	0.3	0.6	0.7	0.2	0.7	-	-	-	-	B
PLCL1	010	1.2	0.51	0.63	3.6	0.61	2.28	0.9	1.38	1.30	0.4	0.3	0.2	0.1	0.3	1.1	0.5	1.1	-	-	001	-	B
PLD4	001	1.2	15.24	18.16	1.2	13.51	17.21	3.1	199.10	626.33	11.5	89.0	9.4	1.3	12.4	314.5	14.6	314.5	010	-	-	-	B
PLXND1	001	1.5	19.82	30.19	1.3	21.20	27.63	2.7	6.98	19.16	19.2	18.9	15.9	6.5	5.8	16.9	5.1	16.9	-	-	001	-	B
POLL	001	1.2	0.71	0.84	1.3	1.02	1.37	4.7	1.81	8.56	23.8	27.0	35.5	15.5	4.5	5.1	5.9	35.5	-	-	-	-	B
POLR1A	010	1.3	9.60	12.29	2.2	9.41	21.63	1.1	15.36	17.08	24.2	32.8	26.1	17.7	17.0	24.5	37.3	37.3	-	-	-	-	B
PPRC1	110	2.7	15.18	40.40	3.3	13.32	44.50	1.1	12.21	14.24	38.5	41.7	45.9	33.8	31.4	31.4	54.3	54.3	-	-	-	-	B
PRDX1	010	1.1	13.06	13.83	2.7	5.57	15.39	1.1	31.64	35.26	30.3	18.2	30.1	80.4	44.9	51.0	102.5	102.5	-	-	-	-	B
PSD	111	3.5	4.17	14.67	3.7	4.53	17.18	4.0	2.34	9.60	2.4	1.0	2.5	1.2	1.3	7.3	1.0	7.3	-	-	-	-	B
PTCRA	111	5.2	81.91	425.87	7.5	54.63	415.18	2.9	257.28	752.12	0	0.1	0	0.2	5.2	365.1	70.0	365.1	-	-	001	-	B
PTPN14	001	1.9	5.20	9.81	1.8	5.46	10.16	2.8	4.04	11.65	0.6	0.3	0.1	0.6	0.6	7.3	6.8	7.3	-	-	-	-	B
QTRTD1	010	1.5	5.11	7.50	2.3	3.95	9.10	1.0	5.84	6.15	8.9	7.7	7.7	8.5	6.8	8.7	9.9	9.9	-	-	-	-	B
RANBP1	010	1.4	61.35	85.74	2.3	49.22	114.56	1.1	129.20	147.63	292.3	193.6	284.1	410.6	371.2	212.8	729.4	729.4	-	-	-	-	B
RASAL1	111	20.7	1.63	33.68	16.2	1.73	28.35	2.8	4.32	12.34	3.8	34.7	9.0	2.0	2.3	12.9	10.9	12.9	010	-	001	-	B
RHOBTB3	110	3.3	3.42	11.24	3.3	2.97	9.86	1.6	9.72	15.67	6.9	1.3	2.3	1.8	1.3	7.4	2.5	7.4	-	-	-	-	B
RHOV	001	18.2	0.85	15.33	21.7	0.76	16.40	44.5	0.62	28.28	0.4	0.2	0.3	0.1	0	1.1	0	1.1	-	-	001	-	B
RNF182	111	3.0	1.05	3.16	3.4	0.49	1.65	12.2	0.58	7.17	0	0	0.1	0	0.1	1.1	0	1.1	-	-	-	-	B
RRP15	010	1.6	11.89	19.17	2.5	11.61	29.71	1.0	27.23	29.04	35.3	32.7	38.9	44.3	39.4	38.0	60.9	60.9	-	-	-	-	B
SCN2B	011	2.3	2.83	6.49	5.3	2.22	11.84	4.4	3.93	17.57	0	0	0.1	0	0.1	8.5	0.9	8.5	-	-	-	-	B
SLC16A10	110	2.2	7.74	17.25	2.1	7.87	16.65	1.0	18.56	18.37	6.7	13.7	8.3	9.1	10.3	21.0	24.5	24.5	-	-	-	-	B
SLC5A6	010	1.5	1.67	2.52	2.5	1.44	3.62	1.2	2.39	2.80	3.9	2.9	3.2	2.6	3.3	4.9	5.7	5.7	-	-	-	-	B
SLC5A9	001	1.1	26.54	28.83	1.7	16.05	27.85	3.3	10.77	35.84	0	0	0	0	0.1	36.2	4.7	36.2	-	-	-	001	B
SMTN	001	1.1	7.57	8.60	1.0	8.44	8.40	2.2	8.02	17.93	13.3	12.7	10.6	7.6	14.3	18.8	16.5	18.8	-	-	-	-	B
SMYD2	010	1.5	11.22	16.32	2.7	9.23	25.75	1.3	24.39	33.22	42.3	30.8	27.6	34.3	26.1	46.1	71.3	71.3	-	-	-	-	B
SOC3	110	2.6	101.92	261.44	2.3	53.15	124.67	0.8	113.73	93.42	9.4	2.6	5.0	5.3	12.9	53.1	4.3	53.1	-	-	-	-	B
TAF4B	010	1.6	1.06	1.70	2.9	0.68	2.05	0.7	2.10	1.55	1.9	1.4	2.0	1.1	0.9	3.0	5.2	5.2	-	-	-	-	B
TIMM10	010	2.1	8.03	17.13	3.0	8.04	24.61	0.9	25.42	24.19	52.2	37.6	35.3	53.8	39.9	21.7	25.2	53.8	-	-	-	-	B
TIMM9	010	1.8	8.34	14.57	2.4	7.03	17.59	0.9	13.80	12.77	19.7	17.5	14.9	23.4	18.6	19.4	32.2	32.2	-	-	-	-	B
TLE3	110	2.2	180.90	405.13	2.2	133.30	294.99	1.3	177.34	234.08	50.4	87.1	119.4	87.4	106.4	174.9	103.1	174.9	-	-	-	-	B
TM6SF1	110	3.7	12.89	47.47	4.3	12.13	52.77	1.6	58.34	96.60	25.1	21.8	20.1	18.3	11.2	62.0	12.0	62.0	-	-	-	-	B
TMED5	010	1.9	7.38	13.64	2.1	6.80	14.63	0.8	24.08	19.85	9.1	9.3	8.0	11.1	9.3	24.6	55.1	55.1	-	-	-	-	B
TNFRSF21	010	2.1	0.96	1.98	3.2	0.97	3.19	1.1	4.33	4.82	29.6	28.5	24.9	16.2	12.2	7.5	7.9	24.9	-	-	-	-	B
TNFRSF8	011	2.2	15.24	32.75	2.7	11.09	30.07	3.4	7.06	24.40	0	0	0.1	0	0.3	9.4	30.9	30.9	-	-	-	-	B
TRP53I13	110	5.2	29.07	151.46	3.0	26.79	82.74	1.5	85.32	133.01	25.3	14.5	25.7	23.7	31.7	71.2	15.9	71.2	-	-	-	-	B
TSPAN9	100	2.4	2.17	5.27	2.0	3.65	7.35	1.1	24.94	28.21	2.9	2.2	31.2	51.5	37.5	26.1	7.9	51.5	-	-	-	-	B
TSR1	010	2.1	6.86	14.24	2.9	5.19	15.50	1.1	8.94	10.40	9.6	11.6	10.9	11.1	9.2	15.3	25.5	25.5	-	-	-	-	B
UACA	011	1.4	4.59	6.21	2.2	5.38	11.90	10.2	6.29	64.88	3.0	9.0	7.0	2.4	8.1	36.7	15.5	36.7	-	-	-	-	B

Supplemental Table 7.1 Continued: Directly Notch-activated genes identified in DN3a cells (135)

Gene	Code	2 h stimulation			8 h stimulation			<i>Notch1^{fl/fl} Rag2^{-/-}</i>			<i>In vivo</i> expression (RPKM)								LMPP		<i>Pax5</i> ^{-/-} pro-B		B _{DN3,B}
		fc	GFP	DL1	fc	GFP	DL1	fc	Cre ⁺	Cre ⁻	LMPP	ALP	DN1	DN2a	DN2b	DN3a	DN3b	Max	↑	↓	↑	↓	
UCK2	001	1.2	148.71	184.95	1.2	117.77	139.55	2.2	399.09	873.26	78.2	84.9	104.9	138.9	295.2	534.3	282.4	534.3	–	–	–	–	B
WDR25	010	2.7	1.76	4.70	4.7	1.15	5.49	1.5	5.99	8.88	3.0	3.2	3.6	3.0	3.1	5.8	5.4	5.8	–	–	–	–	B
WDR43	010	1.7	15.07	26.16	2.1	12.59	26.79	1.0	22.83	22.04	22.8	27.3	24.6	25.5	23.0	36.1	62.6	62.6	–	–	–	–	B
WDR77	010	1.6	8.46	13.54	2.3	6.89	16.35	1.1	17.04	18.74	21.6	15.5	22.1	23.1	18.5	25.5	39.5	39.5	–	–	–	–	B
ZCCHC2	111	2.1	14.78	31.62	2.2	13.50	30.42	3.3	13.94	47.16	10.6	8.4	14.4	8.8	11.6	34.6	15.5	34.6	–	–	–	–	B

Supplemental Table 7.1: List of 135 Notch-activated target genes that were identified by RNA-seq of *in vitro* Notch-stimulated (2 h and/or 8 h) *Vav-Bcl2* transgenic DN3a cells and/or by *Lck*-Cre-mediated deletion of *Notch1* in *Rag2*-deficient DN3a cells. Only the genes with a Rbpj-Bio binding site within 50 kb of the gene-body were included (determined by Bio-ChIP-seq in *Rag2*^{-/-} DN3a cells). Genes which were also identified as Notch-activated by RNA-seq upon *Notch1* deletion in *Rag2*-sufficient DN3a cells are underlined. The 'Code' column shows in which experiment (from left to right in the table) a gene was found to be activated – '0' = no; '1' = yes. The differential expression between non-stimulated (GFP) and Notch-stimulated (DL1), or non-deleted (Cre⁻) and Notch-deleted (Cre⁺) is depicted as moderated fold-change (fc). The absolute expression of each gene in the given cell type is shown as RPKM-normalised expression value. This includes the stimulation and deletion experiments as well as the expression profiling throughout early lymphocyte development (LMPP, ALP, DN1/ETP, DN2a, DN2b, DN3a and DN3b stages). The maximum (Max) intrathymic (DN1 – DN3b) expression level is also shown. If a gene was activated (↑) or repressed (↓) upon Notch-stimulation of LMPPs and/or *Pax5*^{-/-} pro-B cells is shown together with the 'Experiment Code' of the according cell type (see table of according cell type). The 'B_{DN3,B}' column indicates whether the gene was bound (B) by ICN1-Bio and/or Rbpj-Bio (determined by Bio-ChIP-seq in *Pax5*^{Δ/Δ} pro-B cells and/or DN3a cells).

Supplemental Table 7.2: Unbound Notch-activated genes identified in DN3a cells (163)

Gene	Code	2 h stimulation			8 h stimulation			<i>Notch1^{fl/fl} Rag2^{-/-}</i>			<i>In vivo</i> expression (RPKM)									LMPP		<i>Pax5^{-/-}</i> pro-B		B _{DN3,B}
		fc	GFP	DL1	fc	GFP	DL1	fc	Cre ⁺	Cre ⁻	LMPP	ALP	DN1	DN2a	DN2b	DN3a	DN3b	Max	↑	↓	↑	↓		
1200009I06RIK	001	19.2	0.09	1.70	6.6	0.37	2.44	56.8	0.01	0.66	0.2	0.2	0	0	0	0.2	0.1	0.2	-	-	-	-	-	
1810029B16RIK	010	1.3	3.49	4.67	3.2	1.94	6.38	1.1	6.86	7.68	8.2	10.0	7.3	10.6	7.6	9.9	17.4	17.4	-	-	-	-	B	
2300002M23RIK	001	1.6	0.23	0.37	5.3	0.04	0.21	16.2	0.43	7.00	0	0	0	0	0	0.8	0.3	0.8	-	-	-	-	-	
2310042D19RIK	010	2.2	2.47	5.48	2.6	2.95	7.84	2.2	2.31	5.17	0.4	0.5	1.9	1.7	1.6	5.5	1.3	5.5	110	-	-	-	-	
9030409G11RIK	011	1.6	1.16	1.87	2.6	1.00	2.66	2.9	1.22	3.65	0.8	0.2	1.2	1.0	0.6	2.5	1.1	2.5	-	-	-	-	-	
ABCB1B	010	2.0	0.11	0.21	9.6	0.05	0.54	1.0	0.13	0.13	5.1	4.4	3.0	1.9	1.3	0.6	1.7	3.0	-	-	-	-	-	
ABHD14A	010	1.5	2.29	3.33	2.6	4.45	12.05	0.9	11.24	10.13	55.3	43.2	48.7	42.4	21.2	12.2	19.7	48.7	-	-	-	-	-	
AC013548.2	001	1.5	0.31	0.47	1.6	0.22	0.35	14.0	0.12	1.68	0	0	0	0	0	0.6	0	0.6	-	-	-	-	-	
ADAM19	001	2.5	0.31	0.77	2.4	0.39	0.97	7.5	0.37	2.81	0	0.3	0.1	0	0	1.1	0.4	1.1	-	-	-	-	-	
AEN	010	1.7	9.78	16.20	2.4	9.89	24.65	0.8	9.71	8.23	19.2	19.1	17.7	17.8	14.7	12.5	23.5	23.5	-	-	-	-	-	
AGRN	010	2.2	4.18	9.16	3.9	4.98	19.59	1.6	4.72	7.60	3.8	3.4	5.9	5.6	3.8	7.6	3.1	7.6	011	-	-	-	-	
AIMP2	010	2.0	13.35	27.20	2.7	10.66	29.48	1.2	31.94	39.27	63.3	55.2	54.9	76.5	53.7	37.4	88.6	88.6	-	-	-	-	-	
AKAP2	100	2.7	6.10	16.40	1.8	7.21	13.29	1.1	10.72	11.87	0	0.2	1.1	1.8	7.1	11.9	6.6	11.9	-	-	-	-	-	
ALCAM	001	1.1	1.46	1.65	1.8	0.90	1.66	3.3	1.03	3.46	5.7	3.5	4.0	2.3	1.9	2.5	0.5	4.0	-	-	-	-	-	
ANKS1B	001	2.3	0.02	0.04	2.1	0.02	0.05	7.6	0.12	0.91	0	0	0	0	0	0.1	0	0.1	-	-	-	-	-	
APEX1	010	1.8	10.19	18.46	2.5	8.49	21.70	1.0	39.52	42.06	36.1	38.2	46.2	45.5	39.1	29.6	53.3	53.3	-	-	-	-	-	
ATAD3A	010	2.1	14.16	28.97	2.9	13.49	39.80	1.0	18.52	19.69	38.3	35.1	36.3	30.5	26.5	36.0	59.4	59.4	-	-	-	-	B	
ATP8A2	001	1.0	0.59	0.58	1.9	0.36	0.70	9.2	0.23	2.09	0.5	0.5	1.2	0.4	0	0.5	0.5	1.2	-	-	-	-	-	
B3GNT6	001	9.6	0.22	2.09	15.6	0.16	2.52	22.8	0.21	4.88	0.1	0.1	0.2	0.1	0.1	1.5	0.2	1.5	-	-	-	-	-	
BC048355	010	1.4	2.17	3.10	3.6	1.11	4.10	1.9	4.49	8.44	37.0	15.2	25.7	39.1	32.9	13.8	57.7	57.7	-	-	-	-	-	
BEGAIN	010	1.9	4.82	9.01	3.9	4.59	18.25	1.5	4.86	7.60	1.5	0.6	1.3	2.0	4.7	11.1	7.9	11.1	-	-	-	-	-	
BMP1	010	1.5	0.53	0.79	2.8	1.08	3.14	1.4	0.02	0.03	6.7	2.7	7.0	6.2	1.8	0.3	0.3	7.0	-	-	-	-	-	
BTBD19	010	1.9	1.72	3.24	2.7	1.23	3.38	0.9	5.11	4.90	4.6	2.5	3.8	3.3	3.6	6.9	9.8	9.8	-	-	-	-	-	
BYSL	010	1.8	4.48	7.92	2.4	4.29	10.73	1.0	6.72	6.65	12.3	16.7	13.0	14.6	13.5	11.3	31.4	31.4	-	-	-	-	-	
CAD	010	1.5	6.62	9.70	3.6	7.48	27.94	1.2	20.28	23.80	45.5	62.9	59.6	44.4	41.9	41.2	148.2	148.2	-	-	-	-	-	
CAMKK1	110	2.7	9.68	25.66	2.9	5.98	17.46	0.7	5.66	4.19	7.1	4.2	1.2	1.2	2.0	7.9	7.9	7.9	010	-	-	-	-	
CAR12	010	1.7	0.90	1.50	7.2	0.58	4.26	1.9	0.61	1.18	1.5	0.7	1.5	1.8	1.2	4.5	5.0	5.0	-	-	-	-	-	
CBS	010	1.7	3.12	5.17	3.3	1.98	6.63	1.8	0.18	0.33	0.1	0.1	0.2	0.2	0.1	0.8	0.5	0.8	-	-	-	-	-	
CCDC116	010	2.5	0.18	0.45	4.3	0.19	0.84	0.8	0.19	0.15	0.8	0.5	0.7	0.7	0.4	1.0	1.7	1.7	-	-	-	-	B	
CCDC86	010	1.9	6.26	11.73	2.4	5.64	14.04	1.1	9.13	9.93	16.1	14.5	17.3	14.7	12.0	13.6	28.3	28.3	-	-	-	-	-	
CD6	010	1.8	1.53	2.77	2.6	1.93	5.19	1.3	5.34	7.21	2.2	18.1	17.4	11.5	9.8	10.9	24.0	24.0	010	-	-	-	-	
CDC42EP4	001	1.5	29.94	44.68	1.8	17.54	32.35	3.1	11.76	37.18	8.0	6.3	2.2	1.5	2.1	16.1	18.7	18.7	-	-	-	-	-	
CDH11	010	1.5	0.55	0.81	5.0	0.64	3.31	1.9	0.04	0.08	0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	-	-	-	-	-	
CLDN5	001	1.4	0.07	0.10	1.3	0.08	0.10	22.7	0.07	1.64	1.3	3.4	0	0	0	0.1	0	0.1	-	-	-	-	-	
COL3A1	010	0.9	1.09	0.95	5.7	1.14	6.68	2.7	0.01	0.02	0	0	0	0	0	0	0	0	-	-	-	-	-	
COL8A1	010	2.0	0.34	0.67	3.6	0.63	2.32	-	0.00	0.01	0	0	0	0	0	0	0	0	-	-	-	-	-	
CR752656.1	010	1.6	18.44	28.85	2.7	9.53	25.86	0.9	18.58	17.82	30.9	16.4	17.9	19.3	18.7	28.6	37.8	37.8	-	-	-	-	-	
CTPS	010	2.1	3.74	7.86	3.1	3.44	10.97	1.0	6.14	6.10	12.5	14.0	13.1	17.6	14.5	14.8	40.6	40.6	-	-	-	-	-	
CYP27B1	010	4.8	0.15	0.72	5.9	0.20	1.18	0.4	0.49	0.21	0.2	0.1	0.2	0.2	0.3	0.4	0.2	0.4	-	-	-	-	-	
D030028A08RIK	010	1.4	8.71	12.18	3.3	6.34	21.58	1.0	6.91	6.75	7.0	7.2	6.9	5.0	4.3	15.5	8.7	15.5	-	-	-	-	-	
D630045J12RIK	001	0.9	0.24	0.22	1.4	0.41	0.58	2.6	1.24	3.30	3.6	2.9	3.4	2.7	3.0	3.6	2.6	3.6	-	-	-	-	-	
DCTD	010	1.4	0.50	0.68	5.7	0.56	3.26	1.1	2.32	2.51	30.0	22.3	21.3	19.2	9.8	7.1	14.9	21.3	-	-	-	-	-	
DDX21	010	1.8	27.19	48.39	2.5	24.01	62.56	1.1	32.67	37.51	44.5	61.0	55.0	41.6	39.9	60.3	123.8	123.8	-	-	-	-	-	
DKC1	010	1.7	12.22	20.33	2.5	8.17	21.05	1.1	16.94	18.67	25.2	29.0	23.7	30.9	25.1	39.3	67.4	67.4	-	-	-	-	-	
DPH1	010	1.6	4.70	7.43	2.6	4.07	10.82	0.8	14.69	12.39	12.9	13.4	16.4	14.3	12.9	10.1	12.8	16.4	-	-	-	-	B	

Supplemental Table 7.2 Continued: Unbound Notch-activated genes identified in DN3a cells (163)

Gene	Code	2 h stimulation			8 h stimulation			<i>Notch1^{fl/m} Rag2^{-/-}</i>			<i>In vivo</i> expression (RPKM)								LMPP		<i>Pax5^{-/-}</i> pro-B		B _{DN3B}
		fc	GFP	DL1	fc	GFP	DL1	fc	Cre ⁺	Cre ⁻	LMPP	ALP	DN1	DN2a	DN2b	DN3a	DN3b	Max	↑	↓	↑	↓	
DSP	110	12.6	0.02	0.30	51.6	0.01	0.39	7.7	0.02	0.16	0	0.1	0.1	0.1	0.1	0.1	0	0.1	-	-	-	-	-
E130012A19RIK	010	1.5	3.07	4.62	2.3	3.64	8.77	1.0	2.61	2.68	28.5	13.8	38.8	26.1	15.2	8.9	15.6	38.8	-	-	-	-	-
EPAS1	110	2.6	1.10	2.90	2.5	2.22	5.67	1.7	0.09	0.15	0.1	1.3	0.1	0.1	0	0.1	0	0.1	-	-	-	-	-
EPB4.1L4A	001	1.2	1.86	2.18	1.5	1.46	2.18	4.1	0.84	3.56	0.6	0.1	0.2	0.2	0.1	3.5	0.1	3.5	-	-	-	-	-
EPHX3	011	5.6	1.16	6.41	9.0	0.52	4.69	3.5	2.59	9.11	0	0	0	0	0	3.8	0	3.8	-	-	-	-	-
FABP5	111	5.9	1.00	5.87	5.8	1.17	6.91	3.7	3.75	14.01	9.4	14.1	13.1	34.2	17.1	11.5	36.3	36.3	-	-	-	-	-
FAM131A	001	1.1	2.57	2.91	0.6	2.16	1.42	4.4	2.63	11.85	2.0	0.3	0.6	0.3	0.8	4.1	0.2	4.1	-	-	-	-	-
FBL	010	1.4	4.69	6.43	2.5	3.64	9.25	1.3	11.87	15.71	16.0	35.7	14.4	14.7	13.5	14.8	39.9	39.9	-	-	-	-	-
FBN1	010	1.1	0.92	1.03	2.7	2.21	6.08	2.0	0.34	0.69	0.6	2.0	1.3	0.4	0.3	0.4	0.1	1.3	010	-	-	-	-
FBP1	001	1.1	4.99	5.55	1.1	7.48	8.51	3.1	12.84	40.61	9.0	2.1	3.1	5.2	3.1	9.4	1.6	9.4	-	-	-	-	-
FJX1	010	2.2	28.34	63.30	3.0	11.98	36.77	3.2	0.28	0.89	0.4	0	0.4	0.3	0.2	1.4	1.8	1.8	-	-	-	-	B
FTSJ3	010	1.5	19.89	29.59	2.2	16.26	36.48	1.0	24.56	23.82	29.5	45.1	32.2	29.6	30.4	42.1	67.1	67.1	-	-	-	-	-
GAR1	010	1.6	17.29	27.65	2.3	15.63	36.85	1.3	37.61	48.12	61.2	81.3	62.6	55.4	62.6	56.6	133.6	133.6	-	-	-	-	-
GCSH	010	2.0	2.11	4.20	3.7	1.82	6.92	1.3	7.25	9.35	14.2	7.4	11.4	22.7	13.1	17.1	40.9	40.9	-	-	-	-	-
GJB2	010	2.8	0.67	1.88	5.3	0.33	1.77	1.5	0.06	0.09	0	0.1	0.1	0	0	0.1	0	0.1	110	-	-	-	-
GLT1D1	001	1.3	0.13	0.17	3.9	0.05	0.18	15.5	0.17	2.69	0	0.1	0	0	0	0.5	0.2	0.5	-	-	-	-	-
GPATCH4	010	1.9	11.35	21.10	3.0	9.82	29.84	1.1	15.83	17.07	26.0	37.2	28.0	24.9	25.9	30.7	44.0	44.0	-	-	-	-	-
GRWD1	010	2.0	10.65	21.22	2.9	9.58	28.40	1.0	21.91	21.74	38.1	38.8	39.2	38.8	32.0	26.9	67.8	67.8	-	-	-	-	-
GSTO1	010	1.3	35.82	45.71	2.2	26.78	61.47	0.9	68.89	63.17	134.9	76.6	174.6	176.0	82.6	73.3	64.9	176.0	-	-	-	-	-
HEATR1	010	1.5	7.60	11.12	2.4	6.84	16.49	1.0	10.79	10.90	15.3	20.0	18.6	16.6	15.0	16.8	35.9	35.9	-	-	-	-	B
HK2	110	3.7	0.42	1.53	6.2	0.50	3.22	1.8	0.64	1.19	14.6	13.2	14.4	8.8	4.9	3.7	10.6	14.4	-	-	-	-	-
HTRA3	001	1.4	0.68	0.94	1.9	0.81	1.55	-	0.00	1.09	0.1	0.2	1.0	1.8	0.1	0.6	0	1.8	-	-	-	-	-
IFI204	001	1.2	0.11	0.14	5.9	0.07	0.44	8.3	0.22	1.84	0.2	0.2	0.2	0.1	0	0.6	0.2	0.6	-	-	-	-	B
IFRD2	110	2.1	12.72	27.23	2.8	9.09	26.09	1.0	20.13	20.15	45.3	57.1	38.9	38.2	29.6	38.8	86.0	86.0	-	-	-	-	B
IGF2	001	1.0	3.11	3.00	1.0	3.85	3.91	10.4	1.22	12.83	0	0	0	0	0	3.3	0.1	3.3	-	-	-	-	-
IGSF3	001	1.7	1.02	1.76	1.9	0.82	1.57	3.8	0.36	1.40	0.2	1.3	0.1	0.1	0.2	2.1	5.9	5.9	-	-	-	-	-
IL15RA	100	2.8	4.72	13.36	1.2	3.88	4.74	0.7	3.41	2.44	7.1	2.3	3.0	4.3	2.4	3.7	1.9	4.3	-	-	-	-	-
IPO4	010	1.7	7.16	12.13	3.4	7.62	26.57	1.0	20.35	19.96	32.4	34.7	31.9	19.9	18.7	29.1	44.9	44.9	-	-	-	-	-
IPO5	010	1.4	13.11	18.35	2.1	11.59	24.64	1.1	27.81	31.94	52.1	64.4	54.6	47.8	48.1	48.7	128.8	128.8	-	-	-	-	-
KCNIP2	001	1.4	0.32	0.46	0.9	0.46	0.43	19.6	0.16	3.23	0	0.2	0	0	0.1	0.7	0	0.7	-	-	-	-	-
KIF26B	010	13.0	0.00	0.05	14.8	0.04	0.64	-	0.01	0.00	0	0	0	0	0	0	0	0	-	-	-	-	-
LAMA1	010	3.5	0.01	0.02	14.3	0.01	0.21	-	0.00	0.00	0	0	0	0	0	0	0	0	-	-	-	-	-
LAP3	010	1.9	5.13	9.77	3.0	4.95	15.44	1.2	17.08	20.10	36.8	26.7	31.9	32.8	23.8	23.8	44.0	44.0	-	-	-	-	-
LY6D	100	2.7	233.85	634.93	1.7	431.14	758.82	0.9	2514.54	2389.59	4.1	110.1	119.0	882.1	1151.0	991.1	297.9	1151.0	010	-	-	-	B
LYAR	010	1.2	15.57	19.14	2.2	11.35	25.14	1.2	18.36	22.65	39.6	35.5	46.0	41.7	43.0	45.0	167.5	167.5	-	-	-	-	B
LYZ2	001	0.6	1.42	0.82	0.8	1.53	1.19	3.3	9.54	32.01	111.2	51.8	34.9	21.9	19.0	14.8	1.0	34.9	-	-	-	-	-
MAGED1	001	1.0	8.80	8.70	1.0	14.44	15.09	2.7	8.14	22.46	30.8	9.9	18.0	6.9	13.0	13.3	8.2	18.0	010	-	-	-	-
METTL1	010	2.4	4.45	10.52	5.0	3.70	18.72	0.9	15.34	14.60	28.0	23.7	23.3	29.9	25.0	20.8	41.8	41.8	-	-	-	-	-
MFSD2A	010	5.3	0.06	0.32	23.2	0.03	0.77	4.1	0.06	0.26	0.2	0.5	1.1	2.0	1.2	1.7	4.8	4.8	-	-	-	-	-
MIF	010	1.6	45.30	74.19	2.8	55.41	160.18	1.0	159.70	156.07	318.1	118.5	312.9	432.0	336.3	160.2	421.6	432.0	-	-	-	-	-
MLLT4	011	2.2	6.22	13.62	6.4	3.70	23.68	5.2	3.03	16.08	3.5	0.6	0.8	0.2	0.1	11.1	5.3	11.1	010	-	-	-	-
MPZL2	111	2.3	4.50	10.23	4.7	2.51	12.11	4.4	30.37	135.46	0	0	0.6	0.7	10.0	64.4	14.2	64.4	-	-	-	-	-
MTHFD1	010	1.7	7.88	13.56	3.4	6.39	22.46	1.1	16.66	19.12	37.9	61.2	40.8	39.5	34.7	36.2	105.7	105.7	-	-	-	-	-
MTHFD2	010	1.5	1.89	2.79	3.7	1.48	5.65	2.3	3.26	7.48	11.4	18.4	10.5	19.6	24.9	15.4	65.5	65.5	-	-	-	-	-
MTSSL1L	001	1.1	4.84	5.52	1.0	6.65	7.15	2.8	1.75	5.00	3.3	0.7	2.2	3.3	2.7	4.2	1.6	4.2	-	-	-	-	-

Supplemental Table 7.2 Continued: Unbound Notch-activated genes identified in DN3a cells (163)

Gene	Code	2 h stimulation			8 h stimulation			Notch1 ^{fl/m} Rag2 ^{-/-}			In vivo expression (RPKM)									LMPP		Pax5 ^{-/-} pro-B		B _{DN3,B}
		fc	GFP	DL1	fc	GFP	DL1	fc	Cre ⁺	Cre ⁻	LMPP	ALP	DN1	DN2a	DN2b	DN3a	DN3b	Max	↑	↓	↑	↓		
MYBBP1A	010	1.6	46.57	75.07	2.5	40.96	105.18	1.1	73.65	83.47	111.5	147.9	136.3	102.1	94.9	124.2	239.5	239.5	-	-	-	-	-	
NAT10	010	1.7	6.36	10.71	3.6	5.52	20.06	1.0	10.38	10.77	16.7	19.7	19.1	16.1	16.0	21.4	39.0	39.0	-	-	-	-	-	
NDUFA4	010	1.7	2.18	3.65	2.7	1.31	3.61	0.9	2.59	2.50	3.9	3.7	3.1	5.6	4.0	5.4	10.3	10.3	-	-	-	-	-	
NEFH	010	1.5	2.38	3.54	2.6	2.04	5.38	1.0	0.97	0.97	10.2	4.4	5.3	6.0	5.9	8.3	21.5	21.5	-	-	-	-	-	
NFKB2	111	3.6	27.79	98.62	3.7	25.72	96.52	2.6	29.14	76.42	17.2	11.4	19.7	9.4	10.1	41.0	12.3	41.0	-	-	-	-	-	
NGFR	001	77.8	0.02	1.17	42.1	0.04	1.49	17.0	0.79	13.68	0	0.1	0	0	0.1	5.1	0.1	5.1	-	-	-	-	-	
NHLRC1	010	2.3	0.43	0.99	4.6	0.28	1.32	1.3	1.31	1.67	2.9	1.6	3.1	2.1	1.4	1.6	1.5	3.1	-	-	-	-	B	
NKD1	100	5.7	0.16	0.88	2.7	0.21	0.57	6.6	0.04	0.30	0.2	0.3	1.1	0.4	0.4	0.8	0.2	1.1	-	-	-	-	-	
NLE1	110	2.1	16.87	35.25	2.2	11.98	27.39	0.9	18.19	16.93	20.9	21.0	19.7	22.6	19.9	27.2	41.6	41.6	-	-	-	-	-	
NM001081117	001	1.5	0.86	1.33	1.4	0.72	1.05	3.3	0.48	1.62	27.8	44.8	20.7	16.6	44.5	23.8	144.2	144.2	-	-	-	-	B	
NME2	010	1.5	17.71	26.24	2.8	14.17	41.34	1.1	47.12	53.83	113.9	79.2	91.1	193.5	131.7	54.1	144.6	193.5	-	-	-	-	-	
NOL6	010	1.5	21.68	32.23	2.1	16.50	35.79	1.0	37.28	38.55	38.4	53.1	46.5	31.9	30.0	36.3	51.6	51.6	-	-	-	-	-	
NOLC1	110	2.1	26.50	55.67	2.6	25.01	66.91	1.3	26.44	35.06	57.1	76.0	73.8	60.2	62.2	76.4	180.3	180.3	-	-	-	-	-	
NOP2	010	2.0	22.27	45.20	2.5	21.28	54.36	1.1	25.82	27.73	44.3	48.3	47.4	39.3	36.8	49.1	95.5	95.5	-	-	-	-	-	
NOP58	010	1.8	15.39	27.53	2.3	10.05	23.60	1.1	34.67	38.25	39.4	35.0	37.0	51.2	42.4	69.5	149.0	149.0	-	-	-	-	-	
NR001592	001	0.9	10.39	8.87	0.9	9.04	8.89	3.7	514.12	1926.60	0	0	0.2	2.8	13.2	700.5	24.5	700.5	-	-	-	-	B	
NSUN2	010	1.5	23.48	34.83	2.5	19.76	50.02	1.0	42.77	44.59	44.3	51.6	48.4	52.6	44.5	58.2	114.5	114.5	-	-	-	-	-	
ODC1	010	1.5	3.56	5.16	2.8	2.13	6.03	1.0	7.13	7.46	22.3	16.4	19.5	25.6	15.0	10.7	37.0	37.0	-	-	-	-	-	
OGDHL	001	1.0	4.79	4.98	1.1	3.58	3.96	4.1	8.78	36.85	0	0	0	0	0.5	8.6	2.7	8.6	-	-	-	-	-	
OMP	001	4.7	0.62	2.89	7.4	0.60	4.42	6.5	0.67	4.41	0.3	0.6	0.8	0.7	0.5	2.6	0.5	2.6	-	-	001	-	-	
OSBP2	001	1.0	0.69	0.70	1.0	0.36	0.36	7.9	0.15	1.17	0.1	0	0	0	0.1	0.2	0	0.2	-	-	-	-	-	
P2RX1	010	3.6	5.02	18.22	7.1	2.36	16.75	1.3	4.27	5.56	7.8	4.0	4.5	8.4	0.2	7.3	3.9	8.4	-	-	-	-	-	
PAQR5	010	1.6	0.38	0.61	7.4	0.15	1.14	2.0	5.61	11.19	11.7	7.3	1.7	0.9	2.1	6.1	1.0	6.1	-	011	-	-	B	
PCSK4	110	3.1	0.78	2.43	4.0	0.88	3.64	0.9	1.36	1.25	4.9	2.8	3.4	2.2	2.5	4.1	6.8	6.8	-	-	-	-	B	
PDGFA	001	2.6	1.48	3.75	5.7	0.92	5.23	56.5	0.62	35.57	1.9	1.5	0.6	0.1	0.2	8.0	0.4	8.0	-	-	-	-	-	
PHB	010	1.8	6.87	12.20	2.2	6.51	15.00	1.2	14.17	16.89	21.8	16.3	20.2	26.1	19.0	16.1	23.7	26.1	-	-	-	-	-	
PITX3	111	9.8	0.53	5.14	16.5	0.38	6.33	4.5	1.60	7.35	0	0	0	0	0	1.5	0	1.5	-	-	-	-	-	
PLEKHA7	101	2.2	27.88	60.01	1.5	43.05	68.34	2.8	34.26	98.36	2.7	24.8	77.3	69.2	89.9	95.5	17.6	95.5	111	-	011	-	B	
POLR1B	110	2.6	5.23	13.72	4.0	4.81	19.94	1.1	12.05	14.02	24.6	23.7	26.5	22.3	17.1	22.7	47.5	47.5	-	-	-	-	-	
POLR3D	010	1.8	6.48	11.52	2.4	4.96	12.36	1.0	6.83	7.16	10.6	9.3	11.5	8.6	8.5	14.2	19.9	19.9	-	-	-	-	-	
POLR3E	010	1.5	5.67	8.58	2.3	5.07	12.02	1.1	7.78	8.33	12.7	17.2	13.4	12.2	11.9	12.5	19.0	19.0	-	-	-	-	-	
PPA1	010	1.3	3.41	4.49	3.5	3.33	12.00	1.3	16.36	20.88	49.7	36.2	48.6	61.2	47.9	28.8	111.9	111.9	-	-	-	-	-	
PPAN	110	2.2	37.43	81.90	2.3	30.68	72.60	1.0	36.28	37.20	67.0	68.5	65.3	57.9	49.3	77.8	110.1	110.1	-	-	-	-	-	
PSAT1	010	1.2	2.82	3.49	2.6	2.89	7.80	1.3	13.60	17.79	65.9	45.4	48.9	97.5	71.8	38.8	149.2	149.2	-	-	-	-	-	
PTGFRN	001	1.1	0.35	0.40	1.6	0.88	1.43	10.1	0.14	1.47	0.1	0	0.2	0	0.1	0.3	1.2	1.2	110	-	-	-	-	
PUS7	010	1.8	5.03	9.09	3.3	4.48	14.93	1.3	5.68	7.35	11.9	9.9	12.0	11.7	9.4	17.2	29.3	29.3	-	-	-	-	-	
PUS7L	010	2.0	1.30	2.55	3.2	1.14	3.79	0.8	5.32	4.28	6.1	8.4	7.4	6.6	4.8	6.0	11.0	11.0	-	-	-	-	-	
PWP2	010	1.6	11.37	17.96	2.4	9.94	24.49	0.9	18.95	17.91	22.2	24.7	22.2	22.4	21.4	19.1	34.6	34.6	-	-	-	-	-	
RBM9	001	0.8	0.48	0.40	1.3	1.23	1.72	6.2	0.39	2.47	2.2	0.7	1.1	0.1	0.6	1.1	0.1	1.1	-	-	-	-	-	
RCC1	010	1.6	8.69	13.82	2.3	7.06	16.73	1.4	14.97	20.56	43.5	59.9	42.9	47.8	46.4	36.8	163.0	163.0	-	-	-	-	-	
RCL1	010	1.8	17.49	30.68	2.2	14.59	32.94	0.9	33.84	30.78	48.6	30.9	43.2	54.0	41.8	35.8	56.1	56.1	-	-	-	-	-	
RRP1B	010	1.9	8.11	15.25	2.2	8.38	18.49	1.2	8.44	9.94	19.0	19.8	25.6	20.4	17.9	20.9	32.5	32.5	-	-	-	-	B	
RRP9	010	2.0	21.86	44.53	2.3	16.68	39.09	1.0	29.71	29.67	36.5	29.4	35.1	37.7	32.8	41.5	87.1	87.1	-	-	-	-	-	
RRS1	110	2.1	18.23	38.71	2.5	14.99	38.91	1.1	33.76	36.23	39.2	24.2	43.7	41.3	35.9	43.0	96.9	96.9	-	-	-	-	-	
RSAD1	010	2.2	1.23	2.65	2.8	1.32	3.73	1.0	2.77	2.89	8.9	4.7	6.9	4.1	2.6	3.2	4.0	6.9	-	-	-	-	B	

Supplemental Table 7.2 Continued: Unbound Notch-activated genes identified in DN3a cells (163)

Gene	Code	2 h stimulation			8 h stimulation			<i>Notch1^{fl/m} Rag2^{-/-}</i>			<i>In vivo</i> expression (RPKM)								LMPP		<i>Pax5^{-/-}</i> pro-B		<i>B_{DN3B}</i>	
		fc	GFP	DL1	fc	GFP	DL1	fc	Cre ⁺	Cre ⁻	LMPP	ALP	DN1	DN2a	DN2b	DN3a	DN3b	Max	↑	↓	↑	↓		
RUNDC3B	010	2.6	1.80	4.75	3.5	0.50	1.80	0.8	1.82	1.42	1.2	1.0	3.4	3.0	2.3	1.9	3.9	3.9	010	-	-	-	-	-
SDAD1	010	1.4	9.37	13.46	2.3	7.50	17.98	1.1	11.08	12.70	12.0	17.2	14.3	12.7	12.8	18.5	30.6	30.6	-	-	-	-	-	-
SH2B2	001	1.0	0.71	0.69	2.0	0.51	1.05	2.9	3.87	11.54	16.1	35.7	40.6	17.0	7.9	17.0	7.1	40.6	-	-	-	-	-	-
SHMT1	010	1.7	4.56	7.95	4.0	4.78	19.45	1.2	11.40	13.56	42.8	44.7	44.3	50.1	47.4	32.2	101.5	101.5	-	-	-	-	-	-
SHMT2	010	1.7	23.83	41.20	3.1	21.85	69.73	0.9	58.78	54.62	109.5	117.0	128.3	121.5	117.3	87.0	294.7	294.7	-	-	-	-	-	-
SLC26A7	010	14.2	0.01	0.16	0	0.00	0.75	-	0.00	0.00	0	0	0	0	0	0	0	0	-	-	-	-	-	-
SLC29A1	010	1.3	17.64	22.06	2.1	20.12	43.66	1.0	49.09	50.19	79.3	113.3	78.3	82.2	113.4	64.4	132.6	132.6	-	-	-	-	-	-
SLC35F2	110	3.0	1.31	3.86	4.4	1.07	4.87	0.9	5.24	4.90	6.3	3.2	4.7	4.8	5.8	7.4	13.7	13.7	-	-	-	-	-	-
SLC7A1	110	2.1	9.40	19.71	2.6	7.83	20.95	1.0	12.61	13.12	19.4	34.4	25.1	21.6	16.9	19.6	45.6	45.6	-	-	-	-	-	-
SLC7A5	110	3.2	9.18	29.21	4.3	7.20	31.61	1.2	19.64	23.59	44.6	62.3	54.6	46.5	40.3	46.9	149.8	149.8	-	-	-	-	-	-
SMYD5	010	1.3	5.83	7.69	3.2	4.64	15.03	1.1	14.11	15.96	29.4	31.6	33.3	26.8	24.2	25.0	48.2	48.2	-	-	-	-	-	-
SRM	110	3.1	17.93	54.62	4.5	16.02	73.49	1.0	43.66	45.04	127.4	86.3	132.8	132.0	89.9	91.4	277.0	277.0	-	-	-	-	-	-
SYNGR2	001	1.4	70.28	100.82	1.6	64.28	103.66	2.3	131.29	313.32	143.9	177.2	125.5	96.9	82.2	222.4	86.5	222.4	-	-	-	-	-	-
TCFAP4	100	2.1	33.90	69.52	1.9	29.49	57.04	1.0	42.03	41.62	47.6	53.6	53.9	44.8	43.4	38.8	50.0	53.9	-	-	-	-	-	-
TMEM204	001	0.9	2.55	2.41	0.8	2.98	2.47	15.6	0.90	14.22	0.5	0.9	0.3	0.5	0.3	1.9	0.4	1.9	-	-	-	-	-	-
TRIB2	110	3.0	6.46	19.45	2.7	5.41	14.92	0.4	4.02	1.81	1.6	0.8	2.3	1.6	1.6	4.4	9.4	9.4	-	-	-	-	-	-
TRMT61A	010	1.9	6.47	11.96	3.4	5.30	18.40	0.8	10.20	8.73	21.3	21.7	15.8	18.5	14.2	12.4	23.4	23.4	-	-	-	-	-	-
TUSC5	010	0.2	0.06	0.01	7.3	0.17	1.29	-	0.00	0.00	0	0	0	0	0	0	0	0	-	-	-	-	-	-
UHRF1	110	2.3	52.13	122.18	2.8	52.13	148.85	1.6	90.35	148.76	68.4	122.9	93.0	100.4	160.3	177.2	219.2	219.2	-	-	-	-	-	B
UNG	110	2.4	3.60	8.53	3.8	3.67	14.14	1.8	5.93	10.73	43.3	46.4	52.7	73.5	70.4	32.4	66.9	73.5	-	-	-	-	-	B
UTP20	010	1.1	3.87	4.10	2.4	3.16	7.71	1.0	4.58	4.87	6.1	8.5	8.2	6.6	6.4	10.4	19.8	19.8	-	-	-	-	-	-
VASH1	010	2.4	0.39	0.93	4.3	0.42	1.88	1.5	1.75	2.68	3.5	1.9	8.1	9.6	9.5	4.2	5.0	9.6	001	-	-	-	-	-
VWCE	001	2.4	10.69	25.40	3.3	7.76	26.05	7.3	2.41	17.81	0	0.1	0	0	0.2	16.4	1.6	16.4	-	-	-	-	-	-
WDR46	010	1.7	21.56	37.28	2.4	16.72	41.73	1.0	31.75	32.20	40.7	51.5	47.0	37.3	34.4	42.4	76.7	76.7	-	-	-	-	-	-
WFIKK2	010	1.6	0.41	0.64	4.9	0.22	1.12	0.6	0.84	0.53	0.8	0.2	0.4	0.4	0.4	0.8	1.0	1.0	-	-	-	-	-	-
WISP1	010	1.2	0.51	0.63	2.6	0.58	1.54	0.8	0.05	0.04	0	0	0	0	0	0	0	0	-	-	-	-	-	-
YDJC	110	2.7	2.36	6.32	4.8	2.18	10.60	0.9	12.69	11.89	33.7	11.6	24.7	19.0	11.6	15.3	32.2	32.2	-	-	-	-	-	B
ZNHIT6	010	1.6	2.37	3.73	2.5	2.13	5.53	1.2	3.86	4.78	6.9	8.2	6.1	4.7	4.7	6.5	9.8	9.8	-	-	-	-	-	-

Supplemental Table 7.2: List of 163 Notch-activated target genes identified by RNA-seq of *in vitro* Notch-stimulated (2 h and/or 8 h) *Vav-Bcl2* transgenic DN3a cells and/or by *Lck*-Cre-mediated deletion of *Notch1* in *Rag2*-deficient DN3a cells. Only the genes without a Rbpj-Bio binding site within 50 kb of the gene-body were included (determined by Bio-ChIP-seq in *Rag2*^{-/-} DN3a cells). The 'Code' column shows in which experiment (from left to right in the table) a gene was found to be activated – '0' = no; '1' = yes. The differential expression between non-stimulated (GFP) and Notch-stimulated (DL1), or non-deleted (Cre⁻) and Notch-deleted (Cre⁺) is depicted as moderated fold-change (fc). The absolute expression of each gene in the given cell type is shown as RPKM-normalised expression value. This includes the stimulation and deletion experiments as well as the expression profiling throughout early lymphocyte development (LMPP, ALP, DN1/ETP, DN2a, DN2b, DN3a and DN3b stages). The maximum (Max) intrathymic (DN1 – DN3b) expression level is also shown. If a gene was activated (↑) or repressed (↓) upon Notch-stimulation of LMPPs and/or *Pax5*^{-/-} pro-B cells is shown together with the 'Experiment Code' of the according cell type (see table of according cell type). The '*B_{DN3B}*' column indicates whether the gene was bound (B) by ICN1-Bio and/or Rbpj-Bio (determined by Bio-ChIP-seq in *Pax5*^{Δ/Δ} pro-B cells and/or DN3a cells).

Supplemental Table 7.3: Notch-repressed genes identified in DN3a cells (119)

Gene	B _{DN3}	Code	2 h stimulation			8 h stimulation			Notch1 ^{fl/m} Rag2 ^{-/-}			In vivo expression (RPKM)									LMPP		Pax5 ^{-/-} pro-B		B _{DN3,B}
			fc ⁻¹	GFP	DL1	fc ⁻¹	GFP	DL1	fc ⁻¹	Cre ⁺	Cre ⁻	LMPP	ALP	DN1	DN2a	DN2b	DN3a	DN3b	Max	↑	↓	↑	↓		
1190002N15RIK	B	011	1.6	21.62	13.67	2.2	29.92	14.10	3.2	30.45	9.66	5.6	6.8	5.6	8.1	13.9	16.9	22.4	22.4	-	-	-	-	B	
2610019F03RIK	-	010	2.1	5.34	2.55	5.8	5.62	1.01	0	0.03	0.00	0	0	0.1	0	0	0	0.1	-	-	-	-	-		
4632428N05RIK	B	001	1.6	20.17	12.52	1.9	15.25	8.11	2.5	22.88	9.23	16.3	18.2	10.6	3.7	2.9	7.7	2.0	10.6	-	-	-	-	B	
4831426I19RIK	B	010	1.4	11.99	8.38	3.0	14.41	4.96	1.9	4.78	2.54	26.2	28.8	19.0	13.1	19.0	5.2	8.6	19.0	-	-	-	-	B	
5730559C18RIK	-	001	1.5	0.63	0.42	2.7	1.69	0.66	3.0	7.29	2.45	0.4	1.0	4.1	4.8	4.7	6.5	5.3	6.5	-	-	-	-	-	
5830411N06RIK	-	001	0.9	0.38	0.41	1.5	1.27	0.89	8.7	102.38	12.04	0	0.5	1.1	0.5	43.7	24.1	8.0	43.7	-	-	-	-	-	
6430598A04RIK	-	010	1.6	16.02	9.77	2.3	17.77	8.03	1.4	8.05	6.04	0.7	0.4	0.8	1.8	4.6	11.3	4.8	11.3	-	-	-	-	-	
AC140312.1	-	010	1.1	1.46	1.28	3.3	2.46	0.79	1.8	0.12	0.07	4.4	1.3	2.9	2.7	2.1	0.5	0.3	2.9	-	-	-	-	-	
ADAMTSL2	B	001	1.0	22.11	22.53	1.4	14.16	10.31	2.9	8.82	3.06	0.2	0.4	0.5	0.1	0	12.8	0.5	12.8	-	-	-	-	B	
ADRA1B	-	001	2.7	0.30	0.11	1.5	0.90	0.63	17.9	4.81	0.27	0	0	0	0.1	0.2	0.4	0	0.4	-	-	-	-	-	
ALS2CL	-	010	1.2	19.17	15.45	2.0	12.07	5.96	2.6	1.71	0.68	4.9	1.0	5.2	0.9	0.8	1.3	0.5	5.2	-	-	-	-	B	
ARAP2	B	010	1.7	8.66	5.22	2.2	8.66	3.99	1.2	1.59	1.31	1.4	2.9	1.6	2.6	2.3	1.5	0.9	2.6	-	-	-	-	B	
ARHGAP19	-	010	1.2	2.50	2.06	2.4	5.22	2.28	1.9	3.68	2.00	5.7	12.3	3.4	4.8	11.6	4.6	27.4	27.4	-	-	-	-	-	
ATP13A2	B	001	0.8	5.06	6.27	1.3	10.01	7.92	4.6	27.02	6.03	91.6	127.9	141.4	101.8	74.5	26.7	23.0	141.4	-	-	-	-	B	
ATP2A3	B	001	1.0	30.15	30.29	1.8	27.77	16.11	2.4	98.62	42.55	125.1	123.0	91.6	105.5	138.3	76.2	106.4	138.3	-	-	-	-	B	
ATP2B4	B	010	1.2	21.53	18.64	2.1	28.70	14.32	1.4	14.43	10.73	51.5	123.9	41.4	23.7	16.0	13.1	2.0	41.4	-	-	-	-	B	
B230312A22RIK	-	010	1.3	3.11	2.40	2.5	3.72	1.54	1.5	3.51	2.34	3.3	5.5	3.2	2.5	2.4	3.1	0.2	3.2	-	-	-	-	-	
BST2	-	001	1.0	195.76	188.75	1.1	181.15	174.96	2.3	510.46	228.23	143.2	255.9	179.5	231.3	205.1	157.8	93.8	231.3	-	-	-	-	B	
C630004H02RIK	-	001	1.0	78.16	78.38	1.7	72.54	44.32	2.2	86.51	39.15	3.2	1.2	1.0	3.1	24.6	42.8	8.6	42.8	-	-	-	-	-	
CAMK4	-	001	1.0	3.65	3.56	1.7	3.45	2.08	6.8	1.57	0.23	0	0	0.1	0.1	0.3	2.1	12.9	12.9	-	-	-	-	-	
CARD6	-	010	1.7	11.93	7.21	2.1	14.57	7.16	1.6	17.17	10.98	5.9	13.4	9.7	0.9	2.4	6.5	0.3	9.7	-	-	-	-	-	
CCR9	B	011	1.9	170.46	88.64	2.5	130.80	54.24	10.9	114.52	10.71	3.2	2.7	7.8	0.4	4.2	28.9	14.1	28.9	-	-	-	-	B	
CD163L1	B	001	1.1	0.74	0.69	1.6	1.90	1.21	8.2	37.22	4.62	0	0.1	1.4	0.5	16.2	8.4	9.8	16.2	-	-	-	-	B	
CD52	-	010	1.4	48.68	34.25	2.4	40.15	17.40	1.7	48.18	28.10	319.0	318.7	374.4	321.5	95.1	21.0	5.6	374.4	-	-	-	-	-	
CD8B1	-	010	1.6	54.83	34.22	2.4	31.13	13.00	10.0	0.61	0.06	0	0	0.1	0.1	0.1	1.0	13.2	13.2	-	-	-	-	-	
CD96	-	001	1.3	12.82	9.59	1.6	10.76	7.14	2.6	35.36	14.07	3.4	6.9	5.3	15.2	15.8	20.7	8.4	20.7	-	-	-	-	-	
CLEC12A	-	001	1.6	2.80	1.78	1.8	3.65	2.07	5.8	8.23	1.44	7.7	25.5	8.3	21.9	11.2	3.9	3.3	21.9	-	010	-	-	-	
CMPK2	-	001	0.9	0.89	1.01	2.0	0.75	0.38	2.8	5.69	2.03	11.0	7.8	11.9	10.3	5.8	2.1	1.2	11.9	-	-	-	-	B	
CORO7	-	001	1.0	10.07	10.05	1.5	17.76	11.96	2.5	37.83	15.49	114.1	224.4	133.1	52.0	97.1	29.1	37.9	133.1	-	-	-	-	-	
CPA3	-	001	1.9	1.10	0.57	2.2	1.54	0.72	13.2	10.30	0.80	0.2	1.8	11.2	72.5	53.9	6.9	0.7	72.5	-	-	-	-	-	
CST7	-	001	0.5	0.10	0.22	0.5	0.26	0.58	6.2	7.20	1.18	49.7	99.4	100.7	113.3	58.2	3.0	3.7	113.3	-	-	-	-	B	
D10BWG1379E	-	010	1.3	2.59	2.06	2.4	3.42	1.44	1.5	1.56	1.07	0.3	0.3	0.3	0.1	0.1	1.0	0.3	1.0	010	-	-	-	-	
DDHD2	B	010	1.1	5.82	5.47	2.5	5.90	2.44	2.3	2.56	1.11	7.2	5.9	3.5	3.1	3.4	1.9	3.4	3.5	-	-	-	-	B	
DOK2	-	001	1.2	9.27	7.82	1.5	8.76	6.16	3.0	33.76	11.61	64.6	40.5	114.0	90.5	59.3	18.3	20.3	114.0	-	-	-	-	-	
E2F2	B	010	1.5	5.90	3.85	2.7	7.35	2.82	1.2	11.50	9.34	9.9	36.2	8.6	12.2	43.9	30.1	76.7	76.7	-	-	-	-	B	
EFCAB5	-	010	1.2	1.19	0.97	2.8	1.51	0.55	2.1	0.16	0.08	0.1	0.1	0.1	0.2	0.4	0.3	0.3	0.4	-	-	-	-	-	
EMP3	B	001	1.3	63.05	48.01	1.7	53.94	31.98	2.6	76.31	30.17	279.7	228.7	179.2	178.0	126.3	38.4	44.3	179.2	-	-	-	-	B	
EYA2	-	010	1.3	37.91	28.71	2.4	29.13	12.72	2.0	20.53	10.56	21.6	5.0	18.3	9.4	8.1	14.0	2.6	18.3	-	-	-	-	-	
FAAH	-	001	1.0	16.59	16.12	1.2	13.64	12.07	2.5	5.99	2.42	11.5	3.3	2.2	3.0	4.0	6.0	4.4	6.0	-	-	-	-	-	
FAM78A	B	001	1.5	193.66	128.51	1.9	175.17	94.99	2.3	130.43	58.22	26.3	35.8	42.7	39.1	56.0	63.4	46.2	63.4	-	-	-	-	B	
FLRT2	-	100	9.5	0.26	0.03	0.5	0.03	0.07	0	0.00	0.00	0	0	0	0	0	0	0	0	-	-	-	-	-	
FNDC1	-	001	0.9	0.07	0.08	2.4	0.19	0.08	13.1	4.04	0.31	0	0	0.3	1.7	9.1	2.5	0.1	9.1	-	-	-	-	-	
GIMAP8	-	001	1.1	5.34	5.04	1.7	7.97	4.94	3.8	5.34	1.42	9.3	8.6	3.2	2.0	1.6	1.5	0.6	3.2	-	-	-	-	-	
GLCC11	-	010	1.7	26.87	15.56	2.2	30.55	14.35	1.5	6.51	4.52	0.8	0.9	0.4	0.3	1.4	5.1	4.9	5.1	-	-	-	-	-	
GM106	-	010	1.5	7.34	5.01	2.3	11.27	5.07	1.1	2.29	2.15	0.3	0.8	0.1	0.6	1.8	1.1	0.1	1.8	-	001	-	-	-	

Supplemental Table 7.3 Continued: Notch-repressed genes identified in DN3a cells (119)

Gene	BDN3	Code	2 h stimulation			8 h stimulation			<i>Notch1^{fl/fl} Rag2^{-/-}</i>			<i>In vivo</i> expression (RPKM)								LMPP		<i>Pax5^{-/-}</i> pro-B		BDN3B
			fc ⁻¹	GFP	DL1	fc ⁻¹	GFP	DL1	fc ⁻¹	Cre ⁺	Cre ⁻	LMPP	ALP	DN1	DN2a	DN2b	DN3a	DN3b	Max	↑	↓	↑	↓	
GM962	-	001	1.1	16.77	15.44	1.5	19.54	13.20	2.3	13.75	6.12	16.1	13.3	13.1	8.4	8.0	6.7	7.3	13.1	-	-	-	-	B
GPR25	B	001	1.6	44.69	27.41	1.8	64.30	36.89	3.5	215.87	61.81	2.0	17.1	84.9	134.2	126.1	114.3	87.0	134.2	100	-	-	-	B
GPR68	-	001	1.1	0.22	0.20	1.3	0.48	0.38	6.6	1.37	0.21	0.4	0.6	5.1	6.2	9.4	0.8	0.1	9.4	-	-	-	-	-
GPRIN3	B	011	1.8	4.94	2.74	2.9	14.60	5.26	2.4	22.35	9.37	1.8	7.2	8.1	2.6	14.7	7.7	1.2	14.7	-	-	-	-	B
<i>GSN</i>	-	001	1.0	5.53	5.26	0.9	13.11	15.82	4.8	9.59	2.03	21.9	54.5	17.7	13.9	8.2	2.2	2.5	17.7	-	-	001	-	-
<i>GZMA</i>	-	001	1.3	21.66	16.92	1.5	23.76	16.24	3.4	284.20	84.81	0.1	0.1	0.8	10.5	60.6	68.5	257.9	257.9	001	-	011	-	-
H2-OA	B	001	0.9	18.21	19.11	1.5	13.33	8.91	2.6	26.92	10.69	31.5	26.6	21.9	5.8	7.5	6.8	5.2	21.9	-	-	-	-	B
ICOSL	-	011	1.7	1.42	0.81	2.6	3.37	1.34	2.9	6.63	2.28	6.4	7.3	4.3	3.1	7.0	3.1	0.9	7.0	-	-	-	-	-
ID3	-	011	2.0	236.71	117.45	2.2	196.71	91.08	5.6	78.55	14.16	0.6	2.2	5.3	1.8	17.6	21.6	82.9	82.9	-	-	-	001	-
IGFBP4	-	001	1.0	5.11	5.27	1.2	25.21	22.19	6.1	14.78	2.46	372.2	359.4	575.3	283.3	135.8	9.5	9.5	575.3	-	-	-	-	-
IKZF2	B	001	1.3	10.85	8.51	1.9	14.14	7.87	2.3	12.11	5.27	4.9	1.3	5.7	9.3	10.5	6.9	5.0	10.5	-	-	-	-	B
IL10RA	-	001	1.2	40.55	34.60	1.4	41.57	31.23	2.5	33.38	13.80	16.9	29.5	43.5	28.4	23.6	24.2	4.0	43.5	-	-	-	-	-
IL10RB	-	001	1.9	4.32	2.22	2.4	5.18	2.27	4.3	6.42	1.54	46.4	47.6	20.4	11.4	6.4	3.5	3.9	20.4	-	-	-	-	-
IL4RA	-	001	0.8	1.79	2.20	0.9	1.83	2.11	4.1	3.05	0.75	44.6	42.9	45.4	29.7	15.2	2.0	0.6	45.4	-	-	-	-	-
IQGAP2	B	010	1.1	6.65	5.90	2.1	7.46	3.63	1.7	7.69	4.62	37.2	61.7	37.7	23.9	26.4	11.7	16.3	37.7	-	-	-	-	B
<i>IRF7</i>	-	001	0.9	4.37	4.71	1.6	5.98	3.92	2.4	42.60	18.01	33.6	33.0	31.3	11.6	14.4	12.0	2.4	31.3	010	-	-	-	B
ITGAE	B	001	0.5	0.58	1.24	0.6	0.78	1.31	8.0	10.33	1.31	0.1	0.1	0.9	0.1	0.2	3.7	0.3	3.7	-	-	-	-	B
ITGB7	B	001	0.7	0.88	1.19	0.8	1.53	1.96	5.5	3.07	0.57	42.0	49.7	89.5	60.7	26.8	1.8	2.3	89.5	-	-	001	-	B
ITK	B	001	1.5	77.85	53.32	1.7	74.19	45.70	3.1	41.25	13.57	0.2	0.1	1.8	5.0	16.2	30.3	25.5	30.3	-	-	-	-	B
JAKMIP1	B	011	1.3	25.06	19.47	2.3	30.97	14.13	2.3	26.49	11.53	24.5	62.1	37.6	9.7	20.1	19.5	8.2	37.6	-	-	-	-	B
KIT	-	001	1.0	5.44	5.32	1.6	5.94	3.82	2.3	15.87	6.98	133.2	36.2	124.3	110.3	37.1	13.4	5.6	124.3	001	-	001	-	-
LCA5	-	010	1.8	4.76	2.69	2.9	5.65	2.05	1.6	4.63	2.92	1.5	1.4	1.3	1.0	1.6	2.9	0.7	2.9	-	-	-	-	-
LDLRAP1	-	011	1.6	9.93	6.00	2.4	8.11	3.54	4.4	4.52	1.04	43.0	25.7	39.9	12.5	5.3	1.9	3.1	39.9	-	-	-	-	-
LFNG	B	010	1.5	63.00	41.85	2.2	68.03	32.69	1.0	68.54	69.07	45.7	35.1	65.2	50.5	60.9	47.4	9.5	65.2	-	-	-	-	B
LGALS1	-	001	1.4	315.01	226.26	1.7	489.83	299.39	2.6	420.04	160.86	203.3	563.6	299.8	512.2	2009.8	286.5	285.5	2009.8	-	-	-	-	-
LIMD2	-	001	1.2	154.10	124.07	1.5	137.89	92.24	2.1	278.07	132.66	166.8	177.1	127.7	136.1	152.1	122.1	138.0	152.1	-	-	-	-	B
<i>LY6C1</i>	-	001	1.3	8.41	6.30	2.0	15.35	8.16	3.5	76.36	21.97	0.6	0	0.9	3.5	10.8	7.7	1.5	10.8	-	-	-	-	-
MDM1	B	010	1.4	4.23	2.98	2.3	6.10	2.76	0.8	1.62	2.14	4.4	4.9	3.9	5.9	8.2	4.3	13.6	13.6	-	-	-	-	B
MS4A4B	-	001	1.6	2.13	1.31	1.4	3.02	2.29	4.0	16.47	4.15	0.6	1.2	1.2	2.1	6.6	5.6	1.6	6.6	-	-	-	-	-
MS4A6B	-	011	1.6	32.91	20.31	2.4	37.75	16.60	3.0	85.48	29.38	7.2	4.6	4.9	9.7	30.7	28.8	14.0	30.7	-	-	-	-	-
MYO1C	-	011	1.6	26.54	16.26	2.8	24.43	9.16	5.6	8.93	1.63	28.5	41.5	13.4	9.0	12.1	5.4	11.1	13.4	-	-	-	-	-
NEDD9	B	010	1.4	8.16	5.65	2.4	8.85	3.85	2.2	9.56	4.45	5.5	11.3	15.7	9.4	7.9	5.5	5.4	15.7	-	-	-	-	B
NFKB1A	B	001	1.4	88.04	63.13	1.7	94.19	57.89	2.9	58.56	20.83	53.5	62.6	49.1	36.7	31.3	25.3	28.9	49.1	-	-	-	-	B
NOG	-	010	1.4	3.30	2.30	3.4	2.69	0.81	2.6	0.24	0.09	0.1	0.3	0.1	0.2	0.3	0.2	0.3	0.3	-	-	-	-	-
NPHS2	-	011	1.9	5.25	2.72	2.8	4.48	1.64	7.9	3.22	0.42	0	0	0	0	0	0.7	0.1	0.7	-	-	-	-	-
NR003513	B	010	1.6	51.62	32.74	2.1	48.15	23.18	1.7	2.84	1.67	22.2	5.2	11.9	15.2	18.3	7.9	1.2	18.3	-	-	-	-	B
NR3C1	B	010	1.9	104.17	53.87	2.0	122.77	64.26	2.0	115.47	59.44	14.5	18.3	8.8	10.2	24.9	56.5	37.8	56.5	-	-	-	-	B
OLFR524	B	001	0.6	0.22	0.36	1.4	0.27	0.21	4.0	8.37	2.11	0	0	1.1	0.2	9.0	3.5	3.5	9.0	-	-	-	-	B
PADI2	-	011	1.1	30.58	28.90	2.1	46.78	23.45	2.8	32.97	12.04	20.8	50.7	36.3	52.1	39.2	22.3	7.3	52.1	-	-	-	-	-
PAQR7	-	001	1.4	4.10	2.84	1.7	5.43	3.23	3.1	16.04	5.20	42.4	20.2	21.7	8.6	6.6	5.2	3.0	21.7	-	-	-	-	B
PODNL1	-	001	1.0	3.28	3.33	1.5	3.16	2.23	4.0	10.20	2.58	0.8	0.4	7.2	8.0	6.3	10.0	11.8	11.8	-	-	-	-	-
PP11R	-	001	1.2	178.77	153.28	1.5	189.59	134.42	2.5	102.80	41.77	0.6	1.7	4.8	11.6	45.9	78.2	59.4	78.2	-	-	-	-	-
RAB19	-	001	0.8	0.53	0.64	1.1	0.86	0.78	24.3	3.23	0.13	2.2	2.0	2.2	1.4	4.5	0.4	0.8	4.5	010	-	-	-	-
RAB37	-	001	0.9	12.59	14.52	1.7	11.37	6.87	2.2	33.16	15.49	39.8	26.8	31.2	37.2	21.7	11.4	5.2	37.2	-	-	-	-	-
RAP1GAP2	B	001	0.8	6.49	8.04	0.8	6.12	7.37	2.5	9.85	3.93	0.3	0.3	0.5	0.3	0.2	7.4	6.1	7.4	110	-	001	-	B

Supplemental Table 7.3 Continued: Notch-repressed genes identified in DN3a cells (119)

Gene	B _{DN3}	Code	2 h stimulation			8 h stimulation			<i>Notch1</i> ^{fl/fl} <i>Rag2</i> ^{-/-}			<i>In vivo</i> expression (RPKM)								LMPP		<i>Pax5</i> ^{-/-} pro-B		B _{DN3,B}
			fc ⁻¹	GFP	DL1	fc ⁻¹	GFP	DL1	fc ⁻¹	Cre ⁺	Cre ⁻	LMPP	ALP	DN1	DN2a	DN2b	DN3a	DN3b	Max	↑	↓	↑	↓	
RFX2	–	010	1.2	11.73	9.89	2.4	13.06	5.62	2.0	11.19	5.79	10.5	20.8	11.5	7.1	14.1	11.0	10.7	14.1	–	–	–	–	–
RNF39	B	010	1.4	24.79	17.96	2.6	9.07	3.64	4.6	0.65	0.14	0.1	0.1	0.1	0	0.1	0.1	0.1	0.1	–	–	–	–	B
RRAGD	–	010	0.9	3.11	3.52	2.5	2.76	1.14	4.5	0.55	0.12	0	0.2	0	0	0.2	0.4	1.1	1.1	–	–	–	–	–
RSPH9	–	001	1.9	0.63	0.33	1.1	0.65	0.61	7.7	4.48	0.59	4.2	4.7	2.4	1.8	1.5	0.4	0.1	2.4	–	–	–	–	–
SEMA4B	B	011	1.1	18.51	16.71	2.1	22.53	11.26	2.4	22.29	9.24	18.3	28.7	21.5	12.4	15.6	9.9	7.6	21.5	–	–	–	–	B
SEMA7A	B	001	1.2	12.51	10.04	1.8	11.34	6.36	2.7	13.47	5.09	2.4	3.4	1.8	1.8	3.8	6.4	5.6	6.4	–	–	–	–	B
SGK3	B	011	1.1	4.64	4.04	2.2	5.53	2.61	2.2	11.53	5.30	7.5	7.1	5.6	8.5	8.9	8.1	3.6	8.9	–	–	–	–	B
SH2D5	–	001	0.9	9.92	10.66	0.9	9.69	10.84	4.2	10.99	2.63	68.6	94.8	49.4	23.2	27.6	9.6	1.9	49.4	–	–	–	–	B
SIT1	–	001	1.4	48.78	35.55	1.5	19.00	13.24	5.1	46.81	9.38	0	0.2	0.5	0.9	7.3	19.8	26.6	26.6	–	–	–	–	–
SKIL	–	001	1.6	3.89	2.39	1.9	2.55	1.40	3.5	2.82	0.83	2.2	2.4	2.3	1.6	2.1	1.6	2.5	2.5	–	–	–	–	B
SLC14A1	–	001	1.0	5.35	5.45	1.4	7.18	5.42	2.8	12.17	4.45	9.7	29.1	18.5	15.0	9.9	5.4	4.5	18.5	–	–	–	–	–
SLC27A1	–	011	1.2	27.61	23.37	2.3	36.63	16.61	2.8	40.16	14.66	15.2	40.6	17.1	21.6	42.9	29.3	9.7	42.9	–	–	–	–	–
SLC37A3	–	001	0.8	0.61	0.75	1.1	1.35	1.25	13.7	1.29	0.10	0.7	1.1	1.5	0.5	2.5	0.8	2.2	2.5	010	–	–	–	B
SLC6A19	–	010	1.8	10.25	5.74	2.5	5.55	2.30	18.7	1.55	0.08	0	0	0	0	0	0.2	0.1	0.2	–	–	–	–	–
SNAI3	–	001	0.8	15.03	18.09	1.2	15.49	13.59	5.9	27.51	4.75	0.4	1.4	2.0	0.8	5.8	10.9	13.5	13.5	–	–	–	–	–
SORCS2	–	011	0.9	15.91	16.97	2.6	13.03	5.11	2.7	4.25	1.58	0.8	6.6	0.5	0.1	0.1	2.7	0.1	2.7	–	011	–	–	B
SPIB	–	001	0.9	19.18	20.82	1.1	13.06	11.70	2.1	82.83	41.04	0.1	5.7	0.3	1.6	5.7	79.1	6.5	79.1	–	001	–	–	–
SRGAP3	B	001	1.1	0.91	0.85	1.3	1.07	0.90	2.8	2.10	0.77	3.6	4.0	2.9	1.9	1.5	1.5	0.6	2.9	–	001	–	–	B
SSH2	B	010	1.2	20.59	17.25	2.1	25.11	12.47	1.0	16.42	16.49	15.6	30.8	22.3	9.1	17.0	14.8	9.9	22.3	–	–	–	–	B
ST8SIA1	B	001	1.0	10.16	10.50	1.6	9.38	6.05	3.0	12.45	4.17	1.5	5.5	14.1	5.6	9.4	6.5	4.8	14.1	–	100	–	–	B
SYN2	–	010	1.2	2.02	1.73	2.6	2.31	0.92	0	0.00	0.01	0	0	0	0	0	0	0	0	–	–	–	–	–
TBXA2R	B	001	1.1	137.73	130.34	1.7	147.45	88.67	2.3	155.61	69.08	57.5	89.8	107.6	51.4	67.0	71.8	35.2	107.6	–	–	–	–	B
<u>TDRD5</u>	B	011	1.7	51.07	30.15	3.2	55.65	17.96	5.6	10.58	1.92	0	0	0	0.1	0.4	4.7	2.5	4.7	–	–	–	–	B
THBS1	–	010	1.8	69.51	38.63	2.3	115.58	51.37	0.2	0.01	0.07	0.1	0.5	0	0	0	0	0.1	0.1	–	001	–	–	–
TNFAIP8L2	–	001	1.4	6.12	4.44	2.0	6.73	3.51	2.4	31.28	13.35	76.1	94.7	118.9	86.7	45.8	9.7	5.2	118.9	–	–	–	–	–
TNFSF14	–	001	1.3	3.67	2.77	0.9	1.67	1.93	3.8	6.44	1.72	23.8	7.1	19.0	9.9	2.0	3.7	1.6	19.0	–	–	–	–	B
TPCN1	B	001	1.1	31.84	28.70	1.8	37.08	21.60	2.7	37.12	14.00	25.9	48.6	20.3	21.2	31.3	30.5	36.3	36.3	–	–	–	–	B
WFS1	–	010	1.4	8.46	6.00	2.3	10.43	4.62	2.8	3.44	1.26	5.6	7.3	4.8	1.1	2.7	2.1	1.3	4.8	–	–	–	–	B
ZFP467	–	010	1.3	12.33	9.37	2.4	12.22	5.23	0.5	3.56	6.81	22.0	6.2	6.0	0.8	1.1	3.8	0.1	6.0	–	–	–	–	–

Supplemental Table 7.3: List of 119 Notch-repressed genes that were identified by RNA-seq of *in vitro* Notch-stimulated (2 h and/or 8 h) *Vav-Bcl2* transgenic DN3a cells and/or by *Lck*-Cre-mediated deletion of *Notch1* in *Rag2*-deficient DN3a cells. The 'B_{DN3}' column indicates whether the gene was bound (B) by Rbpj-Bio within 50 kb of the gene-body (determined by Bio-ChIP-seq in *Rag2*^{-/-} DN3a cells). Genes which were also identified as Notch-repressed by RNA-seq upon *Notch1* deletion in *Rag2*-sufficient DN3a cells are underlined. The 'Code' column shows in which experiment (from left to right in the table) a gene was found to be repressed – '0' = no; '1' = yes. The differential expression between non-stimulated (GFP) and Notch-stimulated (DL1), or non-deleted (Cre⁻) and Notch-deleted (Cre⁺) is depicted as moderated fold-change⁻¹ (fc⁻¹). The absolute expression of each gene in the given cell type is shown as RPKM-normalised expression value. This includes the stimulation and deletion experiments as well as the expression profiling throughout early lymphocyte development (LMPP, ALP, DN1/ETP, DN2a, DN2b, DN3a and DN3b stages). The maximum (Max) intrathymic (DN1 – DN3b) expression level is also shown. If a gene was activated (↑) or repressed (↓) upon Notch-stimulation of LMPPs and/or *Pax5*^{-/-} pro-B cells is shown together with the 'Experiment Code' of the according cell type (see table of according cell type). The 'B_{DN3,B}' column indicates whether the gene was bound (B) by ICN1-Bio and/or Rbpj-Bio (determined by Bio-ChIP-seq in *Pax5*^{Δ/Δ} pro-B cells and/or DN3a cells).

Supplemental Table 7.4: Directly Notch-activated genes identified in LMPPs (150)

Gene	Code	4 h stimulation			12 h stimulation			4 d stimulation			In vivo expression (RPKM)								Pax5 ⁺ pro-B			DN3		
		fc	GFP	DL1	fc	GFP	DL1	fc	GFP	DL1	LMPP	ALP	DN1	DN2a	DN2b	DN3a	DN3b	Max	↑	↓	↑	↓	B _{DN3}	
201011101RIK	001	1.1	3.73	4.18	1.3	4.83	6.28	2.4	4.63	11.36	4.0	6.1	8.2	9.1	8.1	5.4	3.6	9.1	–	–	–	–	B	
5830405N20RIK	001	0.9	17.22	14.68	0.9	23.25	20.74	2.9	4.66	13.55	25.7	23.5	36.3	52.1	43.1	21.4	17.5	52.1	–	–	–	–	B	
ABCB1A	010	2.8	0.46	1.30	6.5	0.29	1.88	6.2	0.18	1.12	0.2	0.1	0.2	0	0	0	0	0.2	–	–	–	–	B	
ABI3	111	2.9	10.11	28.96	5.3	3.84	20.43	3.2	4.32	13.64	11.2	12.7	23.2	18.7	29.7	83.3	25.0	83.3	001	–	–	–	B	
ABR	010	1.3	22.61	29.61	2.0	23.20	47.05	1.6	34.57	54.92	30.8	46.3	42.1	33.8	44.9	54.0	25.4	54.0	–	–	–	–	B	
ACTN1	010	1.1	35.10	38.69	2.8	13.67	37.94	2.2	8.95	19.58	21.3	3.1	34.4	30.4	54.2	164.2	91.2	164.2	–	–	–	–	B	
ADORA3	010	2.5	0.16	0.39	7.4	0.15	1.07	2.1	2.29	4.82	0.1	0.5	0.3	0.3	0.4	0.3	0.1	0.4	–	–	–	–	–	
AGPAT3	111	2.4	41.89	99.45	4.0	11.82	47.59	2.8	3.12	8.83	18.1	17.2	21.2	9.9	18.7	35.3	38.2	38.2	–	–	–	–	B	
AHR	100	2.7	1.80	4.80	1.9	3.01	5.66	2.2	3.50	7.76	1.2	1.3	3.2	5.0	9.4	24.5	5.7	24.5	–	–	–	–	B	
ALDH3B1	010	1.4	0.73	1.05	5.5	0.48	2.64	1.1	5.80	6.51	0.6	3.3	2.2	0.5	1.4	5.7	3.1	5.7	–	–	–	–	B	
AMZ1	010	2.2	1.48	3.24	3.4	1.48	4.96	1.8	6.64	11.81	1.7	1.3	4.7	2.7	0.5	0.2	0.4	4.7	–	–	–	–	–	
ANGPT1	001	1.0	27.19	27.29	0.8	37.83	30.37	4.9	1.89	9.17	26.7	7.4	42.7	42.0	13.7	6.7	1.9	42.7	–	–	–	–	B	
ANKRD6	011	1.2	1.42	1.62	2.6	1.38	3.55	6.0	0.27	1.62	3.5	2.5	7.5	1.0	0.6	1.0	0.7	7.5	–	–	–	–	B	
ARHGEF3	100	2.5	34.92	86.33	1.5	31.65	46.76	1.2	8.74	10.12	35.1	30.8	30.1	10.5	10.5	10.2	12.3	30.1	–	–	–	–	B	
ARL4C	010	1.8	6.21	10.84	3.7	5.97	21.75	1.2	17.71	21.42	15.2	9.2	44.0	25.8	31.7	40.9	6.5	44.0	–	–	–	–	B	
ASB2	110	8.0	1.21	9.67	107.4	0.03	2.87	2.4	0.38	0.93	0.4	0.1	0.6	0.1	0	0	0	0.6	011	–	–	–	B	
AW011738	110	8.4	2.19	18.34	9.5	2.15	20.25	9.1	0.51	4.68	3.2	1.8	13.6	10.1	10.7	25.0	5.9	25.0	011	–	–	–	B	
BCL11A	110	2.1	86.78	180.84	2.8	54.35	152.83	1.0	98.38	95.52	81.3	96.6	98.4	34.0	9.6	5.8	1.8	98.4	–	–	–	–	B	
BCL11B	001	0.2	0.01	0.00	1.0	0.02	0.02	19.0	0.03	0.50	0.2	0	1.2	44.2	142.2	199.4	107.6	199.4	–	–	–	–	B	
BCL2L14	010	2.5	0.88	2.21	4.5	1.10	4.89	3.9	2.69	10.43	2.3	2.5	9.4	11.3	11.2	17.8	3.8	17.8	001	–	–	–	–	
BCL3	010	2.1	20.34	41.16	2.5	7.43	18.45	1.8	22.26	40.25	21.9	7.6	18.8	6.3	3.2	0.2	0	18.8	–	–	–	–	B	
BLK	110	4.4	4.17	18.40	2.4	9.77	23.58	0.9	23.29	20.87	0.2	11.9	12.1	6.5	15.9	5.7	4.6	15.9	–	–	–	–	B	
BST1	010	4.5	0.04	0.16	58.7	0.08	4.52	1.8	1.60	2.92	0.1	0.9	1.5	4.5	6.6	51.8	5.2	51.8	011	–	–	–	B	
CCDC102A	010	1.4	5.67	7.95	2.9	5.28	15.24	1.6	12.48	20.23	5.6	6.1	10.3	6.7	2.7	0.3	0.3	10.3	–	–	–	–	B	
CD22	010	7.4	0.13	0.92	49.6	0.20	9.75	1.7	13.44	22.93	0.1	1.9	0.1	0	0	0	0	0.1	011	–	–	–	–	
CD276	100	2.8	10.34	28.34	1.8	1.87	3.37	0.9	0.91	0.82	3.2	1.0	4.8	3.6	0.4	0.1	0.2	4.8	–	–	–	–	–	
CD53	010	1.4	14.23	20.25	2.8	6.42	17.82	1.2	13.35	15.79	14.7	19.7	19.1	12.8	16.7	8.7	1.0	19.1	–	–	–	–	B	
CD7	010	1.3	5.64	7.08	18.7	5.35	99.90	1.5	248.71	376.30	4.3	18.1	185.2	53.7	11.6	1.7	0.2	185.2	001	–	–	–	B	
CD82	010	1.1	68.27	76.99	3.2	14.25	45.82	1.6	28.20	46.08	64.5	85.3	126.0	37.0	64.8	155.9	48.4	155.9	–	–	–	–	B	
CD97	100	3.5	19.49	68.41	1.1	50.84	56.90	1.0	34.38	32.86	3.0	5.4	3.5	1.4	2.5	3.2	7.9	7.9	001	–	–	–	B	
CDH23	010	1.2	0.16	0.19	6.1	0.46	2.81	1.9	0.94	1.76	0.1	0	0	0.1	0.3	11.8	0.8	11.8	–	–	–	–	B	
CHN2	110	3.7	5.60	20.80	3.9	3.35	12.93	2.1	8.63	18.59	4.7	4.2	9.4	9.9	2.0	0.2	0.7	9.9	011	–	–	–	–	
CHRM3	010	28.8	0.03	0.77	59.8	0.02	0.93	0.6	0.20	0.12	0	0	0.2	0.1	0	0.1	0	0.2	–	–	–	–	B	
CHST2	110	5.3	0.60	3.18	5.4	0.49	2.61	1.8	0.65	1.21	0.5	0.7	4.2	1.7	0.7	0.5	1.2	4.2	–	–	–	–	–	
CMKLR1	101	8.9	0.22	1.95	5.8	0.10	0.55	6.7	0.65	4.36	0.2	1.2	2.3	1.4	1.4	1.2	0.2	2.3	–	–	–	–	B	
COL15A1	110	7.3	0.57	4.14	16.6	0.26	4.35	3.4	0.41	1.42	0	0	0.1	0.2	0.2	3.8	2.2	3.8	011	–	–	–	B	
CSF1R	110	2.3	75.55	170.95	2.2	71.95	159.62	1.2	255.73	295.12	9.6	52.0	28.7	1.2	0.2	0.1	0	28.7	011	–	–	–	–	
D8ERTD82E	100	3.0	4.58	13.58	2.1	2.84	6.07	0.9	7.02	6.62	3.1	8.2	12.8	2.5	3.2	26.0	11.3	26.0	001	–	–	–	B	
DDIT4	110	3.5	34.12	119.66	2.9	17.58	51.09	1.5	30.31	46.50	28.6	51.6	33.9	18.7	10.4	44.4	11.4	44.4	001	–	–	–	–	
DEGS2	010	1.4	0.26	0.35	5.1	0.25	1.26	1.3	0.74	0.97	0.6	1.6	2.8	1.5	2.7	7.7	2.8	7.7	–	–	–	–	B	
DNASE1L3	010	64.7	0.01	0.51	61.6	0.05	3.06	34.0	0.07	2.34	0.1	1.2	0.4	0	0	0	0	0.4	–	–	–	–	B	
DTX1	111	34.5	1.62	55.49	117.8	1.18	139.19	12.4	1.31	16.29	0.2	1.5	9.6	7.0	2.5	184.1	41.8	184.1	111	–	–	–	B	
DUSP1	100	3.4	7.15	23.95	2.0	4.32	8.69	2.3	2.06	4.74	17.7	6.1	5.2	3.9	5.0	12.9	6.5	12.9	–	–	–	–	–	
DUSP5	110	2.8	86.65	244.11	2.6	59.70	152.51	1.7	56.81	97.31	36.3	30.9	87.6	75.1	60.9	24.5	15.8	87.6	–	–	–	–	B	
DUSP6	001	1.6	41.84	66.22	2.0	19.14	39.07	2.3	7.43	16.89	30.7	35.0	36.1	10.1	6.3	1.9	2.0	36.1	001	–	–	–	B	

Supplemental Table 7.4 Continued: Directly Notch-activated genes identified in LMPPs (150)

Gene	Code	4 h stimulation			12 h stimulation			4 d stimulation			In vivo expression (RPKM)								Pax5 ⁺ pro-B		DN3		B _{DN3}
		fc	GFP	DL1	fc	GFP	DL1	fc	GFP	DL1	LMPP	ALP	DN1	DN2a	DN2b	DN3a	DN3b	Max	↑	↓	↑	↓	
ENDOD1	100	2.9	40.59	116.48	2.0	16.32	32.76	1.0	12.04	11.95	10.6	20.4	15.3	8.3	3.7	0.6	0.9	15.3	–	–	–	–	–
ESAM	110	4.0	3.41	13.73	3.8	4.73	17.74	10.5	0.15	1.54	7.8	3.0	1.7	0.9	0.2	0	0	1.7	001	–	–	–	–
FAM26F	110	3.7	7.14	26.25	2.8	6.41	17.98	1.6	6.01	9.45	17.1	7.8	18.1	13.9	6.1	3.5	2.3	18.1	001	–	–	–	B
FASL	111	13.1	0.75	9.79	67.5	0.09	5.94	192.4	0.01	2.86	0.1	0.1	14.9	8.2	1.5	0.3	0	14.9	111	–	–	–	B
FFAR2	010	2.2	4.77	10.40	6.9	3.34	23.08	3.8	8.81	33.58	12.9	6.8	40.6	15.7	1.4	0	0	40.6	–	–	–	–	–
FILIP1L	010	2.4	1.16	2.71	3.7	1.76	6.53	1.3	8.13	10.91	1.0	3.3	2.2	2.7	3.8	4.5	0.7	4.5	–	–	–	–	B
FYN	010	1.2	34.42	40.72	2.2	13.60	30.16	0.6	53.04	33.42	20.2	28.8	38.2	25.0	24.5	25.2	21.5	38.2	–	–	–	–	B
GATA3	011	1.3	2.38	3.07	2.4	3.43	8.34	10.5	0.82	8.61	4.0	3.0	31.4	46.6	85.5	64.7	78.6	85.5	011	–	–	–	–
GLIS3	010	1.6	1.27	2.03	5.9	0.84	4.95	2.0	1.32	2.65	0.9	2.2	7.4	0.3	0.8	0.5	0	7.4	–	–	–	–	B
GNGT2	010	1.4	24.19	34.23	2.8	9.05	25.27	2.1	16.61	34.53	16.7	15.5	8.8	17.7	33.0	44.0	20.4	44.0	–	–	–	–	B
GPR132	010	1.9	34.51	65.34	2.2	32.26	72.31	1.4	16.89	23.65	33.9	23.5	66.3	30.2	45.0	72.1	30.6	72.1	100	–	–	–	B
GPR18	010	2.6	4.61	11.89	3.1	3.24	9.96	1.9	6.54	12.23	4.0	12.4	12.1	8.0	9.0	3.9	0.4	12.1	110	–	–	–	B
GPR25	100	9.0	8.25	74.28	1.2	0.38	0.47	1.1	15.57	16.40	2.0	17.1	84.9	134.2	126.1	114.3	87.0	134.2	–	–	–	–	B
GRAP2	010	0.6	2.23	1.27	3.3	1.59	5.18	1.6	12.51	19.89	1.6	14.5	25.4	28.7	60.5	97.3	52.0	97.3	–	–	–	–	B
HES1	111	17.8	4.98	88.19	73.7	0.79	58.42	31.7	1.08	34.33	4.5	1.8	94.3	80.8	78.7	159.8	27.7	159.8	–	–	–	–	B
HEY1	001	7.6	0.17	1.29	19.1	0.03	0.62	9.2	0.21	1.93	0.1	0	0.1	0.1	0	0.1	0	0.1	–	–	–	–	B
HEYL	110	99.3	0.03	3.07	64.8	0.01	0.33	25.6	0.01	0.16	0	0	0.1	0.3	0.3	2.9	0.9	2.9	–	–	–	–	B
HPSE	110	3.3	3.37	11.02	3.2	5.99	19.17	1.0	30.48	29.76	5.2	7.5	6.2	4.2	1.0	0.6	0.2	6.2	011	–	–	–	B
I830077J02RIK	111	8.0	2.91	23.05	8.5	4.12	34.94	2.3	23.79	55.21	2.0	14.9	12.8	16.1	5.4	4.5	2.7	16.1	011	–	–	–	B
IFITM3	010	1.5	375.43	547.85	2.6	177.09	459.22	1.3	150.06	200.48	593.2	191.8	232.2	90.1	34.0	8.6	2.6	232.2	–	–	–	–	–
IGF2BP3	001	1.1	1.03	1.12	1.0	1.04	1.00	3.0	1.14	3.43	1.0	5.6	7.8	4.3	4.2	10.3	18.0	18.0	–	–	–	–	B
IL12RB1	010	1.9	4.53	8.68	2.2	6.11	13.56	2.6	2.34	6.18	10.0	5.8	12.2	12.4	8.6	5.7	6.5	12.4	001	–	–	–	B
IRF7	010	2.5	14.29	35.29	4.3	6.73	29.15	0.8	11.36	8.83	33.6	33.0	31.3	11.6	14.4	12.0	2.4	31.3	–	–	–	–	–
ISG15	010	2.9	25.44	71.89	4.0	6.06	24.23	1.1	4.21	4.66	42.7	38.2	50.0	47.6	44.0	32.7	9.3	50.0	001	–	–	–	B
ITGA9	011	2.3	1.94	4.39	3.6	2.63	9.36	9.6	0.28	2.71	4.7	2.3	18.1	6.2	2.2	2.4	1.3	18.1	001	–	–	–	B
ITGAL	010	1.5	114.99	168.33	2.4	70.37	168.13	1.5	82.93	121.67	44.7	101.9	114.8	81.7	94.3	81.6	51.6	114.8	–	–	–	–	B
ITGB3	010	1.8	39.97	70.31	2.6	14.28	36.74	1.5	38.99	59.00	1.6	3.3	4.2	4.8	10.9	4.3	0.6	10.9	–	–	–	–	–
KBTBD11	010	1.3	25.02	33.45	2.0	15.37	31.18	1.0	12.69	12.57	16.0	13.9	25.5	24.4	60.0	52.7	22.6	60.0	–	–	–	–	B
KLF4	110	3.6	13.00	45.76	5.4	5.95	31.83	0.7	26.90	18.75	0.9	2.5	1.3	0.4	0.2	0.7	0.1	1.3	111	–	–	–	–
KLHL18	010	1.6	10.57	16.75	2.3	12.64	29.43	1.7	18.35	31.53	11.5	13.4	16.5	13.0	8.3	6.1	4.4	16.5	–	–	–	–	–
KLRB1C	100	24.5	0.32	7.74	1.4	0.76	1.05	0.6	0.21	0.13	0.1	0.3	0.1	0	0	0.1	0.1	0.1	–	–	–	–	B
KMO	010	1.9	0.60	1.14	2.3	4.65	10.86	1.0	34.28	33.17	1.5	13.6	2.0	1.2	0.5	0.7	1.0	2.0	–	–	–	–	B
LCK	011	1.1	22.08	24.47	2.6	14.50	38.14	2.4	19.39	47.16	37.9	71.6	172.4	385.9	533.8	855.3	574.1	855.3	–	–	–	–	B
LCP2	001	1.7	24.99	42.45	1.9	21.78	41.13	2.2	15.56	34.12	18.6	9.1	40.8	44.2	43.0	50.2	33.5	50.2	001	–	–	–	B
LGMN	010	1.3	6.11	7.71	3.9	2.74	10.75	1.3	28.13	35.50	5.3	45.5	9.7	1.8	1.8	0.7	1.8	9.7	–	–	–	–	B
LY6D	010	1.2	3.15	3.59	4.1	4.15	16.77	0.9	199.77	170.22	4.1	110.1	119.0	882.1	1151.0	991.1	297.9	1151.0	–	–	100	–	–
MAF	010	1.4	1.07	1.54	11.5	0.33	3.81	4.8	1.35	6.49	1.3	1.0	10.4	0.8	1.1	3.4	0.8	10.4	011	–	–	–	B
MARCH1	010	2.2	0.09	0.19	9.7	0.11	1.10	1.3	4.73	6.30	0.1	0.2	0.1	0.1	0	0	0.1	0.1	001	–	–	–	B
MARCKS	110	3.4	9.92	33.04	4.2	6.97	29.01	1.5	22.63	34.63	13.5	11.5	41.7	70.8	136.3	94.7	67.0	136.3	–	–	–	–	B
MDFI	011	2.1	28.75	60.40	2.1	22.18	47.39	4.8	8.09	38.61	31.0	8.6	99.7	62.2	32.4	26.1	8.0	99.7	001	–	–	–	–
MYCN	110	2.5	23.26	58.68	2.5	29.92	73.37	1.6	8.72	13.49	39.6	7.8	103.3	83.3	17.9	16.3	0.7	103.3	–	110	–	–	B
MYL4	010	2.3	3.03	7.00	6.5	2.64	17.13	2.2	14.55	31.71	3.8	26.2	28.5	28.2	29.7	8.7	2.4	29.7	–	–	–	–	B
NDST1	111	3.4	24.01	80.72	3.3	18.96	62.51	2.5	13.73	34.05	25.6	33.8	45.5	35.4	24.3	51.4	34.6	51.4	011	–	–	–	–
NEURL3	001	1.1	63.07	71.04	1.1	41.31	45.93	2.2	12.51	27.10	42.3	7.7	34.4	19.8	1.9	0.2	0.1	34.4	–	–	–	–	B
NM010889	010	2.1	0.05	0.10	6.9	0.32	2.19	2.6	0.09	0.22	0	0	0	0	0.1	0.3	0	0.3	–	–	–	–	B

Supplemental Table 7.4 Continued: Directly Notch-activated genes identified in LMPPs (150)

Gene	Code	4 h stimulation			12 h stimulation			4 d stimulation			In vivo expression (RPKM)								Pax5 ⁺ pro-B		DN3		B _{DN3}
		fc	GFP	DL1	fc	GFP	DL1	fc	GFP	DL1	LMPP	ALP	DN1	DN2a	DN2b	DN3a	DN3b	Max	↑	↓	↑	↓	
NOTCH1	110	4.4	48.35	210.03	3.1	49.21	151.81	1.4	77.74	106.72	68.6	236.3	228.5	163.7	162.1	526.1	230.2	526.1	–	–	–	–	B
NOTCH3	011	4.3	0.59	2.55	4.9	0.60	2.95	13.2	0.35	4.70	0.5	0.3	2.7	12.6	29.8	283.3	33.7	283.3	111	–	–	–	B
NR4A1	110	3.9	16.35	63.16	2.4	15.43	36.66	2.0	12.43	24.94	35.5	3.0	12.1	2.4	1.4	10.6	12.0	12.1	–	–	–	–	B
NRARP	111	33.5	2.92	97.21	14.7	4.13	60.43	6.8	2.56	17.35	4.6	2.1	33.5	20.5	12.2	7.1	9.5	33.5	–	–	–	–	B
NRGN	001	0.6	336.69	212.06	0.8	165.52	138.30	2.2	63.24	138.60	176.0	97.9	343.4	275.3	72.6	3.6	7.0	343.4	–	–	–	–	–
NSG2	010	3.4	1.11	3.71	8.3	1.09	8.99	3.0	0.75	2.21	3.0	7.5	11.5	10.5	13.7	65.0	32.0	65.0	–	–	–	–	B
OSGIN1	110	7.3	7.12	51.29	5.7	4.68	26.67	2.3	0.44	1.02	8.1	0.2	0.2	0.1	0	0.7	0.1	0.7	111	–	–	–	B
PDGFRB	111	2.1	15.99	33.91	2.3	21.47	49.23	3.3	13.80	45.08	23.6	37.7	114.0	49.1	34.0	24.8	10.8	114.0	111	–	–	–	B
PLD4	010	0.9	8.62	7.94	3.6	7.86	28.52	0.9	148.01	128.81	11.5	89.0	9.4	1.3	12.4	314.5	14.6	314.5	–	–	–	–	B
PLEKHA7	111	12.2	0.72	8.79	12.2	2.05	24.87	5.5	3.49	19.24	2.7	24.8	77.3	69.2	89.9	95.5	17.6	95.5	011	–	101	–	–
PRSS2	111	5.8	3.07	17.86	55.6	0.41	22.84	100.6	0.18	18.47	0.3	0.5	3.7	3.2	1.5	0.5	0.2	3.7	001	–	–	–	B
PTAFR	100	2.5	11.33	27.85	1.8	6.04	10.99	0.8	10.23	8.71	10.9	4.9	2.3	0.2	0	0	0	2.3	–	–	–	–	–
PTGIS	010	10.1	0.10	0.99	52.6	0.06	3.04	0.6	0.22	0.13	0	0	0	0	0	0	0	0	–	–	–	–	–
PTPRC	010	1.7	24.76	42.44	2.1	31.63	65.80	1.3	42.28	55.57	24.7	34.4	39.3	46.3	48.6	76.4	66.2	76.4	–	–	–	–	B
RAP1GAP2	110	7.9	0.94	7.45	21.7	0.23	5.04	2.1	1.90	3.99	0.3	0.3	0.5	0.3	0.2	7.4	6.1	7.4	001	–	–	–	B
RASAL1	010	1.4	33.71	46.38	3.6	5.03	18.23	0.9	11.47	9.79	3.8	34.7	9.0	2.0	2.3	12.9	10.9	12.9	001	–	–	–	B
RASGRP1	010	1.1	0.09	0.10	3.0	0.70	2.10	2.0	2.40	4.88	0	0.2	1.3	5.6	28.4	26.2	38.2	38.2	–	–	–	–	B
RBM47	010	1.0	3.54	3.51	3.8	1.33	4.99	1.4	3.85	5.31	4.0	4.4	3.3	0.3	0.4	0.2	0	3.3	–	–	–	–	B
RGS3	110	2.7	1.45	3.95	3.3	0.98	3.23	2.7	0.73	1.96	1.7	1.4	4.5	2.1	1.3	0.6	1.9	4.5	001	–	–	–	B
RHBDF2	010	1.8	9.11	16.19	2.1	7.40	15.79	1.4	12.02	17.11	8.1	7.6	15.5	7.9	7.4	11.4	2.8	15.5	–	–	–	–	–
RIN2	010	0.8	2.52	2.04	2.8	0.95	2.65	0.7	5.10	3.44	1.0	1.0	2.0	0.1	0.1	0.3	0.1	2.0	–	–	–	–	B
RNF149	110	2.5	26.11	64.30	2.4	18.58	44.58	1.9	22.54	43.93	19.9	11.9	28.6	20.4	12.6	16.9	17.5	28.6	–	–	–	–	B
RP23-249P4.1	110	11.1	0.41	4.50	8.4	0.28	2.31	2.7	0.67	1.84	0.1	0.1	0.1	0	0	0	0	0.1	–	–	–	–	B
RXRA	011	1.5	5.88	8.78	3.0	3.69	11.02	3.9	2.78	10.87	10.8	8.0	21.1	4.4	2.5	2.7	5.8	21.1	–	–	–	–	B
SASH1	110	3.6	5.02	17.99	4.7	3.88	18.15	2.4	1.34	3.17	4.7	2.3	5.4	1.8	0.8	1.8	0.3	5.4	001	–	–	–	–
SCIN	001	1.4	4.65	6.36	1.1	1.79	2.05	5.6	3.76	21.05	0.7	0.8	78.7	205.7	129.4	17.3	3.2	205.7	–	–	–	–	B
SDCBP	010	1.7	56.73	93.95	2.5	44.57	111.02	1.1	51.05	54.03	54.6	52.9	46.7	57.9	48.9	46.4	38.8	57.9	–	–	–	–	B
SERPINB6B	110	3.3	3.71	12.26	4.4	1.98	8.65	9.8	0.18	1.76	6.2	1.5	19.2	16.2	10.9	9.9	3.6	19.2	001	–	–	–	B
SH2B3	010	1.4	73.68	102.84	2.3	29.47	66.50	1.2	75.16	92.89	49.4	52.8	66.5	26.4	20.0	18.3	17.6	66.5	–	–	–	–	B
SH3GL3	100	2.9	7.82	22.25	2.8	0.70	1.94	1.3	0.04	0.04	0.5	0	0.1	0	0.1	0.5	0.4	0.5	–	–	–	–	B
SIPA1L2	100	2.3	4.90	11.33	2.2	1.82	4.07	1.1	1.94	2.14	1.1	1.4	1.3	0.4	1.1	1.0	1.5	1.5	–	–	–	–	–
SLC12A7	010	1.7	7.04	11.87	2.4	8.29	20.18	1.9	4.79	9.18	14.9	9.4	19.7	14.4	18.9	60.2	15.6	60.2	–	–	–	–	B
SLC37A3	010	1.7	1.10	1.85	6.5	0.56	3.61	1.9	1.44	2.76	0.7	1.1	1.5	0.5	2.5	0.8	2.2	2.5	–	–	–	–	–
SLC5A5	111	3.1	6.75	20.52	7.4	1.12	8.24	24.5	0.33	7.99	0	0	0.3	0	0.1	0	0	0.3	011	–	–	–	–
SLC8A1	010	1.6	0.62	1.00	3.1	0.76	2.36	2.1	1.89	3.92	0.6	0.8	0.7	0.8	0.3	0.2	0.1	0.8	–	–	–	–	–
SLFN2	010	3.0	4.02	11.69	3.8	2.64	9.90	1.2	17.55	21.60	2.8	3.0	4.1	3.0	8.2	21.5	4.9	21.5	–	–	–	–	B
SMAD7	110	4.6	12.72	58.00	4.1	15.34	62.45	1.7	22.48	38.92	15.3	20.4	60.9	22.3	16.7	5.3	1.4	60.9	011	–	–	–	B
SORL1	010	2.0	8.44	16.97	3.6	5.87	21.21	0.8	42.77	36.15	5.9	16.1	7.5	3.6	2.7	2.0	1.5	7.5	–	–	–	–	–
SPSB1	001	2.1	0.59	1.25	4.2	0.17	0.70	6.8	0.54	3.67	0.4	0.5	2.6	0.1	0.7	1.9	0.8	2.6	100	–	–	–	B
STON2	010	2.0	0.60	1.16	2.9	0.92	2.68	2.0	0.87	1.75	0.5	0.3	0.9	4.7	5.8	4.8	1.7	5.8	–	–	–	–	B
TAGAP	010	0.8	1.66	1.34	0.9	4.46	4.08	0.9	2.53	2.40	4.1	2.2	3.1	4.6	4.9	11.3	4.5	11.3	–	–	–	–	–
TAPBP	010	1.6	17.04	27.64	2.1	18.84	40.30	1.3	16.05	21.69	25.0	30.2	20.5	16.5	19.0	29.3	15.9	29.3	–	–	–	–	B
TCF7	001	0.9	1.35	1.19	1.0	1.30	1.29	12.0	2.82	33.69	2.4	22.1	403.5	709.5	980.7	1049.0	1740.6	1740.6	–	–	–	–	B
TGFB1	100	2.1	365.21	746.24	1.6	238.06	381.39	1.0	372.49	368.56	268.9	190.6	318.9	215.6	191.8	93.4	83.8	318.9	–	–	–	–	B
TGFBR1	110	5.3	6.64	35.17	3.6	6.52	23.17	1.9	9.65	18.32	4.5	4.4	6.3	7.7	8.2	23.5	11.2	23.5	011	–	–	–	B

Supplemental Table 7.4 Continued: Directly Notch-activated genes identified in LMPPs (150)

Gene	Code	4 h stimulation			12 h stimulation			4 d stimulation			In vivo expression (RPKM)								Pax5 ^{-/-} pro-B		DN3		B _{DN3}
		fc	GFP	DL1	fc	GFP	DL1	fc	GFP	DL1	LMPP	ALP	DN1	DN2a	DN2b	DN3a	DN3b	Max	↑	↓	↑	↓	
TGM2	111	5.0	227.82	1135.97	10.3	69.28	708.67	6.1	18.72	114.51	2.9	11.0	0.4	0.1	0	0	0	0.4	111	–	–	–	B
THEMIS2	000	2.1	18.61	38.61	2.4	20.49	48.94	1.0	83.78	87.91	18.7	41.6	45.4	22.5	15.4	9.6	1.3	45.4	–	–	–	–	–
TLE1	010	1.3	4.80	6.34	2.3	3.41	7.84	1.1	8.46	9.47	4.2	4.7	3.5	2.2	2.9	2.4	3.0	3.5	–	–	–	–	B
TNK2	010	1.5	6.55	9.67	2.3	3.48	7.94	1.2	7.89	9.53	3.7	16.4	16.3	12.9	13.4	6.4	26.4	26.4	–	–	–	–	–
TNNI2	010	1.6	9.78	15.43	5.0	3.04	15.23	1.0	21.59	20.71	11.2	17.6	10.8	4.5	0.5	0.3	0.1	10.8	001	–	–	–	–
TPD52	010	1.8	18.59	32.64	2.2	15.50	33.74	1.2	50.91	63.07	16.3	19.0	15.9	12.6	15.7	37.5	5.9	37.5	–	–	–	–	B
TPM4	010	1.0	20.42	19.95	3.5	11.17	39.24	1.2	46.32	54.75	13.5	50.8	39.0	19.5	38.8	11.3	26.5	39.0	–	–	–	–	B
TSPAN2	010	2.1	3.75	7.80	2.4	4.96	11.71	1.1	20.72	23.66	7.3	22.5	21.8	33.3	33.4	22.3	7.0	33.4	–	–	–	–	B
TYROBP	010	1.5	116.58	173.80	3.3	122.52	399.60	1.1	1058.68	1135.05	132.3	287.1	390.3	409.9	18.0	1.1	0.4	409.9	–	–	–	–	–
UBE2E2	010	1.5	5.31	8.13	4.3	3.49	14.80	4.3	0.94	4.05	3.9	1.2	4.7	4.9	1.9	2.0	2.4	4.9	011	–	–	–	B
UNC93B1	010	1.4	78.36	107.57	2.3	87.44	202.04	0.8	348.19	286.75	84.3	229.2	130.0	60.5	42.3	58.4	4.4	130.0	–	–	–	–	–
VAMP5	001	1.2	21.91	25.36	1.4	16.70	23.15	3.5	2.38	8.45	51.6	15.2	36.3	49.9	26.3	9.3	5.2	49.9	–	–	–	–	B
ZC3H12D	010	1.8	2.96	5.20	7.4	0.90	6.67	2.8	3.31	9.29	2.2	1.3	4.8	0.7	0.7	1.7	0.3	4.8	011	–	–	–	–
ZFP385A	010	1.5	32.93	47.95	2.9	21.48	61.66	1.7	65.50	112.10	27.9	16.5	119.0	94.4	75.9	67.9	10.9	119.0	–	–	–	–	B
ZFP658	010	1.5	2.22	3.27	3.6	1.92	6.80	0.7	3.55	2.58	2.7	5.2	3.2	1.1	2.2	2.2	1.3	3.2	–	–	–	–	–

Supplemental Table 7.4: List of 150 directly Notch-activated target genes identified by RNA-seq of *in vitro* Notch-stimulated (4 h, 12 h and/or 4 d) LMPPs. Only the genes with a Rbpj-Bio binding site within 50 kb of the gene-body were included (determined by Bio-ChIP-seq in Pax5^{Δ/Δ} pro-B cells and/or DN3a cells.). The 'Code' column shows in which experiment (from left to right in the table) a gene was found to be activated – '0' = no; '1' = yes. The differential expression between non-stimulated (GFP) and Notch-stimulated (DL1) is depicted as moderated fold-change (fc). The absolute expression of each gene in the given cell type is shown as RPKM-normalised expression value. This includes the stimulation experiments as well as the expression profiling throughout early lymphocyte development (LMPP, ALP, DN1/ETP, DN2a, DN2b, DN3a and DN3b stages). The maximum (Max) intrathymic (DN1 – DN3b) expression level is also shown. If a gene was activated (↑) or repressed (↓) upon Notch-stimulation of Pax5^{-/-} pro-B cells and/or Notch stimulation or deletion in DN3a cells is shown together with the 'Experiment Code' of the according cell type (see table of according cell type). The 'B_{DN3}' column indicates whether the gene was bound (B) by ICN1-Bio and/or Rbpj-Bio (determined by Bio-ChIP-seq in DN3a cells).

Supplemental Table 7.5: Unbound Notch-activated genes identified in LMPPs (117)

Gene	Code	4 h stimulation			12 h stimulation			4 d stimulation			In vivo expression (RPKM)								Pax5 ^{-/-} pro-B		DN3		B _{DN3}
		fc	GFP	DL1	fc	GFP	DL1	fc	GFP	DL1	LMPP	ALP	DN1	DN2a	DN2b	DN3a	DN3b	Max	↑	↓	↑	↓	
2310042D19RIK	110	14.0	0.14	1.99	4.8	0.52	2.46	3.1	0.15	0.46	0.4	0.5	1.9	1.7	1.6	5.5	1.3	5.5	–	–	010	–	–
8430408G22RIK	110	8.0	7.67	61.03	6.7	5.99	39.80	1.5	14.80	22.54	9.4	33.1	30.8	2.8	0.2	0	0	30.8	–	–	–	–	–
9030617O03RIK	010	1.4	4.37	6.22	3.8	3.80	14.34	1.4	17.48	25.33	6.6	15.0	16.6	4.7	2.3	1.3	1.9	16.6	–	–	–	–	–
9930111J21RIK1	010	1.1	0.52	0.57	2.8	0.72	2.02	1.2	1.81	2.20	0.9	0.6	0.6	0.2	0.3	0.4	0.1	0.6	–	–	–	–	–
ADAMDEC1	010	0	0.01	0.00	20.3	0.03	0.58	0	0.00	6.35	0	0	0	0	0	0.1	0	0.1	–	–	–	–	–
AFAP1L1	001	0.8	39.78	31.24	1.2	31.68	38.78	2.9	4.62	13.52	62.9	29.7	57.8	18.4	8.3	4.5	2.2	57.8	–	–	–	–	–
AGRN	011	1.4	2.47	3.43	2.5	2.25	5.62	2.8	1.30	3.71	3.8	3.4	5.9	5.6	3.8	7.6	3.1	7.6	–	–	010	–	–
ALDH3A1	100	6.3	1.56	9.74	2.1	0.10	0.22	5.2	0.02	0.09	0	0	0	0	0	0	0	0	–	–	–	–	–
AOX1	010	3.9	0.05	0.20	14.8	0.02	0.35	1.6	0.02	0.03	0	0	0.2	0.1	0.1	0	0	0.2	–	–	–	–	–
AQP9	010	1.1	16.22	17.91	2.4	5.83	13.90	3.5	0.46	1.61	13.3	2.9	6.7	4.1	0.3	0	0	6.7	–	–	–	–	–
BASP1	001	1.0	71.12	70.72	1.4	35.53	50.42	2.2	20.83	45.16	54.1	6.8	14.5	16.6	20.4	22.8	12.2	22.8	–	–	–	–	–
CADM4	100	3.0	4.12	12.19	1.4	0.44	0.61	0.8	1.00	0.75	0.3	0.8	0.4	0.2	0.1	0.1	0.1	0.4	–	–	–	–	–
CAMK2A	001	1.4	16.19	22.79	1.4	14.49	20.58	3.4	1.88	6.35	30.1	11.8	51.8	25.7	35.6	39.2	20.3	51.8	–	–	–	–	–
CAMKK1	010	2.4	3.22	7.44	2.9	1.90	5.44	1.9	0.77	1.46	7.1	4.2	1.2	1.2	2.0	7.9	7.9	7.9	–	–	110	–	–
CAR8	010	1.1	0.25	0.27	17.2	0.02	0.30	0	0.03	0.00	0	0	0	0	0	0	0	0	–	–	–	–	–
CC2D2A	010	2.7	0.20	0.54	12.1	0.26	3.10	8.3	0.10	0.80	0.2	0.1	0.4	0.1	0.1	0.2	0	0.4	–	–	–	–	–
CCL24	010	4.5	0.11	0.50	41.5	0.04	1.58	10.1	0.14	1.40	0	0	0.1	0	0	0	0	0.1	–	–	–	–	–
CD101	110	68.9	0.05	3.55	60.6	0.03	1.70	0.3	0.56	0.17	0.1	0.1	0.3	0.1	0	0	0	0.3	–	–	–	–	–
CD300LF	010	1.9	0.26	0.49	16.5	0.06	1.03	1.1	5.16	5.52	0	0.3	0.1	0.1	0	0	0	0.1	–	–	–	–	–
CD33	010	1.4	62.91	86.59	2.3	51.13	117.71	0.9	41.65	37.24	18.0	26.0	32.0	6.8	0.7	0.1	0	32.0	001	–	–	–	–
CD38	010	3.1	0.10	0.29	10.6	0.16	1.69	6.7	0.33	2.20	0.1	1.3	0.3	0.3	0	0.1	0	0.3	–	–	–	–	–
CD6	010	2.9	1.44	4.17	2.9	1.99	5.82	2.9	3.74	10.96	2.2	18.1	17.4	11.5	9.8	10.9	24.0	24.0	–	–	010	–	–
CDH1	010	4.0	0.04	0.16	14.0	0.10	1.39	2.4	2.38	5.84	0	0.4	2.8	9.9	7.4	31.9	1.6	31.9	–	–	–	–	–
COX4I2	111	2.7	22.98	60.51	9.4	1.78	16.62	11.6	2.05	23.99	2.3	0.6	18.5	5.1	1.0	0.1	0.4	18.5	–	–	–	–	–
CTSS	010	1.4	23.87	33.49	5.0	11.02	54.78	1.2	95.43	111.07	32.8	74.9	27.4	2.5	2.3	0.6	0.5	27.4	–	–	–	–	–
D10BWG1379E	010	1.0	2.39	2.29	4.4	0.42	1.81	1.2	0.94	1.12	0.3	0.3	0.3	0.1	0.1	1.0	0.3	1.0	–	–	–	–	–
D630003M21RIK	010	11.2	0.05	0.57	9.3	0.05	0.48	3.1	0.07	0.21	0	0	0.2	0.1	0	0	0	0.2	–	–	–	–	–
DDN	010	3.0	0.34	0.99	4.0	0.27	1.10	0.4	1.94	0.68	0.1	0.1	0.1	0.1	0.1	0.6	0.7	0.7	111	–	–	–	–
EMILIN2	010	1.6	11.70	18.40	2.9	8.81	25.17	2.1	64.07	134.97	2.2	11.0	32.5	69.7	25.6	0.9	0.1	69.7	–	–	–	–	–
EPB4.1L3	011	1.0	12.81	13.05	3.0	3.13	9.45	3.8	0.63	2.42	9.8	1.8	10.4	0.7	0.1	0	0	10.4	–	–	–	–	–
EPSTI1	010	1.4	14.49	19.92	2.1	13.48	28.88	1.6	8.57	13.54	32.6	24.5	26.6	19.6	23.7	20.2	4.6	26.6	–	–	–	–	–
EVI2A	010	1.1	10.15	11.42	2.7	3.19	8.53	1.1	10.66	11.88	5.4	2.6	7.8	9.6	8.0	3.6	1.2	9.6	–	–	–	–	–
EVL	010	1.4	7.97	10.67	4.2	3.99	16.89	1.2	17.85	20.59	12.0	47.4	51.3	6.0	7.6	3.6	5.9	51.3	–	–	–	–	–
FAM109B	001	2.1	10.00	20.65	1.9	8.94	17.15	2.7	3.17	8.61	8.5	7.0	19.6	23.8	10.8	7.3	4.3	23.8	–	–	–	–	–
FAM167A	100	2.8	3.74	10.44	1.2	5.99	7.29	0.7	6.78	4.70	5.2	13.8	5.6	3.8	11.1	19.9	7.8	19.9	–	–	–	–	–
FAM20A	001	2.1	0.61	1.31	3.3	0.10	0.34	26.0	0.07	1.89	0.1	0.3	1.5	0.4	0.9	0.3	0	1.5	–	–	–	–	–
FBN1	010	2.8	1.40	3.98	3.3	0.30	0.98	2.3	0.23	0.52	0.6	2.0	1.3	0.4	0.3	0.4	0.1	1.3	–	–	010	–	–
FCRL1	010	2.0	0.79	1.59	10.3	0.51	5.22	2.7	1.12	3.06	5.2	9.1	6.5	0.9	0.2	0	0	6.5	001	–	–	–	–
FGF3	001	0.7	1.05	0.69	1.1	0.57	0.62	7.7	0.49	3.82	0.4	0.2	9.2	114.6	6.9	0	0	114.6	–	–	–	–	–
FHDC1	100	3.8	0.89	3.36	2.1	0.90	1.89	1.5	0.06	0.09	0.1	8.2	0	0	0	0	0	0	–	–	–	–	–
FHOD3	010	8.6	0.04	0.32	10.6	0.10	1.10	0.4	0.57	0.21	0.1	0.1	0.7	0	0	0	0	0.7	–	–	–	–	–
FLNB	011	2.0	17.48	34.50	5.3	10.56	56.06	3.7	11.75	43.65	15.2	14.9	19.4	11.1	17.3	7.9	8.1	19.4	–	–	–	–	–
FUCA2	100	2.6	13.86	36.37	1.8	16.33	29.01	1.1	26.14	27.91	15.4	16.3	13.0	17.9	10.6	7.9	6.5	17.9	–	–	–	–	–
GGT5	011	1.8	2.65	4.81	2.4	6.77	15.88	2.8	2.75	7.65	13.5	6.2	18.8	13.0	3.4	1.4	0.2	18.8	001	–	–	–	–
GJB2	110	35.6	0.13	4.66	0	0.00	0.42	0	0.00	0.05	0	0.1	0.1	0	0	0.1	0	0.1	–	–	010	–	–

Supplemental Table 7.5 Continued: Unbound Notch-activated genes identified in LMPPs (117)

Gene	Code	4 h stimulation			12 h stimulation			4 d stimulation			In vivo expression (RPKM)								Pax5 ^{+/−} pro-B		DN3		B _{DN3}	
		fc	GFP	DL1	fc	GFP	DL1	fc	GFP	DL1	LMPP	ALP	DN1	DN2a	DN2b	DN3a	DN3b	Max	↑	↓	↑	↓		
GM2A	110	2.2	106.67	233.68	3.1	121.46	373.77	1.4	349.64	495.04	254.1	250.0	451.8	267.7	209.2	88.5	27.8	451.8	001	–	–	–	–	–
GPR114	011	1.1	0.29	0.31	21.1	0.31	6.61	3.6	4.59	16.48	0.1	0.5	1.9	4.5	1.3	0.1	0.1	4.5	001	–	–	–	–	–
GPR161	010	2.4	2.34	5.48	3.4	3.32	11.28	1.5	0.63	0.97	2.6	0.9	0.9	0.4	0.3	0.3	0.3	0.9	–	–	–	–	–	
GRM6	100	42.1	0.02	0.84	0	0.00	0.02	0	0.00	0.01	0	0	0.2	0.1	0	0	0	0.2	–	–	–	–	–	
GZMA	001	0.3	0.19	0.06	16.6	0.06	0.93	21.3	0.31	6.65	0.1	0.1	0.8	10.5	60.6	68.5	257.9	257.9	011	–	–	–	–	–
GZMB	011	5.3	0.61	3.19	10.7	0.20	2.13	37.6	0.19	7.23	0	0.1	3.1	6.1	0.3	0	0	6.1	–	–	–	–	–	
HBB-B1	001	1.4	24.99	34.23	1.7	31.74	53.32	41.2	6.61	273.50	36.2	1077.6	92.4	673.2	29.4	0.6	2.5	673.2	001	–	–	–	–	–
HBEGF	010	2.5	1.42	3.55	2.9	2.52	7.26	2.4	0.79	1.90	2.6	1.0	1.2	1.5	0.5	0.3	0.2	1.5	111	–	–	–	–	–
HCK	010	1.7	2.20	3.77	7.5	2.59	19.28	1.6	37.26	58.28	4.1	4.4	10.7	3.9	0.6	0.4	0.1	10.7	–	–	–	–	–	
HIC1	111	100.9	0.60	60.39	63.7	0.58	36.84	8.7	0.32	2.75	0.2	0.2	0.2	0.1	0.2	0.2	0.1	0.2	111	–	–	–	–	–
ID2	010	3.1	17.38	52.74	2.7	3.61	9.74	1.5	46.16	69.25	4.6	9.8	13.8	5.8	5.7	12.5	2.7	13.8	011	–	–	–	–	–
IER3	001	1.3	11.93	15.81	1.6	19.06	29.69	2.8	10.47	29.52	31.5	2.6	25.3	14.8	7.2	2.0	0.4	25.3	–	–	–	–	–	
IFITM1	011	1.6	93.42	147.18	3.5	74.90	261.20	2.3	261.77	603.61	339.4	21.1	202.6	189.0	12.9	0.1	0	202.6	–	–	–	–	–	
IFITM2	010	1.6	512.91	831.68	2.9	286.33	835.16	1.6	272.86	451.91	510.5	98.9	473.3	447.4	267.4	107.6	40.3	473.3	–	–	–	–	–	
IL1R1	001	0.9	18.77	15.99	1.0	17.29	18.05	6.2	1.89	11.77	20.5	9.5	16.2	2.8	0.3	0.1	0.2	16.2	–	–	–	–	–	
ITGAX	110	3.0	3.63	10.70	7.4	3.53	26.08	1.6	23.10	37.96	2.0	12.4	5.7	10.9	1.5	0.2	0	10.9	–	–	–	–	–	
ITGB2	010	1.6	36.91	58.86	3.1	12.93	39.75	0.9	75.59	68.82	21.4	34.7	25.6	9.6	10.9	9.8	22.4	25.6	–	–	–	–	–	
KIRREL	010	1.6	0.63	1.00	5.1	0.17	0.85	0.4	0.68	0.25	0.1	0.1	0	0	0.1	0.8	0.1	0.8	–	–	–	–	–	
KIT	001	1.0	88.34	86.93	0.8	114.10	95.44	2.4	18.01	43.50	133.2	36.2	124.3	110.3	37.1	13.4	5.6	124.3	001	–	–	–	–	–
LAIR1	010	2.7	1.55	4.09	5.4	2.17	11.62	1.8	9.39	16.64	4.0	2.2	2.6	6.5	5.1	3.6	1.9	6.5	001	–	–	–	–	–
LILRB3	010	0.9	2.73	2.51	4.0	2.06	8.29	0.8	12.96	9.82	2.2	0.6	0.4	0	0	0.1	0	0.4	–	–	–	–	–	
LRRC25	010	2.1	13.63	27.91	2.9	9.30	26.83	1.4	19.13	26.90	5.8	4.3	9.8	4.9	1.4	0.1	0	9.8	011	–	–	–	–	–
MAFB	010	8.1	0.03	0.27	12.3	0.04	0.52	0.6	1.26	0.79	1.3	2.2	0.1	0.1	0.1	0.8	0.1	0.8	111	–	–	–	–	–
MAGED1	010	1.0	28.38	28.97	2.3	15.10	34.47	1.2	9.61	11.84	30.8	9.9	18.0	6.9	13.0	13.3	8.2	18.0	–	–	001	–	–	
MARCH3	110	7.9	4.75	37.55	6.3	1.87	11.72	1.0	1.79	1.77	3.2	3.0	4.1	1.7	0.2	0.2	0.1	4.1	–	–	–	–	–	
MLLT4	010	1.2	4.61	5.41	2.8	2.96	8.18	3.8	0.88	3.38	3.5	0.6	0.8	0.2	0.1	11.1	5.3	11.1	–	–	011	–	–	
NAGA	010	2.1	31.71	66.14	2.4	28.74	68.90	0.8	83.05	68.39	46.8	32.8	49.0	32.8	17.5	15.6	9.1	49.0	–	–	–	–	–	
NFATC2	010	1.3	17.06	21.87	2.0	8.41	16.90	1.1	11.19	11.91	14.8	6.8	9.2	2.3	4.5	9.3	2.3	9.3	–	–	–	–	–	
NFKBID	010	1.8	6.50	11.77	2.7	8.12	21.74	1.2	9.18	11.41	15.6	3.7	7.9	5.7	7.4	7.4	6.0	7.9	001	–	–	–	–	–
NKG7	010	1.5	55.90	84.89	2.2	68.65	147.17	2.0	57.65	113.22	159.5	71.8	306.6	321.8	332.0	61.6	6.1	332.0	001	–	–	–	–	–
NLRP1B	010	1.6	0.38	0.62	3.6	0.27	0.99	5.4	0.07	0.37	0.6	0.3	0.5	0.3	0.3	0.5	0.1	0.5	–	–	–	–	–	
NLRP3	010	0.9	7.19	6.30	2.6	4.09	10.69	0.8	24.70	18.69	0.1	1.0	0.1	0	0	0	0	0.1	–	–	–	–	–	
NUAK1	010	1.0	1.03	0.99	3.4	0.45	1.52	0.8	0.51	0.39	0.4	0.4	0.4	0.2	0.1	0.1	0	0.4	010	–	–	–	–	–
P2RX7	010	2.9	0.51	1.46	8.4	0.59	4.98	0.6	5.37	3.22	2.4	9.4	1.6	1.1	2.4	0.1	1.1	2.4	–	–	–	–	–	
PGLYRP2	011	1.7	48.91	81.49	3.1	28.87	90.50	4.3	4.62	19.71	88.1	31.0	118.0	71.2	58.6	45.1	20.7	118.0	–	–	–	–	–	
PLAGL1	010	1.5	0.15	0.23	11.8	0.05	0.55	0.2	0.25	0.05	0	0	0	0	0	0	0.1	0.1	–	–	–	–	–	
PROCR	010	1.2	26.13	32.23	3.1	11.57	35.20	3.8	0.27	1.01	30.4	0.4	2.2	0.5	0.1	0	0	2.2	–	–	–	–	–	
PTGFRN	110	8.2	0.72	5.88	24.5	0.11	2.63	2.6	0.14	0.36	0.1	0	0.2	0	0.1	0.3	1.2	1.2	–	–	001	–	–	
PTPRN	010	2.8	2.05	5.76	10.5	0.19	1.99	1.1	0.10	0.12	0.1	0.8	0.3	1.3	3.1	6.0	1.3	6.0	–	–	–	–	–	
PVR	010	1.2	3.18	3.81	2.9	1.26	3.71	0.9	2.02	1.84	3.3	4.1	2.5	0.4	0.6	3.8	3.9	3.9	–	–	–	–	–	
RAB19	010	0.8	1.69	1.39	5.6	0.91	5.08	1.8	1.74	3.22	2.2	2.0	2.2	1.4	4.5	0.4	0.8	4.5	–	–	–	–	–	
ROBO1	010	4.5	0.05	0.21	16.0	0.03	0.41	7.7	0.01	0.09	0	0	1.2	0.2	0.1	0.8	0.2	1.2	–	–	–	–	–	
RTP4	010	2.3	11.17	25.12	2.3	5.82	13.64	0.7	1.09	0.77	34.9	15.0	30.4	19.7	18.9	19.0	4.3	30.4	–	–	–	–	–	
RUNDC3B	010	2.4	1.17	2.85	5.1	0.88	4.44	1.9	1.28	2.41	1.2	1.0	3.4	3.0	2.3	1.9	3.9	3.9	–	–	010	–	–	
SEMA3D	110	2.2	5.10	10.96	11.9	0.49	5.84	11.1	0.04	0.40	3.9	2.4	1.9	0	0	0	0	1.9	–	–	–	–	–	

Supplemental Table 7.5 Continued: Unbound Notch-activated genes identified in LMPPs (117)

Gene	Code	4 h stimulation			12 h stimulation			4 d stimulation			In vivo expression (RPKM)								Pax5 ^{-/-} pro-B		DN3		B _{DN3}
		fc	GFP	DL1	fc	GFP	DL1	fc	GFP	DL1	LMPP	ALP	DN1	DN2a	DN2b	DN3a	DN3b	Max	↑	↓	↑	↓	
SERPINA12	110	6.7	0.43	2.82	59.5	0.04	2.38	70.2	0.02	1.70	0	0	0.6	0.3	0	0	0	0.6	–	–	–	–	–
SERPINF1	001	1.1	40.19	44.93	1.8	27.76	49.38	2.3	26.47	61.34	54.5	20.4	68.0	38.7	4.8	1.2	0.3	68.0	–	–	–	–	–
SETBP1	010	2.5	0.22	0.55	5.7	0.12	0.66	0.4	2.19	0.77	0.1	0	0.8	0.1	0.1	0.2	0	0.8	–	–	–	–	–
SGMS2	100	4.8	0.36	1.71	2.8	0.33	0.95	0.6	1.08	0.67	0	0.4	0.2	0	0	0	0	0.2	–	–	–	–	–
SIGLECE	010	1.9	1.54	2.95	7.3	0.45	3.26	2.3	0.94	2.21	1.9	3.5	11.6	0.9	0.4	0.2	0	11.6	–	–	–	–	–
SLC15A3	010	1.1	2.53	2.77	3.9	0.75	2.96	1.4	4.93	7.02	1.2	4.5	1.8	0.2	0.1	0.2	0.2	1.8	001	–	–	–	–
SLC22A3	001	1.0	14.21	14.67	1.2	27.50	32.83	2.7	2.39	6.54	36.7	7.6	33.4	21.9	26.4	8.3	11.7	33.4	–	–	–	–	–
SLC7A11	010	3.8	0.62	2.35	9.4	0.13	1.23	3.9	0.12	0.48	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.2	–	–	–	–	–
SLC9A2	011	1.9	0.25	0.46	4.1	0.43	1.76	8.9	0.46	4.03	0.3	1.4	2.4	0.9	2.1	0.6	0	2.4	–	–	–	–	–
STC1	110	15.6	0.13	2.03	83.2	0.01	0.51	10.3	0.01	0.08	0.2	0.1	0.4	0	0	1.5	1.6	1.6	–	–	–	–	–
TCIRG1	010	1.7	11.77	19.38	2.2	19.48	42.28	0.9	34.73	32.92	30.5	31.6	43.5	31.0	29.4	39.3	11.5	43.5	–	–	–	–	–
TGFB1	010	1.4	72.50	100.38	2.6	17.84	46.73	2.6	22.78	60.85	14.5	4.2	0.6	0.1	0	0.1	0	0.6	–	–	–	–	–
TGM3	001	0.6	0.44	0.27	4.7	0.01	0.03	48.9	0.23	11.17	0	0.1	1.1	0.1	0.1	0	0.1	1.1	–	–	–	–	–
THSD1	001	0.7	17.50	11.98	1.4	14.29	20.10	2.6	3.79	9.93	31.2	17.5	34.0	19.7	7.6	4.0	0.5	34.0	–	–	–	–	–
TLR12	010	0.8	1.29	1.05	4.1	1.20	4.85	1.6	10.87	17.76	2.8	6.0	29.1	12.3	11.2	10.2	4.6	29.1	–	–	–	–	–
TMEM109	010	1.3	66.29	87.16	2.7	28.40	75.37	1.2	53.83	62.37	43.1	69.4	42.0	26.3	33.8	18.8	64.0	64.0	–	–	–	–	–
TMEM121	011	1.9	8.63	16.56	2.9	6.25	17.86	3.3	3.28	10.76	24.7	34.8	128.4	223.2	238.5	114.1	58.1	238.5	–	–	–	–	–
TMEM132A	011	1.6	2.00	3.22	3.5	1.68	5.89	3.8	2.82	10.78	2.4	2.7	8.7	4.5	2.6	4.0	4.7	8.7	–	–	–	–	–
TMEM151B	001	1.0	0.54	0.55	1.9	0.50	0.95	3.1	1.02	3.14	0.4	0.7	5.9	6.8	14.1	32.4	25.6	32.4	–	–	–	–	–
TMEM176B	001	0.7	301.40	210.17	0.9	172.98	162.11	2.1	50.04	106.77	205.7	146.9	143.0	79.7	56.4	74.2	12.1	143.0	–	–	–	–	–
TRPM2	010	2.9	0.27	0.79	7.9	0.29	2.32	3.3	0.66	2.20	1.1	0.2	0.5	0.5	0	0	0	0.5	–	–	–	–	–
UST	010	1.1	0.72	0.79	4.3	0.57	2.46	1.8	1.51	2.75	1.0	0.7	4.3	0.5	0.3	0.1	0	4.3	–	–	–	–	–
VASH1	001	1.2	1.93	2.35	2.2	2.05	4.48	2.6	2.14	5.70	3.5	1.9	8.1	9.6	9.5	4.2	5.0	9.6	–	–	010	–	–
VIM	010	1.1	359.08	379.81	2.4	128.38	312.86	0.9	708.52	625.02	322.9	317.4	405.4	183.9	267.0	41.6	42.4	405.4	–	–	–	–	–
VIPR1	110	2.9	1.88	5.48	3.0	0.51	1.54	0.8	0.21	0.17	0.8	0.3	0.5	0	0.1	0.1	0.1	0.5	–	–	–	–	–
ZFP366	010	1.7	0.18	0.30	7.0	0.26	1.83	5.8	2.52	14.66	0.2	0.3	0.5	0	0	0	0	0.5	–	–	–	–	–
ZFP831	001	0.7	6.28	4.57	0.9	9.16	7.83	3.0	0.66	1.99	9.6	5.2	11.6	4.5	2.7	1.2	0.5	11.6	–	–	–	–	–

Supplemental Table 7.5: List of 117 Notch-activated target genes identified by RNA-seq of *in vitro* Notch-stimulated (4 h, 12 h and/or 4 d) LMPPs. Only the genes without a Rbpj-Bio binding site within 50 kb of the gene-body were included (determined by Bio-ChIP-seq in Pax5^{Δ/Δ} pro-B cells and/or DN3a cells.). The 'Code' column shows in which experiment (from left to right in the table) a gene was found to be activated – '0' = no; '1' = yes. The differential expression between non-stimulated (GFP) and Notch-stimulated (DL1) is depicted as moderated fold-change (fc). The absolute expression of each gene in the given cell type is shown as RPKM-normalised expression value. This includes the stimulation experiments as well as the expression profiling throughout early lymphocyte development (LMPP, ALP, DN1/ETP, DN2a, DN2b, DN3a and DN3b stages). The maximum (Max) intrathymic (DN1 – DN3b) expression level is also shown. If a gene was activated (↑) or repressed (↓) upon Notch-stimulation of Pax5^{-/-} pro-B cells and/or Notch stimulation or deletion in DN3a cells is shown together with the 'Experiment Code' of the according cell type (see table of according cell type). The 'B_{DN3}' column indicates whether the gene was bound (B) by ICN1-Bio and/or Rbpj-Bio (determined by Bio-ChIP-seq in DN3a cells).

Supplemental Table 7.6: Notch-repressed genes identified in LMPPs (199)

Gene	B _{DN3,B}	Code	4 h stimulation			12 h stimulation			4 d stimulation			In vivo expression (RPKM)								Pax5 ^{-/-} pro-B		DN3		B _{DN3}
			fc ⁻¹	GFP	DL1	fc ⁻¹	GFP	DL1	fc ⁻¹	GFP	DL1	LMPP	ALP	DN1	DN2a	DN2b	DN3a	DN3b	Max	↑	↓	↑	↓	
1190002H23RIK	B	001	1.3	3.40	2.58	2.2	12.21	5.59	3.0	27.86	9.19	12.2	30.6	24.5	79.9	371.2	456.2	213.2	456.2	–	–	–	–	B
2010300C02RIK	B	001	1.0	1.67	1.63	1.2	1.20	0.99	9.7	5.53	0.57	0.2	2.0	0.3	0.4	0.8	2.6	0.7	2.6	–	–	–	–	B
6330403A02RIK	–	011	2.6	0.15	0.06	26.7	0.83	0.03	63.6	6.90	0.11	0	3.4	0	0	0	0	0	0	–	–	–	–	–
A230083H22RIK	–	001	1.7	0.47	0.28	1.4	0.24	0.16	6.3	0.56	0.09	0.1	0.1	0	0	0	0	0	0	–	–	–	–	–
AATK	–	001	0.6	0.54	0.94	0.6	1.30	2.16	2.3	9.71	4.17	0.9	1.2	1.8	0.9	0	0	0	1.8	–	–	–	–	–
ABCB4	–	001	1.6	1.59	0.96	1.4	3.59	2.56	3.5	6.71	1.93	1.7	10.2	1.3	1.0	0.8	1.2	0.2	1.3	–	–	–	–	–
ABP1	–	110	5.0	12.74	2.51	8.6	2.97	0.34	–	0.04	0.00	0	0	0	0	0	0	0	0	–	–	–	–	–
AC159809.2	–	001	1.3	0.76	0.59	2.0	2.02	1.02	3.4	5.62	1.66	2.7	1.9	0.3	0.1	0	0	0	0.3	–	–	–	–	–
AC172746.1	–	010	1.1	0.50	0.44	3.9	2.79	0.72	–	0.07	0.00	1.3	0.1	0	0	0	0	0	0	–	–	–	–	–
ADC	–	011	1.5	5.20	3.42	2.7	10.35	3.77	3.9	12.86	3.33	3.8	6.9	4.5	5.8	3.4	3.0	0.9	5.8	–	–	–	–	–
ADORA2A	–	100	2.3	24.44	10.42	1.6	13.03	8.36	2.3	15.61	6.74	15.9	11.2	4.6	18.4	32.7	19.9	11.8	32.7	–	–	–	–	–
AI661453	–	001	1.4	3.12	2.16	1.3	4.21	3.32	3.6	2.73	0.75	4.8	5.8	5.4	2.2	2.4	1.6	0.3	5.4	–	–	–	–	–
ALA51	–	001	1.2	31.50	26.52	1.8	41.73	23.37	2.2	58.64	26.06	27.6	29.1	23.4	18.4	17.4	18.7	21.8	23.4	–	–	–	–	–
ANXA3	–	001	0.8	2.08	2.50	1.8	6.31	3.44	3.0	11.01	3.64	3.8	3.4	0.8	0.2	0	0	0	0.8	–	–	–	–	–
APBB1	B	001	1.7	2.09	1.20	1.3	2.12	1.58	4.4	2.88	0.65	2.2	0.5	0.5	0.2	0.3	0.4	0.2	0.5	–	–	–	–	–
ATP6V0A1	B	011	2.0	10.14	5.04	2.9	7.79	2.71	2.8	17.66	6.41	5.8	10.5	3.0	2.5	8.8	0.7	0.1	8.8	–	–	–	–	B
AXL	–	001	0.8	27.90	34.94	0.4	0.96	2.19	4.0	2.44	0.61	4.1	1.4	2.0	1.1	2.6	5.1	4.4	5.1	–	–	–	–	–
BCL2	B	010	1.9	9.32	5.00	2.5	18.83	7.54	1.5	41.50	26.95	14.2	42.8	4.8	9.7	32.7	21.1	8.1	32.7	–	–	–	–	B
BHLHA15	B	001	1.7	2.79	1.66	2.0	9.22	4.50	4.0	9.39	2.37	0.8	1.1	0.4	0.4	0.6	0.7	0.2	0.7	–	001	–	–	–
BLNK	–	001	2.7	1.65	0.60	1.3	1.32	1.02	5.8	14.35	2.48	2.5	64.3	0.5	0.2	0.2	0.2	0.2	0.5	–	–	–	–	–
BPHL	–	010	1.0	12.56	12.14	2.2	33.11	14.77	1.4	36.79	26.92	30.9	39.7	20.2	27.4	25.6	23.0	17.5	27.4	–	–	–	–	–
BTNL9	–	010	2.7	5.24	1.92	2.8	4.11	1.48	1.3	3.94	2.95	3.5	3.5	4.1	3.6	1.2	0.2	0	4.1	–	–	–	–	–
CAMK1	–	001	1.3	21.45	16.29	1.3	20.14	15.64	2.8	13.59	4.93	35.9	14.7	16.5	14.0	6.6	2.7	0.5	16.5	–	–	–	–	–
CARD11	–	001	3.9	16.24	4.17	0.8	2.11	2.66	2.5	33.29	13.08	3.1	13.3	5.8	2.7	5.1	5.5	9.4	9.4	–	–	–	–	–
CCL9	–	010	2.0	16.73	8.39	6.7	35.21	5.20	0.9	19.24	22.03	17.5	2.0	2.2	3.1	0.2	0	0	3.1	–	–	–	–	–
CD180	B	010	1.6	7.31	4.44	2.9	12.06	4.10	1.8	23.49	12.98	6.4	11.4	8.6	8.1	1.0	0	0	8.6	–	–	–	–	–
CD28	B	011	3.8	0.29	0.08	47.2	0.26	0.01	5.7	14.15	2.46	0.1	4.1	0.2	0.3	1.2	6.1	15.5	15.5	–	–	–	–	B
CD300C	–	011	1.9	26.05	13.54	3.4	24.52	7.14	2.9	57.16	19.88	0.3	4.0	0.4	0.3	0	0	0	0.4	–	–	–	–	–
CD300LB	–	010	2.0	46.47	22.78	3.4	42.93	12.45	1.7	44.86	26.95	2.8	6.0	1.5	0.8	0	0	0	1.5	–	–	–	–	–
CD69	B	010	1.4	3.79	2.61	3.2	7.81	2.40	3.0	5.95	2.00	7.4	7.2	1.3	1.5	1.3	1.5	1.3	1.5	–	–	–	–	B
CD72	–	001	1.0	9.25	8.80	1.3	15.36	12.20	3.2	22.25	6.86	23.4	45.1	15.2	20.2	5.9	0.9	0.4	20.2	–	–	–	–	–
CEBPA	–	110	3.0	125.86	42.30	3.0	141.14	46.96	1.2	103.07	89.04	54.9	11.3	5.5	0.4	0.2	0.2	0.3	5.5	–	–	–	–	–
CFP	–	001	1.7	60.68	35.45	0.9	21.12	22.48	2.1	125.96	58.69	28.9	19.7	12.3	7.8	3.2	3.4	1.5	12.3	–	–	–	–	–
CGNL1	–	001	1.5	7.50	5.00	1.4	2.12	1.47	4.5	3.28	0.72	0.1	0.8	0.1	0	0	0	0	0.1	–	–	–	–	–
CHGA	–	010	1.9	1.79	0.95	15.4	3.34	0.22	11.3	1.77	0.16	3.0	1.3	1.3	0.2	0.4	0.2	0.2	1.3	–	–	–	–	–
CHL1	–	010	7.2	0.57	0.08	6.9	0.57	0.08	3.1	0.06	0.02	0.4	0.1	0.1	0	0	0	0	0.1	–	–	–	–	–
CHST3	B	011	3.0	0.26	0.09	5.9	0.72	0.12	10.2	6.68	0.65	0	1.4	0.1	0	0	0	0	0.1	–	–	–	–	–
CITED2	B	100	2.2	73.33	33.12	1.9	59.81	31.49	1.6	106.61	67.66	63.3	46.6	48.3	35.5	22.9	15.7	16.4	48.3	–	–	–	–	B
CITED4	B	001	1.7	8.73	5.25	1.5	18.96	12.90	4.1	24.57	6.06	0.1	6.4	0.6	0.3	0.2	0.3	0.1	0.6	–	–	–	–	B
CLEC12A	–	010	1.4	1.89	1.32	6.7	14.29	2.11	1.9	38.84	20.25	7.7	25.5	8.3	22.0	11.2	3.9	3.3	22.0	–	–	–	–	–
CLNK	–	010	1.5	5.26	3.43	2.5	11.25	4.50	1.6	9.80	6.06	7.1	12.9	3.3	3.0	0.2	0	0	3.3	–	–	–	–	–
CMAH	B	001	1.2	0.37	0.30	3.3	0.65	0.19	3.7	5.74	1.54	1.4	12.1	1.6	2.4	4.3	17.8	3.0	17.8	–	–	–	–	B
CMYA5	B	001	3.6	0.08	0.02	22.8	0.10	0.00	9.0	0.50	0.06	0.2	1.0	0.1	0	0.1	0	0.1	0.1	–	–	–	–	B
CNN3	B	001	1.0	23.04	22.13	1.6	33.70	20.92	2.2	82.45	37.82	34.9	108.6	50.2	47.1	65.5	48.3	35.3	65.5	–	–	–	–	B
CPNE5	–	010	1.5	0.86	0.56	3.9	1.38	0.36	1.5	2.47	1.68	0.9	3.5	0.9	2.8	1.9	10.1	2.5	10.1	–	–	–	–	–

Supplemental Table 7.6 Continued: Notch-repressed genes identified in LMPPs (199)

Gene	B _{DN3,B}	Code	4 h stimulation			12 h stimulation			4 d stimulation			In vivo expression (RPKM)								Pax5 ^{-/-} pro-B		DN3		B _{DN3}
			fc ⁻¹	GFP	DL1	fc ⁻¹	GFP	DL1	fc ⁻¹	GFP	DL1	LMPP	ALP	DN1	DN2a	DN2b	DN3a	DN3b	Max	↑	↓	↑	↓	
CTNND2	B	010	1.9	5.85	3.12	4.5	4.26	0.95	2.1	36.63	17.51	1.3	7.9	0.7	0	0	0	0.1	0.7	–	–	–	–	B
CTR9	B	001	1.3	29.69	21.89	1.4	38.45	26.47	2.2	74.41	34.24	34.7	134.3	35.0	21.6	22.6	25.1	24.6	35.0	–	–	–	–	–
CTSG	–	001	1.0	39.65	38.97	2.0	148.07	74.45	2.9	312.30	107.13	57.8	52.1	32.6	17.0	1.2	0	0	32.6	–	–	–	–	–
DACH1	–	010	1.1	1.03	0.90	4.1	10.17	2.50	2.1	1.30	0.63	5.9	0.8	0.6	1.1	0.2	0	0	1.1	–	–	–	–	–
DENND2A	–	001	1.5	9.00	6.08	1.8	20.40	11.06	3.8	6.17	1.64	1.4	0.9	0.4	0.5	0.4	0.2	0.1	0.5	–	–	–	–	–
DHRS3	B	111	5.0	24.89	4.99	3.2	34.22	10.81	2.6	18.18	6.90	51.2	62.2	24.2	22.9	16.7	60.9	6.5	60.9	–	–	–	–	B
DIRAS2	–	010	1.6	0.13	0.08	8.6	1.16	0.13	7.8	0.93	0.12	0.5	1.9	0.7	1.7	1.2	1.4	0.2	1.7	–	–	–	–	–
DIRC2	–	001	1.0	4.08	4.12	1.3	5.26	4.01	2.2	23.14	10.44	6.8	19.4	5.8	0.5	0.8	1.4	0.6	5.8	–	–	–	–	–
DLK1	–	001	1.7	3.18	1.92	5.4	1.38	0.26	24.1	4.32	0.18	1.0	0.9	0.3	0.2	0.2	0.6	0.8	0.8	–	001	–	–	–
DNTT	B	110	2.4	111.58	46.02	8.3	287.99	34.49	2.2	152.96	70.35	303.0	1620.3	517.1	307.4	920.6	939.6	428.2	939.6	–	–	–	–	B
DOK3	–	011	1.4	4.93	3.42	2.7	20.97	7.69	2.6	75.63	29.21	9.8	17.3	5.2	7.4	4.1	1.6	0.8	7.4	–	–	–	–	–
DPEP2	–	001	1.1	1.30	1.13	1.0	1.52	1.50	3.5	6.63	1.89	0.1	0.2	0.1	0.1	0	0	0	0.1	–	–	–	–	–
DTX4	B	010	1.8	8.01	4.36	2.3	15.63	6.69	1.4	26.14	19.34	5.0	32.9	4.1	4.1	1.9	4.3	0.6	4.3	–	–	–	–	B
EFHD1	–	001	5.2	0.08	0.02	2.6	0.12	0.05	16.2	2.32	0.14	0.2	1.1	0.1	0.1	0.1	0	0	0.1	–	–	–	–	–
EGFR	B	001	0.3	0.11	0.36	0.8	0.04	0.06	14.0	0.81	0.06	0	0.1	0	0	0	0	0	0	–	–	010	–	B
EGR1	B	100	4.2	13.45	3.17	2.0	6.70	3.33	2.1	1.98	0.94	43.8	1.8	1.4	0.4	0.4	12.0	12.3	12.3	–	–	–	–	B
EGR3	B	100	9.2	12.90	1.41	8.1	1.27	0.16	2.7	0.77	0.29	0.8	0	0.3	0	0	1.0	0.4	1.0	–	–	–	–	–
ELANE	–	001	1.1	19.84	17.10	1.0	31.02	30.69	4.0	425.69	106.66	1.8	129.5	34.7	0.1	0.1	0.1	0.1	34.7	–	–	–	–	–
ELTD1	–	010	1.9	178.83	94.32	2.2	150.39	67.13	1.1	5.36	4.96	100.0	24.2	10.6	1.6	0.1	0	0	10.6	–	–	–	–	–
EPB4.1L4B	B	010	1.6	16.55	10.40	3.0	46.89	15.44	1.6	50.33	30.72	24.7	41.3	30.7	43.2	52.0	72.2	12.5	72.2	–	–	–	–	–
EPHA7	B	010	1.4	0.33	0.24	3.4	2.47	0.71	1.6	0.47	0.28	0.9	0.3	0	0	0	0	0	0	–	–	–	–	B
F13A1	–	011	0.9	0.63	0.68	3.0	5.59	1.83	3.9	83.41	21.59	0.7	9.9	0.7	0.5	2.1	2.0	1.6	2.1	–	–	–	–	–
FAM102B	–	010	1.3	11.79	8.76	2.1	19.62	9.14	1.6	22.11	14.05	10.0	12.2	7.7	13.8	10.3	6.2	6.3	13.8	–	–	–	–	–
FAM155A	–	001	0.7	0.04	0.05	0.7	0.09	0.14	30.4	1.07	0.03	0.1	0.1	0.1	0.1	0.1	0	0	0.1	–	–	–	–	–
FAM83F	–	001	3.9	0.28	0.07	1.1	0.07	0.06	5.9	2.68	0.45	0.3	1.6	0.6	0.1	0.4	0.8	0.2	0.8	–	–	–	–	–
FARP1	–	001	0.8	3.04	3.80	0.7	0.53	0.81	4.1	1.79	0.44	0.7	0.8	0.5	0.1	0.2	0.2	0.3	0.5	–	–	–	–	–
FBN2	–	010	–	0.00	0.00	18.3	0.17	0.01	27.9	1.07	0.04	0	0	0.1	0.1	0	0	0	0.1	–	–	–	–	–
FGD4	–	011	1.1	1.81	1.66	2.5	4.95	1.94	3.1	4.34	1.39	1.3	1.0	0.5	0.1	0	0	0	0.5	–	–	–	–	–
FGR	B	001	0.5	2.17	4.62	0.5	0.56	1.02	3.4	42.59	12.55	0.4	7.1	1.4	2.4	1.4	3.7	3.8	3.8	–	–	–	–	B
GAPT	–	001	1.2	1.53	1.29	1.5	5.25	3.43	2.5	24.31	9.65	2.9	11.4	1.3	1.5	0.1	0	0	1.5	–	–	–	–	–
GAS6	–	010	0.8	10.20	12.77	2.5	10.91	4.37	0.9	1.10	1.25	10.9	0.9	0.1	0.1	0	0	0	0.1	–	–	–	–	–
GAS7	B	001	2.5	0.21	0.08	3.4	0.49	0.14	4.0	9.28	2.29	0.1	1.6	0.1	0.3	0	0	0	0.3	–	–	–	–	–
GBP9	–	100	2.5	12.94	5.15	1.4	5.84	4.29	1.1	2.50	2.35	0.3	0.9	0.4	2.2	8.8	26.7	4.3	26.7	–	–	–	–	–
GCET2	–	010	3.5	0.87	0.25	5.4	1.09	0.20	3.5	1.28	0.36	1.5	1.4	0.5	0.4	0	0	0	0.5	–	–	–	–	–
GEM	B	100	2.9	32.24	11.14	1.5	6.64	4.46	1.2	1.00	0.82	13.0	3.9	14.1	3.6	0.8	0.4	1.6	14.1	–	–	–	–	–
GFI1B	–	110	5.2	80.65	15.59	5.6	79.32	14.06	1.8	39.88	22.31	14.7	42.9	30.7	31.7	2.8	1.6	2.0	31.7	–	–	–	–	–
GIMAP6	–	001	1.4	112.79	81.70	1.4	109.44	76.79	2.6	19.44	7.50	144.2	157.6	79.2	41.7	41.2	39.8	18.3	79.2	–	–	–	–	–
GIMAP7	–	001	1.4	3.67	2.58	1.6	7.57	4.84	6.6	4.20	0.64	17.9	30.8	7.6	8.4	7.8	2.2	1.9	8.4	–	–	–	–	–
GM106	–	001	4.4	0.12	0.03	3.2	0.49	0.16	8.7	4.65	0.53	0.3	0.8	0.1	0.6	1.8	1.1	0.1	1.8	–	–	–	–	–
GNG12	B	001	1.2	11.47	9.65	1.8	19.58	10.91	2.2	37.27	16.76	13.4	17.4	7.0	7.3	6.0	3.0	4.5	7.3	–	–	–	–	–
GPX3	–	001	1.1	0.62	0.54	3.3	3.32	1.01	4.3	7.67	1.81	0.7	1.2	0.5	0.1	0.3	0.3	0.2	0.5	–	–	–	–	–
GRB10	–	001	0.9	2.74	2.93	1.8	1.25	0.70	10.1	0.72	0.07	0.9	0.8	0.2	0.3	0.2	0.2	0.1	0.3	–	–	–	–	–
GREB1	–	010	1.4	1.52	1.06	3.2	0.92	0.28	4.5	0.21	0.05	0	0	0	0	0	0.4	0.3	0.4	–	–	–	–	–
H1FX	–	001	1.2	62.73	51.65	1.4	41.28	30.14	2.3	53.26	23.34	89.5	20.5	11.2	14.7	35.2	55.7	66.7	66.7	–	–	–	–	–
H2-OB	–	010	1.2	24.73	20.53	2.1	82.59	39.35	1.3	38.66	28.87	45.4	63.7	37.4	12.4	6.0	1.2	0.3	37.4	–	–	–	–	–

Supplemental Table 7.6 Continued: Notch-repressed genes identified in LMPPs (199)

Gene	B _{DN3,B}	Code	4 h stimulation			12 h stimulation			4 d stimulation			In vivo expression (RPKM)								Pax5 ^{-/-} pro-B		DN3		B _{DN3}
			fc ⁻¹	GFP	DL1	fc ⁻¹	GFP	DL1	fc ⁻¹	GFP	DL1	LMPP	ALP	DN1	DN2a	DN2b	DN3a	DN3b	Max	↑	↓	↑	↓	
H2-Q6	B	001	1.1	76.44	70.18	1.1	38.91	35.61	3.6	10.85	3.06	107.7	29.6	16.3	33.4	12.0	1.0	0.2	33.4	–	–	–	–	B
HAVCR1	–	001	–	0.00	0.01	2.7	0.04	0.01	52.0	3.61	0.07	0	0.3	0	0	0	0	0	0	–	–	–	–	–
HDAC9	–	011	2.0	2.15	1.07	3.9	3.07	0.79	3.4	12.47	3.64	2.0	9.6	0.3	0.3	1.5	0.6	0.4	1.5	–	–	–	–	–
HIP1	B	001	1.7	14.72	8.42	1.6	7.10	4.49	2.4	8.90	3.72	6.0	7.7	1.7	1.2	1.9	0.8	0.4	1.9	–	–	–	–	–
HK3	–	011	1.1	9.93	9.01	2.2	45.17	20.02	3.6	58.12	16.46	35.7	6.1	2.1	0.4	0.2	0.1	0	2.1	–	–	–	–	–
HLF	–	110	2.4	101.36	42.41	2.8	119.70	42.50	2.8	3.97	1.40	98.5	2.6	1.0	0.4	0.1	0.1	0.1	1.0	–	–	–	–	–
HOXA10	–	001	1.5	11.31	7.72	1.5	9.21	6.04	8.0	3.77	0.47	9.8	1.1	0.8	0.1	0	0	0	0.8	–	–	–	–	–
HS3ST1	B	001	3.5	3.18	0.90	4.1	1.91	0.46	3.6	16.68	4.55	1.0	9.8	0.2	0	0.1	0	0	0.2	–	–	–	–	–
HVCN1	–	001	1.9	6.96	3.70	1.8	10.43	5.84	2.6	55.56	20.93	10.9	33.7	18.5	16.0	11.0	11.8	7.6	18.5	–	–	–	–	–
IL12A	–	111	3.6	26.39	7.32	3.8	54.60	14.23	4.0	28.28	7.08	44.5	71.8	12.7	2.1	2.2	0.2	0.1	12.7	–	001	–	–	–
IL18R1	–	010	1.9	0.45	0.23	3.6	3.23	0.90	2.0	1.17	0.58	1.4	4.1	2.6	0.5	0.3	0	0	2.6	–	–	–	–	–
IL4I1	B	001	0.6	1.91	3.04	0.9	1.40	1.55	3.3	9.62	2.90	0.9	1.1	1.4	0.7	1.0	1.3	0.5	1.4	–	–	–	–	B
ILDR2	–	010	0.4	0.03	0.08	30.5	0.19	0.01	–	0.00	0.00	0	0	0	0	0	0	0	0	–	–	–	–	–
IRF4	B	001	1.4	7.10	5.07	0.6	2.24	3.68	3.0	5.52	1.86	0.5	1.8	0.3	0	0	0.5	0.4	0.5	–	–	–	–	B
ITSN1	B	101	2.6	1.96	0.74	1.5	2.26	1.46	2.6	2.90	1.14	3.2	4.3	1.8	0.3	0.2	0.1	0.1	1.8	–	–	–	–	B
KCNG1	–	010	1.7	3.01	1.71	2.3	14.12	6.01	1.5	18.16	12.40	14.5	100.2	7.0	1.5	0.1	0	0.1	7.0	–	001	–	–	–
KHDRBS3	–	011	1.4	11.81	8.69	3.6	34.99	9.83	2.7	51.08	18.71	8.2	5.6	4.2	3.8	3.3	0.8	1.4	4.2	–	–	–	–	–
KLF2	B	110	2.8	24.89	9.02	2.5	14.65	5.73	2.5	57.15	22.41	4.8	4.4	21.5	6.2	27.6	2.2	1.9	27.6	011	–	–	–	B
KLHL30	–	011	1.5	2.00	1.29	3.5	2.98	0.86	3.6	8.81	2.42	1.3	11.6	2.4	3.7	0.8	0.1	0.1	3.7	–	–	–	–	–
L1CAM	–	001	1.2	0.68	0.55	0.7	0.47	0.63	2.8	8.22	2.93	0.2	0.7	0.3	0.1	0.1	0.1	0.1	0.3	–	–	–	–	–
LPAR1	–	011	2.0	1.81	0.92	5.4	6.74	1.25	8.0	4.39	0.55	1.2	3.8	0.2	0.2	0.1	0.1	0	0.2	–	–	–	–	–
LTA	–	010	2.5	1.08	0.42	5.6	10.27	1.82	6.3	9.28	1.46	0.2	1.0	0.9	0.4	0.7	0.4	0.2	0.9	–	001	–	–	–
LY6C1	–	001	1.1	2.92	2.68	2.1	5.84	2.82	3.6	166.01	46.16	0.6	21.9	4.1	1.0	0.3	0.2	0.2	4.1	–	–	–	–	–
MAMDC2	–	010	1.5	0.67	0.45	3.7	2.92	0.79	2.9	1.45	0.50	3.8	0.1	0.5	0.1	0	0.1	0	0.5	–	–	–	–	–
MAN1C1	–	001	1.5	9.04	6.15	1.2	6.88	5.68	2.4	19.81	8.14	13.7	9.8	6.5	0.8	0.2	0.1	0.1	6.5	–	–	–	–	–
MC5R	–	010	1.4	8.60	5.95	2.7	31.17	11.61	0.5	1.42	3.10	41.5	3.9	28.5	23.0	5.4	0.2	0	28.5	–	–	–	–	–
MDGA1	–	010	1.7	4.15	2.47	3.2	12.78	3.95	1.1	0.96	0.86	17.6	0.8	3.0	23.5	18.7	0.3	0	23.5	–	–	–	–	–
MGL1	–	001	2.3	3.32	1.44	1.9	5.38	2.87	3.0	43.57	14.49	5.5	5.2	1.0	0.8	0.4	0	0	1.0	–	–	–	–	–
MLPH	–	010	2.2	1.50	0.67	4.2	4.07	0.96	15.4	0.25	0.02	0.2	0	0	0	0	0	0	0	–	–	–	–	–
MMP19	–	010	1.2	36.67	31.26	3.0	7.71	2.56	2.7	3.41	1.25	0.2	0.1	0.1	0.1	0.1	0.1	0	0.1	–	–	–	–	–
MN1	–	001	1.8	37.72	20.49	1.8	32.18	17.87	2.6	14.33	5.45	25.9	30.0	24.4	2.0	0.5	0.1	0	24.4	–	–	–	–	–
MPEG1	–	011	1.5	3.15	2.08	3.2	23.52	7.23	2.5	179.69	71.03	11.5	197.3	2.3	0.3	0.2	0.1	0	2.3	–	–	–	–	–
MPO	–	011	1.0	274.78	275.59	2.2	444.42	202.97	5.3	1472.94	280.94	574.0	707.2	652.8	199.0	5.1	0.3	0.1	652.8	–	–	–	–	–
MRC1	B	001	1.3	0.03	0.03	2.5	0.73	0.30	4.3	1.69	0.39	0.3	1.6	0	0	0	0	0	0	–	–	–	–	–
MRC2	–	011	1.3	7.60	5.98	3.0	8.18	2.73	4.0	11.46	2.85	0	0.4	0	0	0	0	0	0	–	–	–	–	–
MRVI1	B	010	4.2	0.69	0.16	6.1	0.56	0.09	2.1	0.84	0.40	1.7	5.9	1.9	0.2	0.1	0	0	1.9	–	–	–	–	–
MYO1E	B	001	0.5	0.82	1.52	0.4	0.19	0.49	5.7	1.78	0.32	0.2	0.1	0.3	0.1	0.1	0	0.1	0.3	–	–	–	–	–
NCF1	–	100	2.4	167.13	70.09	1.5	99.57	64.91	1.1	186.59	169.17	74.3	139.8	62.9	46.5	7.2	3.4	0.2	62.9	–	–	–	–	–
NIACR1	–	010	3.0	3.41	1.14	12.5	3.01	0.24	2.6	0.25	0.10	1.2	0	0	0	0	0	0	0	–	–	–	–	–
NOS1AP	–	110	2.4	19.45	8.16	2.9	14.04	4.88	2.2	15.86	7.13	6.0	17.6	1.2	0.2	0.1	0	0	1.2	–	–	–	–	–
NPAS2	B	110	3.2	7.25	2.26	2.6	6.96	2.67	3.2	1.97	0.61	0.8	0	0	0	0.1	2.7	0.3	2.7	–	–	–	–	–
NRXN2	B	011	2.0	0.51	0.26	3.6	1.67	0.46	6.2	27.36	4.40	0.2	0.1	0	0	0	0	0	0	–	–	–	–	–
PACSIN1	B	011	2.2	4.76	2.14	4.0	2.77	0.70	4.4	24.16	5.46	1.1	5.4	0.5	0.1	0.2	0.4	0.4	0.5	–	–	–	–	–
PAPSS2	B	001	0.9	0.97	1.02	1.7	0.69	0.41	2.7	6.72	2.48	0.2	0.7	0.2	0.2	0.4	4.7	1.2	4.7	–	–	–	–	B
PAQR5	B	011	2.3	6.19	2.64	3.7	10.15	2.75	3.2	5.21	1.60	11.7	7.3	1.7	0.9	2.1	6.1	1.0	6.1	–	–	010	–	–

Supplemental Table 7.6 Continued: Notch-repressed genes identified in LMPPs (199)

Gene	B _{DN3,B}	Code	4 h stimulation			12 h stimulation			4 d stimulation			In vivo expression (RPKM)								Pax5 ^{-/-} pro-B		DN3		B _{DN3}
			fc ⁻¹	GFP	DL1	fc ⁻¹	GFP	DL1	fc ⁻¹	GFP	DL1	LMPP	ALP	DN1	DN2a	DN2b	DN3a	DN3b	Max	↑	↓	↑	↓	
PARP8	B	010	1.5	17.54	11.34	2.1	39.95	18.76	1.2	32.89	28.40	20.9	31.9	16.4	13.0	12.7	11.4	1.7	16.4	–	–	–	–	B
PCP4L1	–	111	3.2	104.39	32.06	4.6	93.29	20.40	7.1	10.98	1.55	41.8	1.4	0.2	0.1	0.1	0	0	0.2	–	–	–	–	–
PCYT1A	B	010	1.1	17.63	15.65	2.1	24.80	11.54	1.5	42.59	29.30	16.9	35.9	9.1	14.8	11.9	3.2	7.5	14.8	–	–	–	–	B
PLEK	–	010	1.4	41.64	29.28	2.5	31.84	12.55	1.6	30.80	19.60	22.3	24.6	14.4	4.7	0.8	0.2	0	14.4	–	–	–	–	–
PLTP	–	001	1.4	1.20	0.86	0.6	0.03	0.05	3.9	4.46	1.12	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1	–	–	–	–	–
PLXNA2	–	001	0.8	0.40	0.50	1.4	0.25	0.18	7.1	1.06	0.15	0.3	0.4	0.1	0.1	0.1	0.1	0	0.1	–	–	–	–	–
PRKD2	B	100	3.0	47.31	15.92	1.5	37.76	25.59	2.1	18.48	8.76	44.1	35.9	22.3	14.5	48.5	39.5	33.7	48.5	–	–	–	–	–
PRNP	B	001	1.6	7.25	4.53	1.8	4.06	2.21	3.1	6.08	1.94	2.3	10.5	2.8	2.1	8.2	11.2	2.1	11.2	–	–	–	–	B
PSG17	–	001	–	0.00	0.00	5.6	0.15	0.03	8.2	2.92	0.36	0	0	0	0.4	0	0	0	0.4	–	–	–	–	–
PTPRF	B	011	1.5	2.22	1.43	2.9	1.39	0.47	3.4	14.20	4.15	0.7	2.1	0.7	4.7	17.8	115.9	67.3	115.9	–	–	–	–	B
RAB44	B	010	1.1	2.49	2.20	2.0	23.84	11.64	0.9	26.58	28.64	8.3	8.2	3.3	17.8	13.1	64.9	4.7	64.9	–	–	–	–	B
RAB6B	–	001	1.1	7.70	6.78	1.4	11.12	7.74	2.4	6.14	2.53	9.1	4.4	3.0	3.2	8.4	17.4	2.8	17.4	–	–	–	–	–
RAPH1	–	001	3.2	0.63	0.20	4.6	0.84	0.18	3.1	6.79	2.17	0.2	1.5	0.1	0.1	0	0.1	0.5	0.5	–	–	–	–	–
RASL11B	–	001	1.3	0.42	0.32	3.0	0.94	0.31	6.0	9.91	1.65	0.5	2.2	2.8	3.6	4.3	5.6	2.8	5.6	–	–	–	–	–
RHOB	–	010	1.9	48.88	25.00	2.6	53.10	20.66	2.6	51.76	19.60	66.5	90.8	31.0	13.4	2.4	0.3	0.2	31.0	–	–	–	–	–
RNASE6	–	001	1.0	19.92	19.25	0.7	15.70	21.62	2.3	69.09	29.90	22.1	27.6	8.4	4.0	0.6	0	0.1	8.4	–	–	–	–	–
RNF122	–	001	1.3	12.81	9.47	0.9	10.11	10.81	2.4	15.53	6.58	15.7	11.0	8.8	6.7	3.7	3.2	0.7	8.8	–	–	–	–	–
RUNX1T1	–	001	1.2	0.06	0.05	5.0	0.15	0.03	53.2	1.42	0.03	0	0.1	0	0	0	0	0	0	–	–	–	–	–
RYSR3	–	010	1.7	0.10	0.06	6.2	0.71	0.12	1.1	0.11	0.10	0	0	0	0	0	0	0	0	–	–	–	–	–
SCUBE3	–	010	1.8	3.33	1.86	2.4	7.65	3.13	1.2	0.55	0.45	3.2	0.8	0.9	0.5	0.2	0	0	0.9	–	–	–	–	–
SDC1	B	001	1.0	24.39	25.36	1.5	29.88	19.85	4.9	107.68	21.91	32.9	68.0	0.6	0.1	0	0.1	0.6	0.6	–	–	–	–	–
SDC4	B	001	1.5	46.05	30.55	1.2	13.75	11.00	4.6	23.63	5.10	13.6	31.2	2.7	0.1	0.2	0	0	2.7	–	–	–	–	–
SEC31B	–	010	1.8	0.30	0.17	3.5	2.06	0.58	2.3	6.72	2.91	0.6	5.5	0.4	0.6	0.6	1.5	0.7	1.5	–	–	–	–	–
SELIL3	–	001	1.1	7.20	6.40	1.5	7.09	4.73	6.4	1.71	0.27	0.5	0	0	0	0	0	0	0	–	–	–	–	–
SEMA6D	–	001	2.4	0.04	0.02	1.3	0.02	0.01	15.6	1.32	0.08	0	0.2	0	0	0	0	0	0	–	–	–	–	–
SERPINI1	–	010	1.6	1.42	0.87	2.8	3.27	1.17	2.2	0.79	0.37	2.5	1.4	0.8	1.8	1.0	0.7	0.8	1.8	–	–	–	–	–
SETBP1	–	001	0.4	0.22	0.55	0.2	0.12	0.66	2.8	2.19	0.77	0.1	0	0.8	0.1	0.1	0.2	0	0.8	–	–	–	–	–
SGSM1	B	010	1.8	4.26	2.39	2.3	5.60	2.42	3.7	0.34	0.09	9.8	5.3	3.5	0.1	0	0	0	3.5	–	–	–	–	–
SIGLECH	–	001	2.2	1.03	0.47	1.8	3.15	1.72	3.2	108.87	34.30	0	30.9	0.1	0.1	0	0	0	0.1	–	–	–	–	–
SLC16A11	–	001	1.6	15.83	9.81	4.8	33.37	6.88	5.3	3.43	0.65	39.0	4.4	0.4	0.3	0.2	0.1	0.1	0.4	–	–	–	–	–
SLC7A8	–	010	2.0	5.79	2.89	2.7	5.29	1.94	1.9	2.26	1.20	1.7	0.5	0.6	2.3	2.4	0.1	0.4	2.4	–	–	–	–	–
SLCO2B1	–	110	6.5	5.79	0.89	7.1	4.06	0.57	3.9	0.42	0.11	1.5	0.2	0.1	0	0	0	0	0.1	–	–	–	–	–
SLPI	–	111	4.6	30.09	6.43	16.2	89.68	5.52	6.6	298.20	44.85	4.4	37.0	0.5	0	0	0	0	0.5	–	–	–	–	–
SMPD3	B	001	1.0	0.48	0.50	1.3	0.53	0.40	3.6	1.98	0.56	0.7	2.8	0.4	0.3	0.2	0.2	0.3	0.4	–	–	–	–	B
SOCs2	B	001	1.1	0.68	0.60	1.7	5.22	3.03	3.2	22.97	7.16	1.1	1.8	1.1	2.8	8.7	3.7	0.5	8.7	–	–	–	–	B
SORCS2	B	011	1.5	2.40	1.56	9.5	2.33	0.25	8.3	1.59	0.19	0.9	6.6	0.5	0.1	0.1	2.7	0.1	2.7	–	–	–	–	–
SPIB	–	001	2.6	3.29	1.27	1.9	3.53	1.87	3.6	104.04	28.85	0.1	5.7	0.3	1.6	5.7	79.1	6.5	79.1	–	–	–	–	–
SPP1	–	011	1.5	38.29	24.89	3.2	8.04	2.51	4.9	21.94	4.53	0.7	0.5	0.1	0	0	0	0.1	0.1	–	001	–	–	–
SPRY2	–	100	2.7	13.63	5.03	1.8	10.58	5.71	3.1	5.33	1.74	10.6	7.9	5.1	2.0	1.5	2.9	1.1	5.1	–	–	–	–	–
SRGAP3	B	001	2.3	3.90	1.66	1.5	3.70	2.38	2.5	8.68	3.51	3.6	4.0	2.9	1.9	1.5	1.5	0.6	2.9	–	–	–	–	B
ST3GAL5	–	100	2.5	17.18	6.78	1.7	6.67	3.96	1.2	21.80	17.98	3.9	17.2	4.8	2.8	1.8	0.7	1.9	4.8	–	–	–	–	–
ST8SIA1	B	100	5.3	1.60	0.30	1.2	0.58	0.47	2.2	5.17	2.33	1.5	5.5	14.1	5.6	9.4	6.5	4.8	14.1	–	–	–	–	B
STAMBPL1	B	001	1.4	19.20	13.61	1.2	11.56	9.53	2.8	18.49	6.58	5.9	7.9	5.2	7.4	15.4	15.9	20.2	20.2	–	–	–	–	B
SYNPO2	–	010	1.1	0.24	0.22	3.8	1.10	0.29	1.1	0.34	0.31	0.6	2.3	0	0.1	0.5	0.1	0	0.5	–	–	–	–	–
TANC1	–	001	1.6	3.83	2.33	1.7	4.23	2.45	3.5	4.28	1.22	3.5	5.5	1.8	0.9	1.9	0.5	1.1	1.9	–	–	–	–	–

Supplemental Table 7.6 Continued: Notch-repressed genes identified in LMPPs (199)

Gene	B _{DN3,B}	Code	4 h stimulation			12 h stimulation			4 d stimulation			In vivo expression (RPKM)								Pax5 ^{-/-} pro-B		DN3		B _{DN3}
			fc ⁻¹	GFP	DL1	fc ⁻¹	GFP	DL1	fc ⁻¹	GFP	DL1	LMPP	ALP	DN1	DN2a	DN2b	DN3a	DN3b	Max	↑	↓	↑	↓	
TEX2	B	001	1.1	21.76	19.09	1.8	68.86	37.17	2.1	128.02	59.88	15.1	34.1	10.7	11.7	11.0	13.1	10.2	13.1	–	–	–	–	B
THBS1	–	001	1.0	124.75	124.38	1.4	246.58	180.36	2.1	150.12	70.94	0.1	0.5	0	0	0	0	0.1	0.1	–	–	–	–	–
TIMP3	–	011	1.1	3.96	3.53	2.6	10.55	4.06	3.4	7.53	2.21	0.1	1.5	0	0	0	0	0	0	–	–	–	–	–
TINAGL1	–	100	2.8	17.76	6.39	2.7	4.35	1.62	3.9	0.47	0.12	3.1	6.4	0	0	0	0	0	0	–	–	–	–	–
TMEM65	B	010	1.5	3.85	2.56	2.8	8.85	3.20	1.5	3.78	2.50	3.5	2.0	2.2	1.3	1.2	0.5	1.5	2.2	–	–	–	–	B
TNS1	B	010	1.4	26.35	18.28	2.2	46.56	21.18	1.2	47.31	39.79	32.0	82.8	43.3	20.1	12.6	15.0	7.7	43.3	–	–	–	–	–
TOX	B	100	3.6	18.92	5.25	1.7	7.75	4.46	1.1	4.50	4.26	10.8	21.1	59.6	31.9	28.7	26.0	32.1	59.6	–	–	–	–	B
TPM2	–	001	1.2	5.09	4.27	1.5	4.80	3.27	5.6	11.61	2.06	7.6	20.3	1.9	1.5	0.1	0	0	1.9	–	–	–	–	–
TREM2	–	001	1.1	0.47	0.41	–	0.12	0.00	5.3	6.97	1.32	0.1	0.6	0	0	0	0	0	0	–	–	–	–	–
TRPC6	–	010	2.3	2.17	0.95	3.2	8.28	2.58	4.1	1.64	0.40	0.5	0.1	0	0	0	0	0	0	–	–	–	–	–
TSPAN12	–	001	0.7	0.06	0.09	1.6	0.02	0.01	17.1	1.79	0.10	0	0	0	0	0	0	0	0	–	–	–	–	–
TULP3	–	001	1.3	1.27	0.98	1.1	0.95	0.84	4.9	1.92	0.39	0.7	0.6	0.4	0.1	0.7	2.2	2.8	2.8	–	–	–	–	–
UPB1	B	001	4.8	0.28	0.06	0.4	0.07	0.17	4.8	33.93	7.04	0	1.2	0	0	0.3	0.7	0.2	0.7	–	–	–	–	B
VIT	–	010	0.7	0.02	0.03	7.1	1.22	0.17	0.7	0.44	0.64	0.8	0.1	0	0	0	0	0	0	–	–	–	–	–
VLDLR	–	010	1.3	1.52	1.19	3.0	4.18	1.40	1.5	1.06	0.72	6.6	0.3	0.1	0.1	0	0	0	0.1	–	–	–	–	–
XKRX	–	001	2.4	0.32	0.14	3.5	1.90	0.54	3.3	9.16	2.80	0.2	1.7	0.2	1.1	1.1	1.8	0.3	1.8	–	–	–	–	–
ZBTB16	–	010	2.2	0.31	0.14	4.6	1.34	0.29	1.0	0.72	0.75	0.3	1.6	1.3	7.3	1.1	0	0	7.3	–	–	–	–	–
ZEB2	–	010	1.8	6.73	3.61	2.3	15.13	6.43	1.4	23.04	16.58	6.0	16.9	6.6	5.7	5.3	4.4	0.5	6.6	–	–	–	–	–
ZFP608	B	100	2.1	21.89	10.17	1.6	37.73	24.22	1.6	18.72	11.41	40.0	32.6	17.2	5.1	3.1	2.4	0.2	17.2	–	–	–	–	B

Supplemental Table 7.6: List of 199 Notch-repressed genes identified by RNA-seq of *in vitro* Notch-stimulated (4 h, 12 h and/or 4 d) LMPPs. The 'B_{DN3,B}' column indicates whether the gene was bound (B) by Rbpj-Bio within 50 kb of the gene-body (determined by Bio-ChIP-seq in Pax5^{Δ/Δ} pro-B cells and/or DN3a cells). The 'Code' column shows in which experiment (from left to right in the table) a gene was found to be repressed – '0' = no; '1' = yes. The differential expression between non-stimulated (GFP) and Notch-stimulated (DL1) is depicted as moderated fold-change⁻¹ (fc⁻¹). The absolute expression of each gene in the given cell type is shown as RPKM-normalised expression value. This includes the stimulation experiments as well as the expression profiling throughout early lymphocyte development (LMPP, ALP, DN1/ETP, DN2a, DN2b, DN3a and DN3b stages). The maximum (Max) intrathymic (DN1 – DN3b) expression level is also shown. If a gene was activated (↑) or repressed (↓) upon Notch-stimulation of Pax5^{-/-} pro-B cells and/or Notch stimulation or deletion in DN3a cells is shown together with the 'Experiment Code' of the according cell type (see table of according cell type). The 'B_{DN3}' column indicates whether the gene was bound (B) by ICN1-Bio and/or Rbpj-Bio (determined by Bio-ChIP-seq in DN3a cells).

Supplemental Table 7.7: Directly Notch-activated genes identified in Pax5-deficient pro-B cells (91)

Gene	Code	2 h stimulation			4 h stimulation			8 h stimulation			In vivo expression (RPKM)								LMPPs		DN3		B _{DN3}
		fc	GFP	DL1	fc	GFP	DL1	fc	GFP	DL1	LMPP	ALP	DN1	DN2a	DN2b	DN3a	DN3b	Max	↑	↓	↑	↓	
ABCG3	001	1.0	1.29	1.26	2.0	1.09	2.09	2.9	1.62	4.64	1.0	0.7	0.5	1.6	4.8	15.0	6.0	15.0	–	–	–	–	B
ABI3	001	1.6	15.05	23.48	1.7	15.87	27.37	2.3	12.36	28.99	11.2	12.7	23.2	18.7	29.7	83.3	25.0	83.3	111	–	–	–	B
ARHGEF17	011	1.6	2.50	4.08	2.5	2.47	6.07	2.2	2.86	6.32	0.9	0.4	0.6	0.5	0.5	0.6	0.4	0.6	–	–	–	–	B
ARNT2	011	2.7	0.07	0.19	5.0	0.14	0.70	5.2	0.12	0.65	0	0.1	0.2	0.5	0.2	0.2	0	0.5	–	–	001	–	B
ASB2	011	2.5	0.22	0.54	4.6	0.23	1.06	6.2	0.19	1.16	0.4	0.1	0.6	0.1	0	0	0	0.6	110	–	–	–	B
ATP1A3	001	1.4	1.09	1.55	1.6	1.25	1.92	2.7	0.79	2.12	13.4	61.9	3.3	3.9	1.6	1.5	0.9	3.9	–	–	–	–	B
AW011738	011	3.6	0.21	0.73	6.1	0.28	1.70	7.4	0.22	1.62	3.2	1.8	13.6	10.1	10.7	25.0	5.9	25.0	110	–	111	–	B
BCL2L11	100	2.2	67.14	144.63	1.7	48.57	83.93	1.7	27.23	45.46	6.3	9.3	9.1	6.4	6.6	15.2	8.7	15.2	–	–	–	–	B
BCL2L14	001	1.6	0.89	1.43	1.8	0.93	1.70	4.6	0.72	3.33	2.3	2.5	9.4	11.3	11.2	17.8	3.8	17.8	010	–	–	–	–
BST1	011	1.6	9.15	14.77	2.6	5.85	14.74	2.2	6.82	15.32	0.1	0.9	1.5	4.5	6.6	51.8	5.2	51.8	010	–	111	–	B
CAPN5	011	1.4	21.03	30.06	2.5	15.37	38.11	3.4	11.39	38.26	26.1	30.9	70.1	42.7	34.5	147.6	23.2	147.6	–	–	111	–	B
CCDC109B	110	2.7	2.98	7.83	2.9	3.14	9.11	2.6	1.36	3.51	0.2	0.4	0.6	17.4	112.5	88.2	31.4	112.5	–	–	001	–	B
CCL3	001	0.7	2.20	1.59	2.8	1.53	4.21	3.6	3.60	12.91	27.3	3.1	3.8	0.7	0	0.4	0	3.8	–	–	–	–	B
CD22	011	1.1	8.50	8.90	2.5	6.72	16.47	3.1	8.62	26.39	0.1	1.9	0.1	0	0	0	0	0.1	010	–	–	–	–
CD300A	001	1.0	0.71	0.69	2.7	0.34	0.91	2.9	0.49	1.41	8.7	5.4	3.1	0.7	0	0	0	3.1	–	–	–	–	–
CD7	001	1.3	79.56	98.61	1.6	74.58	119.17	2.1	89.96	190.42	4.3	18.1	185.2	53.7	11.6	1.7	0.2	185.2	010	–	–	–	B
CD97	001	1.3	10.82	14.43	1.9	8.42	15.53	3.0	5.03	15.02	3.0	5.4	3.5	1.4	2.5	3.2	7.9	7.9	100	–	–	–	B
CHN2	011	1.1	8.93	9.64	2.4	8.76	20.50	3.1	8.70	26.87	4.7	4.2	9.4	9.9	2.0	0.2	0.7	9.9	110	–	–	–	–
CLN8	011	1.7	2.94	4.85	2.2	2.46	5.33	3.5	2.65	9.22	7.2	7.4	7.8	5.4	5.2	5.4	4.5	7.8	–	–	–	–	–
COL15A1	011	1.6	0.30	0.49	7.5	0.15	1.13	9.5	0.22	2.05	0	0	0.1	0.2	0.2	3.8	2.2	3.8	110	–	–	–	B
CSF1R	011	2.6	0.65	1.63	4.5	1.19	5.29	10.2	1.27	12.98	9.6	52.0	28.7	1.2	0.2	0.1	0	28.7	110	–	–	–	–
CTLA4	100	3.1	17.23	52.17	2.1	9.64	19.93	1.0	7.19	7.38	0.1	0.4	0.1	0.1	3.9	25.2	11.3	25.2	–	–	–	–	B
D8ERTD82E	001	1.1	8.14	8.73	2.0	6.95	13.73	2.4	5.53	13.28	3.1	8.2	12.8	2.5	3.2	26.0	11.3	26.0	100	–	–	–	B
DDIT4	001	1.5	24.48	36.61	1.9	27.95	52.41	2.5	21.86	54.67	28.6	51.6	33.9	18.7	10.4	44.4	11.4	44.4	110	–	–	–	–
DTX1	111	4.5	0.96	4.24	18.5	1.17	21.40	51.6	1.31	67.61	0.2	1.5	9.6	7.0	2.5	184.1	41.8	184.1	111	–	111	–	B
DUSP6	001	1.6	3.25	5.24	1.6	3.69	5.87	2.3	3.97	9.28	30.7	35.0	36.1	10.1	6.3	1.9	2.0	36.1	001	–	–	–	B
ESAM	001	0.9	0.52	0.48	2.7	0.23	0.61	7.8	0.17	1.33	7.8	3.0	1.7	0.9	0.2	0	0	1.7	110	–	–	–	–
FAM26F	001	1.3	2.16	2.80	1.9	2.36	4.40	4.2	2.29	9.74	17.1	7.8	18.1	13.9	6.1	3.5	2.3	18.1	110	–	–	–	B
FASL	111	2.9	1.22	3.45	2.9	1.98	5.65	6.5	1.57	10.22	0.1	0.1	14.9	8.2	1.5	0.3	0	14.9	111	–	–	–	B
GATA3	011	2.1	1.20	2.52	3.2	1.65	5.20	4.8	1.14	5.48	4.0	3.0	31.4	46.6	85.5	64.7	78.6	85.5	011	–	–	–	–
GBP2	001	1.2	6.35	7.59	1.4	8.35	11.44	2.7	4.78	12.77	14.9	2.6	9.2	12.1	12.0	16.4	2.1	16.4	–	–	–	–	B
GPBAR1	001	14.9	0.03	0.39	7.1	0.18	1.24	11.4	0.11	1.29	0.1	0	0.5	0.1	0.2	0	0	0.5	–	–	–	–	–
GPR132	100	2.1	13.26	27.01	1.4	16.53	22.81	1.5	12.60	18.98	33.9	23.5	66.3	30.2	45.0	72.1	30.6	72.1	010	–	–	–	B
GPR18	110	2.4	30.60	73.78	2.1	32.05	65.11	1.8	40.66	72.63	4.0	12.4	12.1	8.0	9.0	3.9	0.4	12.1	010	–	–	–	B
HPSE	011	1.2	32.24	37.81	2.6	34.27	87.37	3.8	41.85	159.16	5.2	7.5	6.2	4.2	1.0	0.6	0.2	6.2	110	–	–	–	B
I830077J02RIK	011	1.8	14.12	24.87	3.1	12.73	39.31	3.1	17.36	53.25	2.0	14.9	12.8	16.1	5.4	4.5	2.7	16.1	111	–	–	–	B
IL12RB1	001	1.5	1.69	2.57	1.8	1.91	3.43	3.1	1.10	3.43	10.0	5.8	12.2	12.4	8.6	5.7	6.5	12.4	010	–	–	–	B
IL12RB2	001	1.4	3.55	5.02	1.3	2.52	3.31	2.7	2.18	5.87	4.0	2.0	12.8	26.4	17.5	9.4	22.4	26.4	–	–	–	–	B
ISG15	001	1.4	4.61	6.57	1.5	9.28	13.75	2.9	8.36	23.89	42.7	38.2	50.0	47.6	44.0	32.7	9.3	50.0	010	–	–	–	B
ITGA9	001	1.0	1.24	1.18	2.4	0.57	1.36	7.0	0.46	3.22	4.7	2.3	18.1	6.2	2.2	2.4	1.3	18.1	011	–	–	–	B
ITGB7	001	1.2	23.89	27.21	1.3	25.51	33.51	2.9	16.15	46.54	42.0	49.7	89.5	60.7	26.8	1.8	2.3	89.5	–	–	–	–	B
KCNJ16	011	4.8	0.05	0.23	23.5	0.02	0.35	17.1	0.03	0.51	0	0	0.7	0.3	0.1	0.1	0	0.7	–	–	–	–	B
KLF2	011	1.8	16.61	28.96	2.1	13.36	27.73	3.1	5.55	17.28	4.8	4.4	21.5	6.2	27.6	2.2	1.9	27.6	–	110	–	–	B
KLF4	111	2.9	8.92	25.82	2.9	6.13	17.61	2.2	7.50	16.39	0.9	2.5	1.3	0.4	0.2	0.7	0.1	1.3	110	–	–	–	–
LCP2	001	1.1	2.43	2.73	2.1	2.06	4.31	3.6	1.66	6.00	18.6	9.1	40.8	44.2	43.0	50.2	33.5	50.2	001	–	–	–	B

Supplemental Table 7.7 Continued: Directly Notch-activated genes identified in Pax5-deficient pro-B cells (91)

Gene	Code	2 h stimulation			4 h stimulation			8 h stimulation			In vivo expression (RPKM)								LMPPs		DN3		B _{DN3}
		fc	GFP	DL1	fc	GFP	DL1	fc	GFP	DL1	LMPP	ALP	DN1	DN2a	DN2b	DN3a	DN3b	Max	↑	↓	↑	↓	
LRP1	001	1.0	5.55	5.71	1.4	4.68	6.32	2.7	3.09	8.37	3.1	11.6	20.4	14.0	5.7	13.1	0.9	20.4	–	–	001	–	B
LRP8	001	1.3	3.85	5.05	1.4	4.12	5.59	2.3	4.67	10.56	4.0	4.3	4.1	5.5	7.3	6.7	9.1	9.1	–	–	–	–	–
LY6I	001	2.2	0.48	1.02	2.0	0.55	1.11	6.7	0.55	3.68	0	0.1	0.2	0.1	0.1	0.1	0	0.2	–	–	–	–	B
MAF	011	2.2	1.42	3.10	4.1	1.35	5.52	4.4	2.20	9.68	1.3	1.0	10.4	0.8	1.1	3.4	0.8	10.4	010	–	–	–	B
MARCH1	001	0.7	0.35	0.26	1.5	0.26	0.40	4.9	0.38	1.86	0.1	0.2	0.1	0.1	0	0	0.1	0.1	010	–	–	–	B
MDFI	001	1.5	3.25	4.89	2.1	5.12	10.69	2.9	3.77	10.76	31.0	8.6	99.7	62.2	32.4	26.1	8.0	99.7	011	–	–	–	–
MMP13	001	0.9	1.84	1.71	1.6	1.66	2.70	2.2	3.83	8.41	0	0	0	0	0	0	0	0	–	–	–	–	B
NCF2	001	1.2	20.72	25.49	1.6	21.66	33.30	2.1	20.34	41.87	21.0	18.6	18.7	20.3	8.8	5.0	2.0	20.3	–	–	–	–	–
NDST1	011	1.9	14.45	26.54	2.6	15.58	39.91	3.8	11.77	44.97	25.6	33.8	45.5	35.4	24.3	51.4	34.6	51.4	111	–	–	–	–
NOD1	001	1.7	1.40	2.40	2.1	1.51	3.07	3.8	0.81	3.03	10.1	7.9	9.4	3.1	3.1	2.6	0.6	9.4	–	–	–	–	B
NOTCH3	111	3.0	2.06	6.17	4.0	3.43	13.54	7.6	2.09	15.83	0.5	0.3	2.7	12.6	29.8	283.3	33.7	283.3	011	–	110	–	B
OSGIN1	111	6.2	0.75	4.61	9.1	0.60	5.38	40.2	0.07	2.96	8.1	0.2	0.2	0.1	0	0.7	0.1	0.7	110	–	110	–	B
PDE4B	100	2.2	11.21	24.38	1.2	10.87	12.44	1.0	11.94	11.63	18.7	13.0	15.6	10.7	11.7	13.0	4.8	15.6	–	–	–	–	B
PDGFRB	111	3.7	1.51	5.59	9.3	1.55	14.27	19.6	1.27	24.95	23.6	37.7	114.0	49.1	34.0	24.8	10.8	114.0	111	–	–	–	B
PLCL1	001	1.2	0.12	0.14	2.5	0.21	0.52	6.0	0.13	0.80	0.4	0.3	0.2	0.1	0.3	1.1	0.5	1.1	–	–	010	–	B
PLEKHA7	011	1.5	4.52	6.59	3.9	4.06	15.52	4.2	6.57	27.80	2.7	24.8	77.3	69.2	89.9	95.5	17.6	95.5	111	–	101	–	–
PLXND1	001	1.4	20.94	28.67	1.8	28.57	49.77	2.1	21.30	44.25	19.2	18.9	15.9	6.5	5.8	16.9	5.1	16.9	–	–	001	–	B
PMEPA1	100	2.9	6.65	19.20	1.0	6.88	7.11	0.6	3.96	2.46	1.1	3.2	1.1	1.6	6.3	17.1	6.1	17.1	–	–	–	–	B
PRKCQ	001	1.2	0.60	0.70	1.8	0.43	0.77	5.3	0.12	0.62	11.2	3.1	6.9	9.8	18.5	33.9	14.0	33.9	–	–	–	–	B
PRSS2	001	1.4	13.07	17.77	2.0	15.78	31.73	3.0	22.62	68.40	0.3	0.5	3.7	3.2	1.5	0.5	0.2	3.7	111	–	–	–	B
PTCRA	001	2.1	0.27	0.58	4.6	0.32	1.46	8.2	0.28	2.31	0	0.1	0	0.2	5.2	365.1	70.0	365.1	–	–	111	–	B
PTP4A3	011	1.4	46.60	63.50	3.3	48.85	159.01	2.9	36.79	106.62	90.6	131.2	89.6	58.2	37.5	39.4	61.1	89.6	–	–	–	–	B
RAP1GAP2	001	1.7	0.06	0.10	2.8	0.16	0.44	8.4	0.05	0.41	0.3	0.3	0.5	0.3	0.2	7.4	6.1	7.4	110	–	–	–	B
RASAL1	001	1.1	4.01	4.22	1.7	3.49	5.92	3.6	2.53	9.22	3.8	34.7	9.0	2.0	2.3	12.9	10.9	12.9	010	–	111	–	B
RGS10	011	1.4	5.12	7.31	2.8	2.97	8.20	3.8	4.14	15.81	28.9	37.7	49.6	35.5	21.7	37.0	41.2	49.6	–	–	–	–	B
RGS3	001	1.3	0.76	0.97	2.1	0.82	1.73	2.5	0.72	1.81	1.7	1.4	4.5	2.1	1.3	0.6	1.9	4.5	110	–	–	–	B
RHOV	001	3.2	0.36	1.14	2.5	0.75	1.82	8.7	0.11	0.94	0.4	0.2	0.3	0.1	0	1.1	0	1.1	–	–	001	–	B
RND3	010	2.1	7.37	15.31	2.3	7.69	17.22	1.9	8.28	15.52	0.6	0.3	0.1	0	0	0.1	0	0.1	–	–	–	–	B
SASH1	001	1.2	3.34	3.88	1.5	2.96	4.47	2.3	3.29	7.51	4.7	2.3	5.4	1.8	0.8	1.8	0.3	5.4	110	–	–	–	–
SEPT14	100	2.1	60.34	124.33	1.4	73.84	105.30	1.3	58.32	77.53	59.9	63.0	76.3	51.4	55.0	34.8	58.1	76.3	–	–	–	–	–
SERPINB6B	001	1.2	11.82	14.24	1.9	6.28	11.76	2.4	8.25	19.41	6.2	1.5	19.2	16.2	10.9	9.9	3.6	19.2	110	–	–	–	B
SLC35D3	011	1.9	2.70	5.17	4.1	1.21	4.90	4.2	2.76	11.56	19.0	15.8	15.6	4.2	0.2	0.1	0	15.6	–	–	–	–	–
SLC5A5	011	1.2	5.20	5.92	2.8	4.17	11.53	5.7	3.06	17.40	0	0	0.3	0	0.1	0	0	0.3	111	–	–	–	–
SMAD7	011	2.0	8.76	17.43	3.2	7.03	22.36	3.8	6.93	26.57	15.3	20.4	60.9	22.3	16.7	5.3	1.4	60.9	110	–	–	–	B
SMTNL2	111	4.3	0.37	1.58	4.7	0.71	3.29	5.9	0.30	1.76	0.3	0.1	0.2	0.1	0.1	0.1	0.2	0.2	–	–	–	–	–
SPSB1	100	2.5	1.70	4.18	2.2	2.08	4.50	1.7	1.84	3.05	0.4	0.5	2.6	0.1	0.7	1.9	0.8	2.6	001	–	–	–	B
SVEP1	010	2.9	0.08	0.23	4.8	0.05	0.22	2.5	0.06	0.14	0	0	0.1	0	0	0	0	0.1	–	–	–	–	B
TGFBR1	011	1.9	13.66	25.81	3.3	9.22	30.20	3.4	13.70	47.00	4.5	4.4	6.3	7.7	8.2	23.5	11.2	23.5	110	–	–	–	B
TGM2	111	5.7	8.76	48.95	4.3	12.70	54.57	15.1	2.51	37.95	2.9	11.0	0.4	0.1	0	0	0	0.4	111	–	–	–	B
TNNI2	001	1.1	51.78	56.15	1.7	52.66	87.80	2.3	55.39	126.44	11.2	17.6	10.8	4.5	0.5	0.3	0.1	10.8	010	–	–	–	–
TPST2	100	2.1	30.80	63.39	1.6	35.14	55.08	1.4	29.30	40.93	60.4	34.8	56.8	32.9	26.1	33.3	23.0	56.8	–	–	–	–	B
TTYH2	100	3.2	3.21	10.13	2.2	1.98	4.24	1.5	1.90	2.86	5.5	12.4	11.8	11.5	2.2	1.1	0.3	11.8	–	–	–	–	B
UBE2E2	011	3.3	0.27	0.88	13.1	0.17	2.19	25.3	0.36	9.01	3.9	1.2	4.7	4.9	1.9	2.0	2.4	4.9	010	–	–	–	B
VEGFC	011	1.7	0.94	1.59	3.2	0.94	2.94	6.5	0.80	5.15	3.1	2.4	0.6	0.3	0.3	0	0	0.6	–	–	–	–	–
VPS37B	100	2.4	33.09	77.46	1.3	35.10	44.70	1.2	24.87	31.05	35.4	17.1	35.2	26.6	27.1	15.1	19.8	35.2	–	–	–	–	B

Supplemental Table 7.7 Continued: Directly Notch-activated genes identified in Pax5-deficient pro-B cells (91)

Gene	Code	2 h stimulation			4 h stimulation			8 h stimulation			In vivo expression (RPKM)								LMPPs		DN3		B _{DN3}
		fc	GFP	DL1	fc	GFP	DL1	fc	GFP	DL1	LMPP	ALP	DN1	DN2a	DN2b	DN3a	DN3b	Max	↑	↓	↑	↓	
ZC3H12D	011	1.3	15.48	20.42	2.3	9.40	21.27	2.5	12.37	31.17	2.2	1.3	4.8	0.7	0.7	1.7	0.3	4.8	010	–	–	–	–

Supplemental Table 7.7: List of 91 directly Notch-activated target genes identified by RNA-seq of *in vitro* Notch-stimulated (2 h, 4 h and/or 8 h) Pax5-deficient pro-B cells . Only the genes with a Rbpj-Bio binding site within 50 kb of the gene-body were included (determined by Bio-ChIP-seq in *Pax5*^{Δ/Δ} pro-B cells and/or DN3a cells.). The 'Code' column shows in which experiment (from left to right in the table) a gene was found to be activated – '0' = no; '1' = yes. The differential expression between non-stimulated (GFP) and Notch-stimulated (DL1) is depicted as moderated fold-change (fc). The absolute expression of each gene in the given cell type is shown as RPKM-normalised expression value. This includes the stimulation experiments as well as the expression profiling throughout early lymphocyte development (LMPP, ALP, DN1/ETP, DN2a, DN2b, DN3a and DN3b stages). The maximum (Max) intrathymic (DN1 – DN3b) expression level is also shown. If a gene was activated (↑) or repressed (↓) upon Notch-stimulation of LMPPs and/or Notch stimulation or deletion in DN3a cells is shown together with the 'Experiment Code' of the according cell type (see table of according cell type). The 'B_{DN3}' column indicates whether the gene was bound (B) by ICN1-Bio and/or Rbpj-Bio (determined by Bio-ChIP-seq in DN3a cells).

Supplemental Table 7.8: Unbound Notch-activated genes identified in Pax5-deficient pro-B cells (73)

Gene	Code	2 h stimulation			4 h stimulation			8 h stimulation			In vivo expression (RPKM)								LMPPs		DN3		B _{DN3}
		fc	GFP	DL1	fc	GFP	DL1	fc	GFP	DL1	LMPP	ALP	DN1	DN2a	DN2b	DN3a	DN3b	Max	↑	↓	↑	↓	
1300014106RIK	101	4.0	0.96	3.82	2.3	1.68	3.78	3.3	0.96	3.16	0	0.1	0.1	0	0	0	0	0.1	–	–	–	–	–
2810459M11RIK	100	11.9	0.08	0.97	7.1	0.04	0.25	4.5	0.02	0.09	0	0	0.1	0	0	0	0.2	0.2	–	–	–	–	–
AC016791.1	001	1.5	0.22	0.34	3.3	0.15	0.49	6.5	0.20	1.30	0.5	0	0.9	1.5	1.1	1.5	0.2	1.5	–	–	–	–	–
ADRBK2	001	1.4	0.78	1.05	1.7	0.75	1.26	2.5	0.83	2.03	9.0	4.9	7.5	4.1	3.0	2.7	0.5	7.5	–	–	–	–	–
AL928857.1	001	1.2	8.62	10.38	1.6	7.60	12.07	2.5	4.72	11.62	16.6	14.6	11.8	13.5	7.8	5.3	1.0	13.5	–	–	–	–	–
B3GALT5	100	2.2	3.54	7.59	1.7	3.02	4.98	1.2	2.44	2.90	0.3	1.0	1.8	1.4	0.3	3.0	0.8	3.0	–	–	–	–	–
B3GNT8	011	2.3	1.98	4.54	3.5	2.17	7.58	3.0	2.55	7.65	11.3	8.6	10.6	2.8	3.0	3.0	0.6	10.6	–	–	–	–	–
BATF3	001	0.9	0.44	0.37	2.2	0.53	1.15	–	0.00	0.79	0.5	0.2	0.7	0	0	0	0	0.7	–	–	–	–	–
CASZ1	001	0.8	1.29	0.99	1.3	0.95	1.21	2.4	0.59	1.39	4.0	1.7	1.7	0.9	0.5	0.6	0.1	1.7	–	–	–	–	–
CCL6	001	0.9	1.00	0.91	1.2	0.59	0.73	6.9	0.24	1.69	1.1	2.1	0.3	0.3	0.2	0.2	0.1	0.3	–	–	–	–	–
CD300E	001	5.1	0.05	0.26	2.7	0.10	0.26	5.3	0.20	1.05	0	0.1	0	0	0	0	0	0	–	–	–	–	–
CD33	001	1.3	22.11	28.61	2.0	15.47	31.19	2.1	19.89	41.32	18.0	26.0	32.0	6.8	0.7	0.1	0	32.0	010	–	–	–	–
CHIT1	001	1.1	5.50	6.00	1.6	4.09	6.33	10.3	2.51	25.88	0.1	0.7	2.4	2.2	0.3	0	0	2.4	–	–	–	–	–
CNN2	001	1.1	34.37	37.23	1.3	40.70	53.55	2.4	33.12	77.89	149.5	141.4	185.5	154.4	122.7	65.6	51.0	185.5	–	–	–	–	–
COL17A1	011	1.0	0.78	0.78	2.7	0.56	1.51	5.2	0.54	2.82	0	0.1	0.2	0	0	0	0	0.2	–	–	–	–	–
CSF1	100	3.6	1.33	4.70	1.6	2.73	4.42	0.6	1.09	0.69	3.0	1.2	0.6	0.1	0	0	0	0.6	–	–	–	–	–
CSPG4	001	0	0.02	0.00	26.4	0.00	0.09	–	0.00	0.32	0	0	0	0	0	0	0	0	–	–	–	–	–
CTGF	001	1.7	0.23	0.38	5.8	0.10	0.55	38.6	0.01	0.53	0.1	2.2	0.2	0	0	0	0	0.2	–	–	–	–	–
DDN	111	2.7	0.79	2.13	2.9	0.79	2.30	5.7	0.61	3.48	0.1	0.1	0.1	0.1	0.1	0.6	0.7	0.7	010	–	–	–	–
DSE	001	1.2	0.96	1.12	1.5	1.32	1.92	2.5	1.27	3.17	0.5	1.2	4.1	0.6	0.3	0	0.1	4.1	–	–	–	–	–
EDNRB	100	3.2	0.51	1.60	2.6	0.24	0.61	2.3	0.16	0.37	0	0.1	0	0	0	0	0	0	–	–	–	–	–
EIF2AK3	100	2.5	17.47	43.40	1.2	21.53	25.23	0.8	19.51	16.28	5.2	7.2	4.9	4.7	4.7	10.8	9.9	10.8	–	–	–	–	–
FCRL1	001	1.1	8.70	9.04	1.4	5.18	7.04	2.3	5.71	12.87	5.2	9.1	6.5	0.9	0.2	0	0	6.5	010	–	–	–	–
GAL	011	3.5	0.25	0.86	6.2	0.52	3.16	14.5	0.67	9.75	0	0.1	0.3	0.2	0.2	0.3	0.4	0.4	–	–	–	–	–
GGT5	001	1.3	1.09	1.44	2.0	1.13	2.22	2.5	1.11	2.76	13.5	6.2	18.8	13.0	3.4	1.4	0.2	18.8	011	–	–	–	–
GM2A	001	1.2	221.49	251.75	1.5	196.32	291.16	2.1	240.42	512.31	254.1	250.0	451.8	267.7	209.2	88.5	27.8	451.8	110	–	–	–	–
GPR114	001	1.3	20.82	26.66	1.6	23.13	36.09	2.1	19.51	40.37	0.1	0.5	1.9	4.5	1.3	0.1	0.1	4.5	011	–	–	–	–
GPR160	001	1.2	2.21	2.61	1.4	1.44	1.99	2.8	1.06	2.93	6.9	7.0	5.8	7.2	5.1	2.0	0.2	7.2	–	–	–	–	–
GPR35	001	1.4	0.71	0.95	1.0	1.08	1.10	3.2	0.78	2.50	0.2	0.6	0.2	0	0	0	0	0.2	–	–	–	–	–
GSN	001	1.0	38.06	38.87	1.2	36.95	43.58	2.8	33.08	93.21	21.9	54.5	17.7	13.9	8.2	2.2	2.5	17.7	–	–	–	–	–
GUCY1A3	001	0.9	1.50	1.26	2.2	1.36	2.95	3.0	0.76	2.26	4.3	1.1	2.7	0.8	0.7	0.2	0	2.7	–	–	–	–	–
GZMA	011	1.2	2.33	2.79	4.1	1.22	4.93	4.3	1.90	8.07	0.1	0.1	0.8	10.5	60.6	68.5	257.9	257.9	001	–	–	–	–
HBB-B1	001	1.3	0.52	0.65	3.7	0.31	1.14	7.1	0.71	5.06	36.2	1077.6	92.4	673.2	29.4	0.6	2.5	673.2	001	–	–	–	–
HBEGF	111	4.5	0.73	3.23	5.7	0.56	3.18	7.5	0.24	1.78	2.6	1.0	1.2	1.5	0.5	0.3	0.2	1.5	010	–	–	–	–
HIC1	111	3.8	0.31	1.14	5.9	0.50	2.88	26.3	0.17	4.46	0.2	0.2	0.2	0.1	0.2	0.2	0.1	0.2	111	–	–	–	–
ID2	011	1.7	15.43	26.59	2.4	12.63	29.59	2.8	10.42	29.18	4.6	9.8	13.8	5.8	5.7	12.5	2.7	13.8	010	–	–	–	–
KIT	001	1.5	18.29	27.35	1.7	15.68	26.66	2.0	15.61	31.22	133.2	36.2	124.3	110.3	37.1	13.4	5.6	124.3	001	–	–	–	–
LAIR1	001	0.7	0.68	0.49	1.0	0.44	0.43	4.3	0.71	3.03	4.0	2.2	2.6	6.5	5.1	3.6	1.9	6.5	010	–	–	–	–
LRP4	100	2.6	0.30	0.79	1.5	0.27	0.41	1.2	0.18	0.20	0.4	0.3	0.2	0.1	0.1	0.1	0	0.2	–	–	–	–	–
LRRC25	011	1.6	4.15	6.68	2.4	4.10	9.85	3.2	4.37	13.94	5.8	4.3	9.8	4.9	1.4	0.1	0	9.8	010	–	–	–	–
MAFB	111	33.6	0.02	0.52	71.0	0.03	2.30	–	0.00	5.77	1.3	2.2	0.1	0.1	0.1	0.8	0.1	0.8	010	–	–	–	–
MARCH4	001	0.9	0.24	0.21	1.8	0.34	0.59	13.2	0.06	0.75	0	0	0	0	0	0	0	0	–	–	–	–	–
MS4A6C	001	0.9	0.19	0.18	1.7	0.10	0.16	10.8	0.07	0.76	6.4	13.2	3.0	1.6	3.0	1.3	0.2	3.0	–	–	–	–	–
MXD1	100	2.8	8.11	22.65	1.4	6.12	8.53	1.1	5.57	5.93	7.8	11.4	8.2	4.7	4.6	6.2	2.5	8.2	–	–	–	–	–
MYADM	001	1.4	18.83	25.35	1.8	16.99	29.45	2.7	18.20	48.55	18.5	6.9	16.1	2.8	0.3	0	0	16.1	–	–	–	–	–

Table 7.8 Continued: Unbound Notch-activated genes identified in Pax5-deficient pro-B cells (73)

Gene	Code	2 h stimulation			4 h stimulation			8 h stimulation			In vivo expression (RPKM)								LMPPs		DN3		B _{DN3}
		fc	GFP	DL1	fc	GFP	DL1	fc	GFP	DL1	LMPP	ALP	DN1	DN2a	DN2b	DN3a	DN3b	Max	↑	↓	↑	↓	
NFKBID	001	1.4	8.44	11.91	1.7	10.52	18.04	2.3	8.95	20.28	15.6	3.7	7.9	5.7	7.4	7.4	6.0	7.9	010	–	–	–	–
NKG7	001	1.2	7.04	8.34	1.6	6.11	9.66	2.4	6.03	14.30	159.5	71.8	306.6	321.8	332.0	61.6	6.1	332.0	010	–	–	–	–
NM001109914	100	3.7	0.35	1.27	1.9	0.14	0.27	0.8	0.08	0.06	0.1	0.2	0.1	0.1	0.3	0.2	0.3	0.3	–	–	–	–	
NOD2	100	2.9	1.44	4.09	1.4	1.96	2.71	1.2	1.57	1.96	0.9	0.2	0.8	0.5	0.3	0.3	0.1	0.8	–	–	–	–	
NQO1	001	1.7	0.27	0.45	2.8	0.28	0.77	9.9	0.07	0.71	3.1	1.7	1.1	3.0	1.5	0.4	0.1	3.0	–	–	–	–	
NUAK1	010	2.1	6.73	13.65	2.5	2.55	6.18	1.4	1.16	1.56	0.4	0.4	0.4	0.2	0.1	0.1	0	0.4	010	–	–	–	–
OMP	001	1.8	0.91	1.62	2.9	0.63	1.81	5.9	0.28	1.66	0.3	0.6	0.8	0.7	0.5	2.6	0.5	2.6	–	–	001	–	–
PGF	111	3.6	2.33	8.28	5.5	1.84	9.98	10.5	0.62	6.49	0.2	0	0.1	0.1	0.1	1.4	0	1.4	–	–	–	–	
PLK2	100	2.3	2.07	4.75	1.0	2.26	2.30	1.6	2.63	4.32	0.2	0.3	0.1	0	0.1	0.1	0.1	0.1	–	–	–	–	
PRKCC	001	1.1	13.44	15.27	1.3	14.07	18.72	2.1	12.37	26.43	0	0	0	0	0	0	0	0	–	–	–	–	
RAPGEF2	001	1.1	5.28	5.96	1.6	4.67	7.22	2.5	4.46	11.31	6.7	9.2	23.6	23.7	16.2	12.3	9.5	23.7	–	–	–	–	
RBM24	111	3.4	1.75	5.90	6.2	1.11	6.77	4.9	1.71	8.41	0	0	0	0	0	0.2	0	0.2	–	–	–	–	
RHBG	001	1.0	0.08	0.08	1.3	0.49	0.64	13.3	0.05	0.71	0.1	0	0	0	0	0	0	0	–	–	–	–	
RHOD	100	9.5	0.11	1.02	1.1	0.37	0.39	0.9	0.27	0.24	3.2	0.5	6.0	3.0	0.9	0.1	0.1	6.0	–	–	–	–	
RSAD2	001	1.5	0.37	0.54	2.0	0.26	0.51	3.0	0.51	1.52	4.3	6.6	2.7	2.4	1.6	1.7	0.3	2.7	–	–	–	–	
RTN4RL1	001	1.2	1.41	1.64	2.0	1.64	3.23	2.6	1.47	3.80	1.1	15.8	5.8	11.1	8.6	5.3	4.0	11.1	–	–	–	–	
SESN3	001	1.5	34.43	52.19	1.9	30.21	57.42	2.1	36.19	77.75	13.1	10.8	14.2	6.2	6.8	14.5	7.2	14.5	–	–	–	–	
SIGLEC5	001	1.1	1.06	1.12	1.0	1.12	1.07	2.7	1.27	3.47	2.2	1.6	14.8	13.0	2.0	0.1	0	14.8	–	–	–	–	
SLC15A3	001	1.7	8.10	13.65	1.5	5.91	8.61	2.3	4.99	11.60	1.2	4.5	1.8	0.2	0.1	0.2	0.2	1.8	010	–	–	–	–
SLC26A9	100	12.3	0.04	0.51	11.3	0.02	0.17	0.3	0.03	0.01	0	0.1	0	0	0	0	0	0	–	–	–	–	
SLC44A2	001	1.0	57.10	56.88	1.6	42.03	66.78	2.2	44.98	100.67	70.2	104.3	89.3	78.9	106.6	106.5	57.4	106.6	–	–	–	–	
SPON1	011	2.6	0.23	0.58	3.8	0.17	0.61	4.9	0.07	0.36	0.1	0.1	0.1	0.1	0.3	0.3	0.2	0.3	–	–	–	–	
SSTR2	111	6.5	0.95	6.10	7.6	1.85	13.89	5.4	3.09	16.61	29.0	38.9	70.0	76.5	52.4	30.3	4.4	76.5	–	–	–	–	
SYTL3	011	1.8	2.43	4.30	2.5	1.90	4.72	2.7	1.52	4.12	0	0.1	2.8	9.1	16.6	31.1	7.7	31.1	–	–	–	–	
TGTP1	001	1.2	1.42	1.72	2.0	1.18	2.33	2.7	1.02	2.74	2.9	0.5	2.6	2.4	2.7	4.3	0.4	4.3	–	–	–	–	
TLR1	001	1.0	0.28	0.29	1.7	0.27	0.44	9.3	0.13	1.17	0.1	0.1	0	0	0	0	0	0	–	–	–	–	
UGCG	001	1.5	13.22	19.95	2.0	11.27	21.89	2.1	15.54	32.76	10.9	9.3	28.3	28.5	16.5	24.8	15.5	28.5	–	–	–	–	
VDR	100	3.9	0.55	2.13	0.9	0.47	0.43	0.8	0.10	0.08	5.2	1.5	2.0	1.0	0.4	0.3	0.7	2.0	–	–	–	–	

Supplemental Table 7.8: List of 73 Notch-activated target genes identified by RNA-seq of *in vitro* Notch-stimulated (2 h, 4 h and/or 8 h) Pax5-deficient pro-B cells. Only the genes without a Rbpj-Bio binding site within 50 kb of the gene-body were included (determined by Bio-ChIP-seq in Pax5^{Δ/Δ} pro-B cells and/or DN3a cells.). The 'Code' column shows in which experiment (from left to right in the table) a gene was found to be activated – '0' = no; '1' = yes. The differential expression between non-stimulated (GFP) and Notch-stimulated (DL1) is depicted as moderated fold-change (fc). The absolute expression of each gene in the given cell type is shown as RPKM-normalised expression value. This includes the stimulation experiments as well as the expression profiling throughout early lymphocyte development (LMPP, ALP, DN1/ETP, DN2a, DN2b, DN3a and DN3b stages). The maximum (Max) intrathymic (DN1 – DN3b) expression level is also shown. If a gene was activated (↑) or repressed (↓) upon Notch-stimulation of LMPPs and/or Notch stimulation or deletion in DN3a cells is shown together with the 'Experiment Code' of the according cell type (see table of according cell type). The 'B_{DN3}' column indicates whether the gene was bound (B) by ICN1-Bio and/or Rbpj-Bio (determined by Bio-ChIP-seq in DN3a cells).

Supplemental Table 7.9: Notch-repressed genes identified in Pax5-deficient pro-B cells (29)

Gene	B _{DN3,B}	Code	2 h stimulation			4 h stimulation			8 h stimulation			In vivo expression (RPKM)								LMPP		DN3		B _{DN3}
			fc ⁻¹	GFP	DL1	fc ⁻¹	GFP	DL1	fc ⁻¹	GFP	DL1	LMPP	ALP	DN1	DN2a	DN2b	DN3a	DN3b	Max	↑	↓	↑	↓	
A430110N23RIK	B	001	1.1	21.64	19.45	1.2	27.58	21.97	2.6	21.98	8.43	0	0	0	0	0	0	0	0	–	–	–	–	–
AA467197	B	001	1.3	58.42	46.14	1.6	55.70	35.09	2.4	35.49	14.79	2.2	2.4	0.1	0.3	1.7	6.0	1.0	6.0	–	–	–	–	–
ANTXR1	B	001	0.9	0.77	0.84	0.7	0.50	0.68	3.4	1.09	0.32	0.1	0	0.2	0	0	0	0	0.2	–	–	–	–	–
BHLHA15	B	001	1.3	20.40	15.40	1.6	25.29	15.78	2.9	17.92	6.09	0.7	1.1	0.4	0.4	0.6	0.7	0.1	0.7	–	001	–	–	–
CABLES1	B	001	0.5	11.09	21.14	1.4	7.24	5.14	2.9	3.64	1.25	7.0	1.3	4.7	1.4	0.6	0.4	0.3	4.7	–	–	–	–	B
CACNA1B	B	001	1.0	7.62	7.73	1.5	11.80	7.88	2.2	6.51	2.99	0	0	0	0	0	0	0	0	–	–	–	–	–
CDH13	–	001	1.1	1.79	1.58	1.1	1.61	1.49	3.6	2.48	0.69	0	0	0	0	0	0	0	0	–	–	–	–	–
CPLX2	–	001	1.3	7.00	5.20	1.7	12.02	6.79	2.4	5.44	2.30	1.1	0.3	0	0	0	0	0	0	–	–	–	–	–
DLK1	–	001	0.9	11.61	12.57	1.5	16.27	11.02	3.7	11.41	3.06	1.0	0.9	0.3	0.2	0.2	0.6	0.8	0.8	–	001	–	–	–
DPYSL5	–	001	0.9	1.98	2.25	1.4	3.80	2.77	2.9	1.74	0.60	0	0	0	0	0	0	0.1	0.1	–	–	–	–	–
ELOVL7	B	100	2.2	3.94	1.73	1.0	2.61	2.64	0.9	2.21	2.53	0.4	0.1	0.2	0.1	0.1	0.1	0.1	0.2	–	–	–	–	–
EMID2	B	001	1.1	4.73	4.31	1.2	5.39	4.34	2.4	6.15	2.60	0	0	0	0	0	0	0	0	–	–	–	–	–
EPHA4	–	001	3.2	0.17	0.05	4.4	0.28	0.06	13.0	0.85	0.07	0	0.1	0	0	0	0	0	0	–	–	–	–	–
GJB3	–	001	1.3	11.46	8.94	1.6	12.84	7.89	2.7	13.17	4.80	7.2	1.8	1.3	0.7	0.1	0	0	1.3	–	–	–	–	–
ID3	–	001	1.8	10.20	5.49	2.0	13.53	6.82	3.0	16.43	5.56	0.6	2.2	5.3	1.8	17.6	21.6	82.9	82.9	–	–	–	–	–
IL12A	–	001	1.5	10.00	6.71	2.1	9.76	4.64	2.7	15.77	5.93	44.4	71.8	12.7	2.1	2.2	0.2	0.1	12.7	–	111	–	–	–
IL2RB	–	001	0.6	3.56	5.78	1.9	5.04	2.61	2.8	2.64	0.96	0.2	4.7	11.0	0.1	0.1	0.3	0.8	11.0	–	–	–	–	–
KCNG1	–	001	1.1	33.47	29.24	1.5	48.54	32.75	2.5	46.82	18.92	14.5	100.2	7.0	1.5	0.1	0	0.1	7.0	–	010	–	–	–
LTA	–	001	1.7	150.97	89.59	1.5	98.11	63.90	2.2	21.63	9.96	0.2	1.0	0.9	0.4	0.7	0.4	0.2	0.9	–	010	–	–	–
MEGF10	–	001	1.4	0.72	0.52	1.7	0.61	0.35	3.4	0.96	0.28	0	0	0	0	0	0	0	0	–	–	–	–	–
MYC	B	100	2.1	249.38	116.54	1.6	210.83	126.69	1.8	159.62	86.67	278.7	169.7	245.8	132.9	109.4	276.6	344.5	344.5	–	–	110	–	B
MYCN	B	110	2.3	13.40	5.72	2.4	16.87	7.03	1.8	8.01	4.54	39.6	7.8	103.3	83.3	17.9	16.3	0.7	103.3	110	–	110	–	B
NM001167937	–	001	0.9	0.58	0.66	1.2	1.16	0.97	8.1	1.96	0.24	0	0	0	0	0	0.1	0.1	0.1	–	–	–	–	–
PXDN	–	001	1.0	5.45	5.63	1.4	6.24	4.30	2.9	3.72	1.28	0	0.7	0	0	0	0	0	0	–	–	–	–	–
RAMP3	–	001	0.7	32.10	42.54	1.3	35.90	27.57	2.3	20.72	9.12	0	0.2	0	0	0	0	0.1	0.1	–	–	–	–	–
SLC5A9	B	001	1.0	15.95	15.69	1.2	21.47	17.51	2.1	24.69	11.55	0	0	0	0	0.1	36.2	4.7	36.2	–	–	001	–	B
SPP1	–	001	0.9	21.54	22.55	1.7	15.03	8.84	3.0	21.58	7.19	0.7	0.5	0.1	0	0	0	0.1	0.1	–	011	–	–	–
SYTL2	–	001	0.9	4.96	5.24	1.9	2.16	1.10	4.1	1.90	0.46	0	0.1	0.1	1.7	5.5	2.3	0.2	5.5	–	–	–	–	–
TMPRSS6	B	001	0.6	0.42	0.69	1.4	0.82	0.59	11.3	0.44	0.04	0	0.2	0	0	0	0	0	0	–	–	–	–	B

Supplemental Table 7.9: List of 29 Notch-repressed genes identified by RNA-seq of *in vitro* Notch-stimulated (2 h, 4 h and/or 8 h) Pax5-deficient pro-B cells. The 'B_{DN3,B}' column indicates whether the gene was bound (B) by Rbpj-Bio within 50 kb of the gene-body (determined by Bio-ChIP-seq in Pax5^{Δ/Δ} pro-B cells and/or DN3a cells). The 'Code' column shows in which experiment (from left to right in the table) a gene was found to be repressed – '0' = no; '1' = yes. The differential expression between non-stimulated (GFP) and Notch-stimulated (DL1) is depicted as moderated fold-change⁻¹ (fc⁻¹). The absolute expression of each gene in the given cell type is shown as RPKM-normalised expression value. This includes the stimulation experiments as well as the expression profiling throughout early lymphocyte development (LMPP, ALP, DN1/ETP, DN2a, DN2b, DN3a and DN3b stages). The maximum (Max) intrathymic (DN1 – DN3b) expression level is also shown. If a gene was activated (↑) or repressed (↓) upon Notch-stimulation of LMPPs and/or Notch stimulation or deletion in DN3a cells is shown together with the 'Experiment Code' of the according cell type (see table of according cell type). The 'B_{DN3}' column indicates whether the gene was bound (B) by ICN1-Bio and/or Rbpj-Bio (determined by Bio-ChIP-seq in DN3a cells).

Supplemental Table 7.10: ChIP-seq, DHS mapping and CAGE tracks generated in this study

Experiment	MBU ID	#	Cell type	Treatment	Genotype
Input	9908	1	DN3a	wTHY	<i>Notch1</i> ^{Bio/Bio} <i>Rosa26</i> ^{BirA/BirA} <i>Rag2</i> ^{-/-}
H3K9ac ChIP-seq	8502	1	DN3a	wTHY	<i>Rag2</i> ^{-/-}
	9871	2			
H3K4me2 ChIP-seq	8501	1			
	9872	2			
H3K4me3 ChIP-seq	8338	1			
	8488	2			
H3K27me3 ChIP-seq	8337	1			
	8489	2			
ICN1-Bio ChIP-seq	10618	1	DN3a	wTHY	<i>Notch1</i> ^{Bio/Bio} <i>Rosa26</i> ^{BirA/BirA} <i>Rag2</i> ^{-/-}
	14264	2			
	14265	3			
	8861	1	pro-B	<i>in vitro</i> OP9-DL1	<i>Notch1</i> ^{Bio/Bio} <i>Rosa26</i> ^{BirA/BirA} <i>Pax5</i> ^{Δ/Δ} <i>Vav-Bcl2</i>
Rbpj-Bio ChIP-seq	8873	1	DN3a	wTHY	<i>Rbpj</i> ^{Bio/Bio} <i>Rosa26</i> ^{BirA/BirA} <i>Rag2</i> ^{-/-}
	14266	2			
	14267	3			
	15078	1	DN3b/DN4	CD3 stimulated wTHY	<i>Rbpj</i> ^{Bio/Bio} <i>Rosa26</i> ^{BirA/BirA} <i>Rag2</i> ^{-/-}
	10489	1	pro-B	<i>in vitro</i> OP9-GFP	<i>Rbpj</i> ^{Bio/Bio} <i>Rosa26</i> ^{BirA/BirA} <i>Pax5</i> ^{Δ/Δ}
	10490	1	pro-B	<i>in vitro</i> OP9-DL1	<i>Rbpj</i> ^{Bio/Bio} <i>Rosa26</i> ^{BirA/BirA} <i>Pax5</i> ^{Δ/Δ}
DHS mapping	8846	1	DN3a	wTHY	<i>Rag2</i> ^{-/-}
	14271	2			
TSS mapping (CAGE)	14645	1	DN3a	wTHY	<i>Rag2</i> ^{-/-}

wTHY = material prepared from unsorted whole thymocyte suspensions; '# ' = replicate number

Supplemental Table 7.11: RNA-seq tracks of *in vitro* Notch-stimulated cells generated in this study

Cell type	Genotype	Treatment	MBU ID	#
LMPP	wild-type	4 h OP9-GFP	14962	1
			14964	2
		4 h OP9-DL1	14963	1
			14965	2
		12 h OP9-GFP	14200	1
			14204	2
		12 h OP9-DL1	14201	1
			14205	2
		4 d OP9-GFP	14202	1
			14966	2
		4 d OP9-DL1	14203	1
			14977	2
pro-B	<i>Pax5</i> ^{-/-} <i>Vav-Bcl2</i>	2 h OP9-GFP	14083	1
			14084	2
		2 h OP9-DL1	14085	1
			14086	2
		4 h OP9-GFP	14087	1
			14088	2
		4 h OP9-DL1	14089	1
			14090	2
		8 h OP9-GFP	14091	1
			14092	2
DN3a	<i>Vav-Bcl2</i>	2 h OP9-GFP	14320	1
			14322	2
		2 h OP9-DL1	14321	1
			14323	2
		8 h OP9-GFP	14196	1
			14198	2
		8 h OP9-DL1	14197	1
			14199	2
DN3b/DN4	<i>Vav-Bcl2</i>	2 h OP9-GFP	14246	1
			14248	2
		2 h OP9-DL1	14247	1
			14249	2

' #' = replicate number

Supplemental Table 7.12: *Ex vivo* RNA-seq tracks generated in this study

Experiment	Cell type	Genotype	MBU ID	#
<i>Notch1</i> deletion (<i>Rag2</i> ^{+/+})	DN3a	<i>Notch1</i> ^{fl/fl}	12521	1
			12522	2
	DN3a CD25 ^{hi}	<i>Notch1</i> ^{fl/fl} <i>Lck</i> -Cre	12519	2
	DN3a CD25 ^{int}	<i>Notch1</i> ^{fl/fl} <i>Lck</i> -Cre	12520	2
<i>Rbpj</i> deletion (<i>Rag2</i> ^{+/+})	DN3a	<i>Rbpj</i> ^{fl/fl}	13326	1
			13327	1
		<i>Rbpj</i> ^{fl/fl} <i>Lck</i> -Cre	13329	2
	DN3b	<i>Rbpj</i> ^{fl/fl}	13324	1
			13330	2
		<i>Rbpj</i> ^{fl/fl} <i>Lck</i> -Cre	13325	1
			13331	2
<i>Notch1</i> deletion (<i>Rag2</i> ^{-/-})	DN3a	<i>Notch1</i> ^{fl/fl} <i>Rag2</i> ^{-/-}	15069	1
			15072	2
	DN3a CD25 ^{hi}	<i>Notch1</i> ^{fl/fl} <i>Rag2</i> ^{-/-} <i>Lck</i> -Cre	15070	1
			15073	2
	DN3a CD25 ^{int}	<i>Notch1</i> ^{fl/fl} <i>Rag2</i> ^{-/-} <i>Lck</i> -Cre	15071	1
			15074	2
<i>In vivo</i> expression profiling	LMPP	wild-type	14587	1
			14592	2
	DN1/ETP	wild-type	14588	1
			14593	2
			14591	3
	DN2a	wild-type	14589	1
			14594	2
			14596	3
	DN2b	wild-type	14590	1
			14595	2
			14597	3
CAGE reference	DN3a wTHY	<i>Rag2</i> ^{-/-}	8277	1

'#' = replicate number

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11/2006 – 08/2007	Part time research assistant: "Microscopical classification of murine lung tissue", group of Prof. Dr. Heinz Fehrenbach, faculty of medicine, department of pneumology, Philipps-Universität Marburg, Germany
08/2007 – 11/2007	Internship: "Adenylate cyclase mutagenesis screen in <i>Candida albicans</i> ", group of Prof. Dr. Fritz Mühlischlegel, department of biosciences, medical microbiology, University of Kent in Canterbury, UK
05/2009 – 08/2009	Research assistant: "Promoter analysis of the <i>Saccharomyces cerevisiae</i> cell surface flocculin gene FLO11", group of Prof. Dr. Hans-Ulrich Mösch, department of genetics, Philipps-Universität Marburg, Germany

Courses & Conferences

April 2014	ThymOz Conference; Heron Island, Australia (Talk)
October 2013	Annual IMP/IMBA Recess; Vienna, Austria (Poster presentation)
October 2012	Birthday Symposium - "Immunity, Epigenetics & Cancer"; Vienna, Austria
September 2012	Idibell Cancer Conferences (ICC) - "Epigenetics in Lymphocyte Biology & Disease"; Barcelona, Spain (Poster presentation)
April 2012	Cold Spring Harbor (CSH) meeting - "Gene Expression & Signalling in the Immune System"; Cold Spring Harbor, USA (Poster presentation)
October 2011	Annual IMP/IMBA Recess; Vienna, Austria (Poster presentation)
September 2011	Federation of European Biochemical Societies (FEBS) 16 th International Summer School - "Immune System: Genes, Receptors & Regulation"; Hvar, Croatia, (Poster presentation)
March 2011	European Transcriptome, Regulome & Cellular Commitment Consortium (EuTRACC) - "Transcription Networks Symposium"; Berlin, Germany (Poster presentation)
November 2010	Vienna Biocenter PhD Symposium - "Origin of Life"; Vienna Austria (Organizer)
May 2010	IMP Conference - "Epigenetic Regulation in Cell Fate & Disease"; Vienna, Austria

Publications

Kollmann K, Heller G, Schneckenleithner C, Warsch W, Scheicher R, Ott RG, **Schäfer M**, Fajmann S, Schleder M, Schiefer AI, Reichart U, Mayerhofer M, Hoeller C, Zöchbauer-Müller S, Kerjaschki D, Bock C, Kenner L, Hoefler G, Freissmuth M, Green AR, Moriggl R, Busslinger M, Malumbres M, Sexl V. *A kinase-independent function of CDK6 links the cell cycle to tumor angiogenesis*. Cancer Cell. 2013 Aug 12;24(2):167-81.