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„Control of B cell development and function by the SAGA complex“

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Zusammenfassung

Der SAGA-Komplex, ein ubiquitär exprimierter Transkriptions-Co-Aktivator mit mehreren Untereinheiten, reguliert die Genexpression durch Acetylierung von Histonen. Trotz umfangreicher Forschung zu SAGA-Komplex in Hefe sind seine direkt regulierten Gene in Säugetierzellen weitgehend unbekannt. Da epigenetische Histonmodifikatoren eine Schlüsselrolle bei Entscheidungen über das Zellschicksal in der Hämatopoese spielen, untersuchten wir die Rolle des SAGA-Komplexes in der B-Zell-Entwicklung.
Wir identifizierten mehrere Komponenten des Histon-Acetyltransferase (HAT)-Moduls von SAGA in einem CRISPR/Cas9-basierten sgRNA-Screening unter Verwendung eines *in vitro* Plasmablasten-Zelldifferenzierungssystems, um neue Plasmablasten-Regulatoren zu identifizieren. Der Verlust der SAGA-HAT-Komponente TADA3 zeigte eine starke Auswirkung auf die terminale B-Zell-Differenzierung. Daher konzentrierten wir uns bei der weiteren Analyse auf TADA3. Da SAGA mit der allgemeinen Transkriptionsmaschinerie interagiert, untersuchten wir die Auswirkungen des Verlusts von *Tada3* in der B-Zell-Linie durch konditionale Mutagenese in Mäusen. Nach *Cd79a*-Cre-induzierter Deletion eines *loxP*-Stellen flankierten *Tada3*-Allels beobachteten wir ein Problem in der Entwicklung vom Pro-B-Zell zum Pre-B-Zell-Stadium. Die Deletion des *loxP*-Stellen flankierten *Tada3*-Allels in reifen B-Zellen durch *Cd23*-Cre beeinträchtigte jedoch nicht die Marginalzonen- oder die follikulären B-Zellen, sondern führte zu einem Verlust von Plasmablasten und Plasmazellen. Als diese Mäuse mit NP-KLH immunisiert wurden, beobachteten wir nicht nur den Verlust von Plasmablasten und Plasmazellen, sondern auch einen Mangel an Keimzentrum-B-Zellbildung. Dies deutet auf eine frühe Rolle des SAGA-Komplexes in der Immunantwort hin. *In-vitro*-Aktivierungsstudien bestätigten außerdem, dass TADA3-defiziente B-Zellen weder proliferieren noch Plasmablasten bilden können. Um die direkt regulierten Gene zu identifizieren, haben wir ein Auxin-induzierbares-Degron (AID)-markiertes *Tada3*-Allel generiert, das zu lebensfähigen homozygoten $Tada3^{AID/AID}$ Mäusen führte. In Zukunft wollen wir die unmittelbare Auswirkung der Auxin-induzierten Degradation des TADA3-AID-Proteins auf die Genexpression in aktivierten B-Zellen mithilfe der Analyse von neugebildeten Transkripten untersuchen. Dieses Mausmodell gibt Aufschluss über die Rolle des SAGA-Komplexes bei der Transkriptionsregulation in B-Zellen.

Abstract

The SAGA complex, a ubiquitously expressed transcriptional co-activator with multiple subunits, regulates gene expression by acetylating histones. Despite extensive research on SAGA in yeast, its direct target genes in mammalian cells are largely unknown. Since epigenetic histone modifiers play a key role in cell fate decisions in hematopoiesis, we investigated the role of the SAGA complex in B cell development. We identified several components of the histone-acetyltransferase (HAT) module of SAGA in a CRISPR/Cas9-based sgRNA screen using an *in vitro* plasmablast (PB) cell differentiation system to identify novel PB regulators. Loss of the SAGA-HAT component, TADA3, had a strong impact on terminal B cell differentiation. Therefore, we focused on *Tada3* for further analysis. Because SAGA interacts with the general transcription machinery, we studied the effect of *Tada3* loss in the B cell lineage using conditional mutagenesis in mice. Upon *Cd79a*-Cre driven deletion of a floxed *Tada3* allele, we observed a defect in the pro-B cell to pre-B cell transition. However, deletion of the floxed *Tada3* allele in mature B cells with *Cd23*-Cre did not affect marginal zone or follicular B cells but resulted in the loss of PBs and plasma cells (PCs). When these mice were immunized with NP-KLH, we did not only observe the loss of PBs and PCs but also a lack of germinal center (GC) B cell formation. This indicated an early role for the SAGA-HAT in the immune response. *In vitro* activation studies further confirmed that TADA3-deficient B cells could not proliferate or form PBs. To identify direct targets of the SAGA-HAT, we created an auxin-inducible degron (AID)-tagged *Tada3* allele, which resulted in viable *Tada3*$^{AID/AID}$ mice. We aim to study the immediate effect of auxin-induced TADA3-AID degradation on gene expression in activated B cells using nascent transcript analysis. This mouse model provides insights into the role of the SAGA complex in the transcriptional regulation of B cells and other cell fate decisions.

1 Introduction

1.1 The adaptive immune system and B cells

The immune system protects the host against invading pathogens, and vertebrates have established several mechanisms to fulfill this task. From a simplistic view, two lines of defense can be distinguished: innate immunity and adaptive immunity, with the latter being antigen-dependent and antigen-specific, which takes some time to build up and can establish immunological memory (Marshall et al. 2018). Neither line should be viewed as mutually exclusive, but rather as complementary mechanisms. Within the adaptive immune system, B cells are responsible for antibody-mediated immunity, and have been a subject of fascination since as early as 1884, when germinal centers were first observed (Flemming 1884). Unlike T cells, which depend on antigen-presenting cells (APCs), B cells can recognize antigens directly via antigen receptors. These receptors are immunoglobulins called B cell receptors (BCR) when located on the cell membrane, or they can be secreted as highly specific antibodies that are key to eliminating pathogens (Marshall et al. 2018). Each B cell has a unique BCR and the enormous diversity of the receptor repertoire is remarkable, allowing antibody production against almost any foreign surface (Cyster and Allen 2019). Efficient antibody production is the main task of B cells, and they must undergo a series of tightly controlled proliferation, differentiation, and selection processes to prevent harmful effects on the host.

1.1.1 Early B cell development

In mice, B cells are first generated from pluripotent hematopoietic stem cells (HSCs) in the fetal liver, and this process continues after birth in the bone marrow. HSCs have self-renewal capabilities and can give rise to all types of blood cells throughout life. The generation of blood cells is a stepwise differentiation process towards more lineage-restricted progenitor cells that can no longer self-renew. Each differentiation stage is defined by unique gene expression signatures that involve induction and suppression of lineage-relevant genes. HSCs differentiate into the common lymphoid progenitor (CLP) stage, which is the source of NK, B, and T cells (Nemeth and Bodine 2007). CLPs are a heterogeneous population that can be further divided into two subpopulations based on Ly6D-expression. The $Ly6D^-$ all-lymphoid

progenitors (ALP) give rise to T cells, B cells, NK cells, dendritic cells, and Ly6D$^+$ B-cell biased progenitors (BLP) that develop into B cells (Inlay et al. 2009). BLPs are restricted to the B cell lineage and are considered the earliest B cell progenitors. The subsequent expression of the B-cell-associated marker B220 defines the pre-pro-B cell stage that precedes CD19 cell surface expression in pro-B cells and full commitment to the B cell lineage (Hardy & Hayakawa, 1991; Li et al., 1996). The transition from the still uncommitted pre-pro-B cell stage to the B cell lineage-committed pro-B cell stage coincides with full expression of the B cell lineage commitment factor PAX5. Without PAX5, B cell development is abrogated at an uncommitted progenitor stage, and cells can differentiate into other lymphoid and myeloid lineages, highlighting the utmost importance of PAX5 for B cell generation (Busslinger 2004). The expression of PAX5 is preceded by the sequential activation of the transcription factors E2A and EBF1, which together with FOXO1, induce the expression of B cell genes in uncommitted pre-pro-B cells (Busslinger, 2004; Lin et al., 2010; Mansson et al., 2012). Co-expression of these transcription factors is a prerequisite for PAX5 expression. Once PAX5 expression is initiated, its expression can further strengthen B cell commitment and fulfill its role by repressing inappropriate B cell lineage genes and at the same time activating the expression of B cell-specific genes. The latter are involved in V-DJ recombination, pre-BCR signaling, and mature B-cell development (Busslinger 2004).

B cells possess a diverse antigen receptor repertoire that allows for recognition of almost any possible antigen. BCR diversity is achieved via a mechanism called V(D)J recombination, which involves recombination and assembly of antigen receptor segments to express covalently bound immunoglobulin heavy chains (IgH) and two light chains (IgL). The variable region at the N-terminus of the immunoglobulin is responsible for antigen specificity, whereas the C-terminal region is called the constant region (C_H) and defines antibody effector function (Tonegawa 1983). While the *IgH* locus contains variable (V), diversity (D), joining (J), and constant gene segments, the *IgL* locus lacks the D segments. To recombine these gene segments to a functional BCR, the lymphocyte-specific recombinases Recombination Activating Gene (RAG) 1 and RAG2 are required (Oettinger et al. 1990). These enzymes generate double-stranded breaks (DSB) that are repaired by non-homologous end-joining (NHEJ). First, D-J recombination of the *IgH* locus is initiated at the CLP stage, followed by V-DJ recombination in committed pro-B cells, resulting in an assembled *IgH* locus (Alt et al. 1984). The successfully assembled *IgH* locus is transcribed and expresses the *Igμ* heavy chain, which,

together with the surrogate light chains, forms the pre-BCR that can be expressed on the surface of large pre-B cells. The expression of pre-BCR is a checkpoint in B cell development that controls the transition from the pro-B to pre-B cell stage, followed by positive selection (Burrows et al. 2002). Positively selected cells undergo clonal proliferation to increase the number of cells that have a successfully recombined *IgH* gene (Herzog, Reth, and Jumaa 2009). Subsequently, they downregulate the pre-BCR and recombination of the *IgL* locus occurs. Once the *IgL* locus has successfully recombined, the entire IgM BCR can be expressed on the surface, and B cells reach the immature B cell stage. From this point on, only immature B cells with a non-autoreactive BCR should exit the bone marrow via the bloodstream to migrate to secondary lymphoid organs, which is controlled by tonic BCR signaling and additional survival signals (Herzog et al. 2009; Nemazee 2017).

1.1.2 Late B cell development

Immature B cells are the first type of B cells capable of antigen recognition. However, they do not proliferate and differentiate in response to antigens but home to the spleen to complete maturation. The maturation process is another checkpoint where B cells with a high-affinity BCR to self-antigens undergo apoptosis, or B cells that recognize self-antigens with lower affinity can undergo further receptor editing and then develop into mature B cells (Nemazee 2017). The developmental steps towards maturity comprise short-lived transitional T1 and T2 B cell stages, which are marked by the upregulation of IgD. While IgM and IgD contain the same antigen-binding domain, both BCR isotypes differ in their Fc domains and their expression patterns; IgM expression starts once heavy and light chains have recombined and persists until class switch recombination (CSR) takes place (Blum, Stevens, and DeFranco 1993; Chen and Cerutti 2010). IgD, on the other hand, is uniquely co-expressed with IgM on late transitional and mature naïve FO B cells (Pioli et al. 2014).

At the T1 B cell stage, BCR and Notch2 signaling determine whether cells become follicular (FO) B cells or marginal zone (MZ) B cells, both of which are mature B cell populations found in the spleen (Pillai and Cariappa 2009). Whereas FO B cells express a diverse BCR repertoire and circulate between lymphoid organs, MZ B cells reside in the marginal zone of the spleen. MZ B cells express high levels of IgM, CD21, and CD1d, and lower levels of IgD and CD23, and can respond to bacterial cell wall components and T cell-independent (TI) antigens, which

initiates differentiation into short-lived plasmablasts (PBs) that produce low-affinity antibodies. This is the first line of defense in B cell immunity directed at blood-borne antigens that are trapped in the spleen and is a way to bridge the temporal gap until high-affinity antibodies can be produced in a T-cell-dependent reaction (Cerutti, Cols, and Puga 2013).

In contrast, FO B cells express high levels of IgD and CD23 and lower levels of IgM, CD21, and no CD1d. Since they can recirculate, FO B cells make up the majority of B cells in the peripheral lymph nodes and in addition, make up the vast majority of mature B cells in the mouse (Cerutti et al. 2013; Hoek et al. 2010). Once naïve FO B cells encounter an antigen in secondary lymphoid organs and receive additional T-cell help, they become activated. This can, similar to MZ B cells, give rise to short-lived, low-affinity antibody producing PBs or, alternatively, initiate the germinal center (GC) reaction. GCs are transient structures in secondary lymphoid follicles that give rise to plasma cells (PCs) and memory B cells. Highly activated and proliferative GC B cells can undergo rounds of somatic hypermutation (SHM) of variable (V) gene segments of *IgH* and *IgL* gene loci to increase BCR affinity for the given antigen. The GC is a very dynamic entity that can be divided into a light zone (LZ) and a dark zone (DZ). GC B cells circulate between these two zones and are exposed to antigens in the LZ, where they also receive T cell help, while they extensively proliferate in the DZ. Successful affinity maturation results in the egress of high-affinity antibody-producing PCs and a smaller fraction of memory B cells, which can generate new high-affinity antibody-secreting PCs during a secondary response (Lebien and Tedder 2008; Victora and Nussenzweig 2022).

1.1.3 B cell activation

B-cell activation is an important step that initiates the transition from a naïve to an effector cell stage. Activation can either occur via the BCR or by recognition of pathogen-associated molecular patterns (PAMPs), such as bacterial lipopolysaccharide (LPS), by pathogen recognition receptors (PRRs), such as Toll-like receptors (TLRs). *Ex vivo* anti-IgM stimulation stimulates the BCR and activates the B cells whereas *ex vivo* stimulation with LPS engages TLR4 signaling and initiates PB differentiation. However, most complex antigens will not only engage the BCR, but also additional receptors, such as TLRs or complement receptors, underlining the connective nature of adaptive and innate immunity.

Upon antigen encounter by the BCR, the signalosome, an intracellular assembly of signaling molecules such as the SYK kinase and phosphoinositide 3-kinase (PI3K), will form, and the interplay of several signaling cascades leads to an amplification of the activation signal. Common downstream signaling pathways comprise mitogen-activated protein (MAP)-kinases and NF-κB, which result in expression changes of numerous genes and internalization of antigen. The antigen will be processed and loaded onto major histocompatibility complex class II (MHC-II) to be presented on the B-cell surface for T cells to help complete B-cell activation. This T cell-dependent (TD) activation of B cells applies primarily to FO B cells, which recognize complex antigens via their BCR and can develop into extrafollicular PBs or enter the GC reaction, whereas MZ B cells preferentially recognize antigens that are poor activators of T cells and develop into PBs independent of T cell help (Cerutti, Cols, and Puga 2012; Cyster and Allen 2019; Harwood and Batista 2010; Hoffman, Lakkis, and Chalasani 2016; Otipoby et al. 2015; Schweighoffer et al. 2017).

Regardless of cell type, the initial signaling of B cell activation rapidly induces immediate early genes (IEGs), also called primary response genes (PRGs), whose products initiate secondary waves of transcription. This triggers egress from the G_0 state, followed by proliferation and effector functions. IEGs include *c-Fos*, *c-Jun, Egr1, Egr2, Egr3,* and *Myc*, which are upregulated shortly after stimulation (Calderón et al. 2021; Fowler et al. 2015; Fowler, Sen, and Roy 2011; Li et al. 2012). While the expression of IEGs precedes that of secondary response genes (SRGs), some IEGs show delayed induction and have been termed “delayed primary response genes,” which share many similarities with IEGs but are, however, a different group of genes with distinct functions and genomic architecture (Bahrami and Drabløs 2016; Tullai et al. 2007). Interestingly, most IEGs and some delayed early genes are poised genes, meaning that they are expressed as mRNAs but not as proteins in mature B cells prior to activation, which adds to a state of readiness of the cell. This includes the early activation markers, CD69 and MYC (Fowler et al. 2011; Salerno et al. 2023). While the type of antigen defines the involved signaling cascades, the majority of differentially induced genes are shared between different modes of activation (Fowler et al. 2015). Many IEGs are involved in cellular differentiation, proliferation and lineage specificity and often encode for transcription factors but due to their rapid expression, their transcriptional regulation and differences in chromatin architecture compared to later expressed genes that require de novo protein synthesis are challenging questions to address (Fowler et al. 2011). The latter and far

more numerous group of genes that are expressed during the secondary wave of transcription are the SRGs. Unlike SRGs, IEG expression is transient, their protein products are usually unstable, and their promoters have more TATA-box motifs than other genes, suggesting high affinity for TATA-binding protein (TBP) and easy unwinding of DNA. Additionally, RNA polymerase II accumulates at these poised genes. The chromatin architecture at the promoters of IEGs is often bivalent, meaning that they carry active and repressive marks that keep them poised for rapid activation. It has also been shown that there is a dynamic turnover of histone acetylation marks by the action of histone acetyltransferases and deacetylases, and that the maintenance of histone acetylation seems to be important for IEGs (Bahrami and Drabløs 2016; Tullai et al. 2007).

1.2 The SAGA-complex

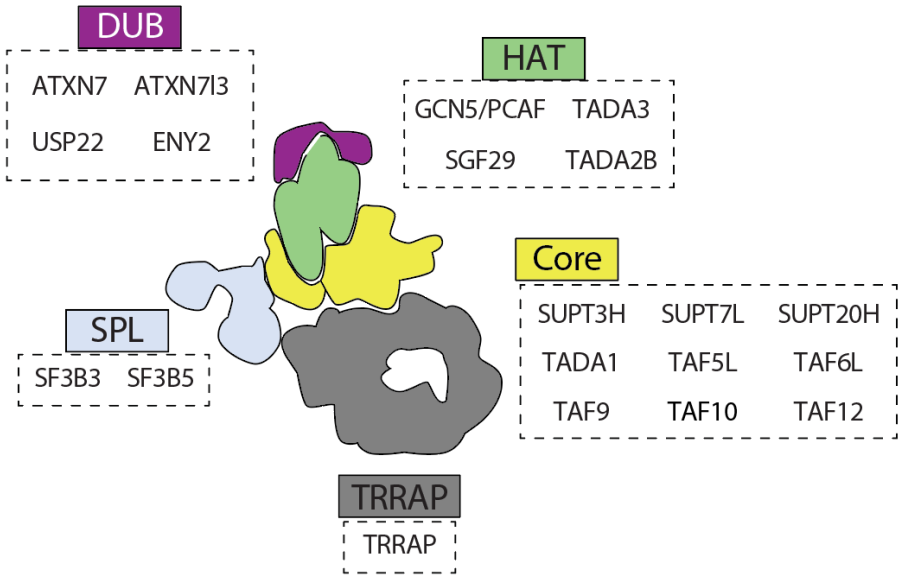
The SAGA-complex is an evolutionarily conserved transcriptional co-activator. It was first discovered in Tetrahymena and yeast (Brownell et al. 1996; Massari et al. 1999). To date, several distinct variants of SAGA have been described among organisms and even within species (Chen et al. 2022). The general control non-repressed protein 5 (Gcn5), which is part of the SAGA-complex, was the first described histone acetyltransferase (HAT) that was linked to transcription (Brownell et al. 1996; Brownell et al. 1995; Georgakopoulos et al. 1992). Despite its histone-acetyl-transferase activity, SAGA also shows deubiquitylase capacity via its deubiquitylation (DUB) module (Henry et al. 2003; Zhang et al. 2008). Both enzymatic activities have an impact on transcriptional initiation and elongation. In addition, the SAGA-complex can bind to specific histone modifications and interact with some transcription factors such as TATA-binding protein (TBP) (Sterner et al. 1999). The different properties of this large complex make it a potent and interesting co-factor and regulatory hub to study in the context of gene regulation.

1.3 The structure of SAGA-complex

The SAGA-complex is a rather large complex which amounts in humans to 1.4-MDa, depending on its composition, which can vary between species (Herbst et al. 2021). It consists of several modules that come together to form a complete complex and some

complex variants entailing functional differences have been described (Chen et al. 2021). Most structural analyses have focused on yeast SAGA (ySAGA), and it was believed that the 19-subunit ySAGA complex may also be representative of the human SAGA (hSAGA) architecture (Papai et al. 2020; Wang et al. 2020). This has been confuted because of the latest revolutionizing power of cryogenic-electron microscopy (cryo-EM) in structural biology, which has also had an impact on the SAGA field. The cryo-EM structure of the hSAGA-complex revealed how the arrangement of structural components results in a globally divergent organization from that of yeast (Herbst et al. 2021). Notably, hSAGA has not only compositional differences, but also some functional differences, one of which is the incorporation of a U2 splicing subunit (Helmlinger et al. 2017). That there might be differences between ySAGA and hSAGA might have come as no real surprise considering its conservation rate. While vertebrate SAGA is highly conserved (~ 95–58% sequence identity) it is much less when compared to yeast (~18% sequence identity) (Herbst et al. 2021). Owing to domain insertions, gene duplications, and deletions, hSAGA subunits are subfunctionalized (Antonova et al. 2019). In addition, hSAGA is essential for development in vertebrates, as opposed to ySAGA, further indicating the differences between these complex variants (Cheon et al. 2020; Wang et al. 2014).

The hSAGA-complex is composed of 20 subunits when completely assembled and can be divided into five functional modules (Herbst et al. 2021). These modules comprise a core, Transformation/Transcription Domain Associated Protein (TRRAP), deubiquitinase (DUB) module, histone acetyltransferase (HAT) module, and metazoan-specific splicing (SPL) module (Herbst et al. 2021).



Introductory Figure | Structure of the hSAGA complex.
Adapted from (Herbst et al. 2021). Schematic of proteins and modules of the SAGA-complex in human. HAT, Histone-acetyltransferase module; DUB, Deubiquitinilation module; Core module; TRRAP, Transformation/Transcription domain Associated Protein module; SPL, splicing module.

1.3.1 Core module

The core module acts as a scaffold and consists mostly of TATA-box binding protein (TBP)-associated factors (TAFs) (Herbst et al. 2021; Wang et al. 2020). Nine proteins (TAF5L, TAF6L, TAF9B, TAF10, TAF12, TADA1, SUPT20H, SUPT7L, and SUPT3H) make up the core of hSAGA around which the other modules are assembled (Herbst et al. 2021). The core forms a pseudo-octamer which has also been observed in ySAGA (Papai et al. 2020; Wang et al. 2020). Notably, several components of the SAGA-complex can also be found in other complexes, for instance in TFIID (Cheon et al. 2020). TFIID shares the structural components of the SAGA core (Chen et al. 2021; Kolesnikova et al. 2018; Patel et al. 2018). This makes it sometimes difficult to allocate them in traditional mass spectrometry experiments when separated and sub-functional subunits on their own may be identified, which can be part of multiple complexes. However, there are unique differences in these complexes, such as the proteins TAF5L and TAF6L, which are preferentially found in the SAGA-complex, whereas TAF5 and TAF6 are part of TFIID (Cheon et al. 2020; Ogryzko et al. 1998). In TFIID, there are two copies of several subunits, whereas there is only one copy of each protein in the SAGA architecture (Kolesnikova et al. 2018; Patel et al. 2018). The TAF proteins, which are shared

between SAGA and TFIID, are thought to be needed for promoting the preinitiation complex (PIC) formation (Cheon et al. 2020).

On its concave side, the core module connects the SPL module and on its convex side, most likely the HAT module (Herbst et al. 2021). SUPT20H was thought to be an integral protein of the core, which forms the largest interface with the rest of the complex and plays a central role in its assembly (Elías-Villalobos et al. 2019; Nagy et al. 2009; Sterner et al. 1999). In *S. cerevisiae*, Spt20p (ortholog of SUPT20H) has been shown to be essential for SAGA assembly (Han et al. 2014; Lee et al. 2011; Sterner et al. 1999). Interestingly, studies in *S. pombe* have shown that the loss of Spt20p does not significantly affect the integrity of the SAGA-complex, and almost the complete complex can be assembled despite its absence in this model organism (Elías-Villalobos et al. 2019). This was more recently confirmed in mammalian cells by deletion experiments of SUPT20H, which showed mild effects on SAGA subunit composition and SAGA core complexes were still forming (Fischer et al. 2021).

An important protein of the core module is SUPT7L, whose ortholog Spt7 has also been described as important for SAGA integrity in yeast (Han et al., 2014; Lee et al. 2011; Wu et al., 2002). When *Supt7l* was deleted from mammalian cells, no SAGA subunits were detected, suggesting disorganization of the complex in the absence of SUPT7L (Fischer et al. 2021). This would make *Supt7l* an interesting candidate for studying the effects of its loss and the concomitant absence of the SAGA-complex and resulting phenotypes. It is possible that certain subunits, which may contain some sort of remaining functionality, can assemble in the absence of other subunits. However, once the core of the SAGA-complex cannot assemble any potentially remaining HAT module might not be able to exert its function. It would lack the other subunits needed to bring the HAT module to its target locations on the genome. The same is true for the opposite case when the HAT module is lacking, but the core and structural modules might still be assembled. These mutant complexes would lack the histone-acetyltransferase activity of the HAT. In that case, SAGA would still be able to bind chromatin and interact with other transcription factors (TFs), but could not acetylate histones, leading to the loss of one of its major capabilities.

Before transcription can take place, RNA Polymerase II (Pol II) needs to be recruited to promoters, which involves the formation of a pre-initiation complex (PIC) (Farnung et al. 2022). In the classical model, a multi-subunit complex such as TFIID recognizes promoter DNA and deposits TBP, which further leads to the recruitment of the mediator complex and Pol II

(Chen et al. 2021; Schier et al. 2020; Schilbach et al. 2017). In hSAGA, the protein SUPT3H was expected to bind and/or deliver TBP at gene promoters, which in yeast is accomplished by its ortholog Spt3 together with the yeast-specific subunit Spt8, which is missing in hSAGA (Hahn et al. 2011; Han et al. 2014; Helmlinger et al. 2017). Surprisingly, cryo-EM analysis of the hSAGA-complex failed to detect a stable TBP-hSAGA interaction, which indicates either that hSAGA does not bind TBP at all or that the interaction is very dynamic and could require additional factors to stabilize the interaction, which is in stark contrast to the ySAGA or TFIID data (Herbst et al. 2021; Sermwittayawong et al. 2006). In yeast, Spt8 has been shown to be sufficient to form a stable TBP-SAGA interaction, whereas Spt3 alone failed to do so (Sermwittayawong et al. 2006). In hSAGA, SUPT3H is not required for complex stability and overall Pol II transcription was not affected in its absence (Fischer et al. 2022). To date, it is unclear whether SAGA interacts with TBP and the PIC (Farnung et al. 2022).

In summary, while Spt7 and Spt20p are required for the complex integrity of ySAGA in *S. cerevisiae*, only SUPT7L has been shown to be essential for the assembly of the hSAGA core in mammals.

1.3.2 TRRAP-module

The TRRAP module of hSAGA is cradle-shaped and is found below the core module (Herbst et al. 2021). In yeast, the corresponding protein is called Tra1, which is the largest subunit of ySAGA (Cheon et al. 2020). TRRAP and Tra1 belong to the family of phosphoinositide 3 kinase-related kinases (PIKK), but show no catalytic activity, making them the only pseudokinases in the family (McMahon et al. 1998; Saleh et al. 1998). While TRRAP lacks kinase activity, the recent release of the cryo-EM structure of hSAGA led to speculations of a possible undiscovered function, as has been observed for other pseudokinases (Herbst et al. 2021). The Tra1-module is important for the recruitment of the SAGA-complex by TFs to genomic target locations to exert its function as a co-activator. For Tra1 to bind an activator and subsequently to DNA, it has been suggested that the module undergoes conformational change upon binding to an activator (Cheon et al. 2020). This conformational change could provide a second layer of target specificity control, meaning that only then SAGA can bind to the target DNA, together with recruitment control via selective activator interaction. This supposition of conformational change as a control of target specificity and function can also

be applied to the HAT module, which might become even more flexible when the SAGA-complex is recruited by TFs to the DNA (Cheon et al. 2020; Eberharter et al. 1999). Binding to nucleosomes induces structural changes of SAGA, which leads to the displacement of the HAT and DUB modules from the core, so that they can act on nucleosomes in the proper orientation (Wang et al. 2020).

One TF that has been shown to interact with the TRRAP module of hSAGA is MYC (Feris et al. 2019; McMahon et al. 1998). In addition, a different region of MYC has been postulated to interact with TBP through cooperative interactions with the transcription initiation factor, TAF1 (Wei et al. 2019). This could indicate that some activators might play a role in TBP recruitment in metazoans (Cheon et al. 2020; Wei et al. 2019), and that MYC could be the link between TBP and the hSAGA complex (Zhang et al. 2022).

1.3.3 DUB-module

The hSAGA DUB module is flexibly attached to the core module via its anchor subunit, ATXN7 (Herbst et al. 2021). In hSAGA, the DUB and HAT modules were confirmed in the analyzed sample but failed to be resolved via cryo-EM, which was attributed either to their flexible tethering or labile attachment (Herbst et al. 2021). This is consistent with observations in yeast, where the flexibility of these modules was thought to be responsible for the poorer resolution, although the structure of the DUB module could be fitted (Morgan et al. 2016; Papai et al., 2020; Wang et al., 2020).

The DUB module contains a histone H2B deubiquitinase whose role in transcription has been extensively studied (Cheon et al. 2020). A co-transcriptional H2B ubiquitylation-deubiquitylation cycle has been shown to be important for stimulating transcriptional elongation (Weake et al. 2008). For ySAGA, the crystal structure of the DUB module revealed the composition of four proteins (Ubp8, Sgf11, Sgf73, Sus1), and the basic features of yeast the DUB module are conserved in the human DUB module (Morgan et al. 2016; Spedale et al. 2012).

Ubp8, a ubiquitin-specific protease of ySAGA, is responsible for the deubiquitylation of histone H2B and integrity of the SAGA-complex is a prerequisite for its function (Daniel et al. 2004; Henry et al. 2003). The other proteins, Sgf11, Sgf73, and Sus1, partially hold scaffolding functions to ensure proper conformation and function of Ubp8 (Samara et al. 2012; Yan et al.

2015). The human DUB module consists of USP22, ATXN7L3, ATXN7, and ENY2, which are homologues of the ySAGA DUB proteins (yeast Ubp8, Sgf11, Sgf73, and Sus1, respectively) (Zhang et al. 2008). USP22, a human ubiquitin-specific protease, can act like its yeast counterpart on the core histone H2B, once hSAGA is recruited to individual genes via sequence-specific activators (Bhaumik et al. 2001; Larschan et al. 2001). The regulation of transcription via alterations in the levels of histone ubiquitylation is an important mechanism that has been intensely studied also for other complexes such as polycomb group protein complexes (Fang et al. 2004; Li et al. 2006; de Napoles et al. 2004). However, Polycomb-complexes are associated with transcriptional repression, whereas deubiquitylation by USP22 is linked to transcriptional activation (Zhang et al. 2008). Transcriptional regulation by USP22 has been observed at MYC target genes (Zhang et al., 2008).

The protein Ataxin-7 (ATXN7) of the DUB module is integrated into the core of hSAGA by interacting with the core protein SUPT20H, which corresponds to ySAGA data (Herbst et al. 2021; Wang et al. 2020). Therefore, the DUB module is an independently folded subcomplex that is flexibly connected to the overall SAGA-complex via ATXN7 (Morgan et al. 2016).

ENY2, which is another component of the DUB-module and the human ortholog of yeast Sus1 (Cheon et al. 2020), is a protein found in both the SAGA-complex and the Three prime repair exonuclease 2 (TREX-2)-complex (Rodríguez-Navarro et al. 2004). The TREX complex is a multiprotein complex that couples transcription with the nuclear export of mRNA, which suggests that there is a link between SAGA-complex transcriptional activation and a role in transcription-coupled mRNA export (Köhler et al. 2006; Lim et al. 2013; Pascual-García et al. 2008; Rodríguez-Navarro et al. 2004).

1.3.4 HAT-module

Studying the histone acetyltransferase (HAT)-module was the starting point of the SAGA-complex field following the discovery of the nuclear histone acetyltransferase Gcn5 (Brownell et al. 1996). Initial studies showed HAT activity on free histones by Gcn5 alone but failed to produce similar results on nucleosomal histones (Grant et al. 1997). This led to the discovery of the SAGA-HAT-complex in yeast (Grant et al. 1997; Grant et al. 2021). The attachment of the HAT module to the core of the SAGA-complex was expected to be very flexible, similar to the DUB module, which made it impossible to resolve the HAT cryo-EM structure (Herbst et

al. 2021; Wang et al. 2020). Traditional genetic studies in yeast and subsequent electron microscopy studies have revealed that the ySAGA HAT module comprises four proteins, Gcn5, Ada2, Ada3, and Sgf29 (Grant et al. 1997; Roberts et al. 1997; Wu et al. 2004). Gcn5 is the catalytic enzyme of the module that requires other proteins to be able to unfold its function by undergoing conformational changes once it is bound in the module (Cheon et al. 2020). In humans, there are two paralogs of yeast Gcn5, named GCN5 and PCAF; the latter can be found in both hSAGA and the p300/CBP complex (Nagy et al. 2007). GCN5 is a synonym for KAT2A (encoded by the *Kat2a* gene) and PCAF is a synonym for KAT2B (encoded by *Kat2b*). For simplicity, we will use the gene names *Gcn5* and *Pcaf* instead of *Kat2a* and *Kat2b*. PCAF is approximately 75% identical to GCN5 (Nagy et al. 2007) and is not only a HAT but also has intrinsic E3 ligase activity within the so-called PCAF homology domain (Linares et al. 2007). Although some studies have suggested that PCAF can also be found in hSAGA, it is primarily GCN5, which is discussed as the main enzyme acting in the hSAGA HAT module (Herbst et al. 2021; Wang et al. 2020). Some have even named PCAF only in the context of interacting with TADA2a, an alternative ADA2 protein that is not found in the SAGA-HAT-module but only in a related complex, the ATAC-complex (Nagy et al. 2007; Ogryzko et al. 1998). Despite numerous studies, it is still not entirely clear whether both enzymes (although mutually exclusive) can be part of SAGA-HAT and what the unique and shared functions of these lysine acetyltransferases (KATs) are (Koutelou et al. 2021).

The differences between PCAF and GCN5 are further implied by different phenotypes upon mutation of *Gcn5* or *Pcaf* in mice (Xu et al. 2000; Yamauchi et al. 2000). Despite their possibly different functions, GCN5 and PCAF both contain a bromodomain in their structure, which is a motif found in almost all known nuclear HATs (Jeanmougin et al. 1997). Bromodomains function as acetyl-lysine binding domains and are important for highly specific histone acetylation by tethering HATs to specific chromosomal sites (Brownell et al. 1996; Manning et al. 2001; Travers 1999). Once the SAGA-complex is recruited to its target sites by a TF, the HAT module can further bind to acetyl-lysine with its bromodomain to bring the HAT module in the correct position to exert its function. Bromodomains are largely conserved in order to bind to lysine-acetylated proteins but some important changes to the sequence can add needed ligand-selectivity (Zeng et al. 2002). In addition, bromodomains are needed for the assembly and activity of multi-protein complexes such as the SAGA-complex, which was shown by deletion of the bromodomain module in yeast, which proved to be indispensable

for the function of Gcn5 (Brown et al. 2001; Georgakopoulos et al. 1995; Syntlchakl et al. 2000).

The central HAT domain of GCN5 is responsible for catalyzing the transfer of an acetyl group from acetyl coenzyme A (AcCoA, the “donor”) to a primary amine (the “acceptor”), and there are many ways to bind AcCoA (Dydaet al. 2002). Interestingly, it has been suggested that the prior binding of AcCoA and the subsequent formation of a GCN5/AcCoA complex are required to bind the target peptide, but this involves no major conformational change of GCN5 (Dyda et al. 2002).

Ada2 has been shown to be an important interaction partner by facilitating Gcn5 binding to the enzymatic co-substrate AcCoA (Sun et al. 2018). In *Drosophila*, two Ada2 variants have been discovered, namely Ada2a and Ada2b (Muratoglu et al. 2003), which are called TADA2a and TADA2b in human. Without TADA2b, the HAT module could not form and lost its capacity for histone acetylation when *Tada2b* was deleted from mouse embryonic stem cells (mESCs) (Fischer et al. 2021). Nevertheless, the core SAGA-complex remained intact (Fischer et al. 2021). Similar results were obtained from studies in yeast (Han et al. 2014; Lee et al. 2011; Sterner et al. 1999; Wu et al. 2002).

The Alteration in activation 3 (Ada3) protein is a structural protein of the HAT, which is the yeast ortholog of TADA3 in humans (Pina et al. 1993). Based on studies in yeast, it was expected that TADA3 would be as important to the HAT integrity as TADA2b (Balasubramanian et al. 2002). Once the TADA3 protein was depleted from mESCs, the enzymatic function of the SAGA-complex was significantly reduced, indicating a loss of HAT-module functionality, whereas the core would most likely remain intact (Fischer et al. 2021).

In summary, without TADA3 and TADA2b, the HAT module is most likely not formed and loses its capacity for histone acetylation, while the core complex may remain intact.

The latest adaptor protein found to be part of the HAT module is Sgf29 (Sanders et al. 2002). Similar to most SAGA proteins, Sgf29 is conserved from yeast to humans; however, little is known about its function (Bian et al. 2011). Within the HAT module, SGF29 can directly bind to TADA3 (Kurabe et al. 2007). Studies in rat (r) tumor cells further showed that rSGF29 was recruited together with MYC to MYC-target genes adding to the interaction of the SAGA-complex and MYC (Kurabe et al. 2007). Sgf29 contains a C-terminal Tudor domain, which has been shown to bind methylated histones, raising the possibility that this protein can add

histone methyllysine-binding ability to the already versatile functions of the SAGA-complex (Adams-Cioaba et al. 2009; Bian et al. 2011). Many chromatin-associated proteins or protein complexes have both histone writing and reading activities, and the SAGA-complex is no exception (Bian et al. 2011). While the GCN5 bromodomain is another reader-domain of the HAT-module, also the Tudor-domain of Sgf29 can recognize methyl marks for its HAT complex and might lead to acetylation at H3 (Bian et al. 2011). SGF29 has been shown to link the hSAGA complex to H3K4me3 modifications (Vermeulen et al. 2010), which mark the transcription start sites of active genes (Benayoun et al. 2014). It was suggested that H3K4me3 is required for subsequent acetylation of histone H3 and SAGA primarily acts on H3 (Jiang et al. 2007). Binding assays in yeast suggested the following mechanism: the SAGA-complex can read and bind to H3K4me3 via the Sgf29 protein, which allows the HAT-module to act on promoters where Gcn5's bromodomain adds another layer of reader specificity and can eventually modify H3. Despite of the observation that Sgf29 is not essential for the integrity of the HAT-module, it is needed for the complete and efficient activity of the HAT-module (Bian et al. 2011). This was further confirmed by mutant Gcn5, Ada3, and Sgf29 yeast strains, where global acetylation levels of the known SAGA modification histone 3 lysine 9 acetylation (H3K9ac) were decreased, further suggesting that the integrity of the HAT module is vital for its function (Bian et al. 2011).

1.3.5 Splicing-module

The splicing module (SF3B3 and SF3B5) of the SAGA-complex has been more recently identified in higher eukaryotes (Brand et al. 2001; Martinez et al. 2001; Stegeman et al. 2016). It is a subcomplex of SAGA and has been shown to activate the proper splicing of some SAGA-regulated transcripts (Brand et al. 2001; Martinez et al. 2001; Stegeman et al. 2016). However, the specific role of this subcomplex in SAGA requires further investigation. SF3B3 functions as part of the SF3B complex, which plays a well-defined role in the first step of splicing (Golas et al. 2003; Will et al. 2011). Splicing occurs co-transcriptionally, and multiple mechanisms couple transcription to splicing (Stegeman et al. 2016). In addition to its role as a HAT at promoters, ySAGA has also been shown to recruit TBP to some promoters, leading to RNA Pol II recruitment, which allows for speculation of possible involvement in splicing (Laprade et al. 2007; Larschan et al. 2001). However, since it was not possible for the hSAGA-

complex to detect a stable TBP-hSAGA interaction, which is in stark contrast to the ySAGA data (Herbst et al. 2021; Papai et al. 2020; Sermwittayawong et al. 2006), it is unclear what this means with regard to its possible role in splicing. To date, it is unclear whether hSAGA can interact with the PIC (Farnung et al. 2022), and further structural analyses are needed to determine if and how the SAGA-complex can deliver TBP, possibly relying on MYC (Wei et al. 2019).

1.4 Existing HAT-module specific alleles of SAGA-complex

1.4.1 *Gcn5* and *Pcaf*

While GCN5 dominates hematopoiesis, neural tissue, and development, PCAF is more prevalent in skeletal muscles (Arede et al. 2021; Xu et al. 2000; Yamauchi et al. 2000). During development, GCN5 is ubiquitously expressed early on but decreases after E16.5, which suggests a reduced contribution to terminal differentiation, whereas PCAF shows minimal early expression and upregulation in adult tissue (Arede et al. 2021; Xu et al. 2000). The importance of *Gcn5* in development was demonstrated by analyzing mice null for *Gcn5,* which died by 10.5 d.p.c, whereas *Pcaf*-null embryos were viable and fertile with no obvious abnormal phenotype, indicating that individual histone acetyltransferases are required as cofactors for specific developmental processes (Xu et al. 2000). Interestingly, *Gcn5 and Pcaf* double-knockout mice showed earlier embryonic lethality than *Gcn5* null mice (Xu et al. 2000).

To analyze the role of GCN5 in different cell types and developmental stages, floxed *Gcn5* alleles were generated (Addicks et al. 2022; W.Lin et al. 2008). The inducible conditional knockout of the *Gcn5* allele by crossing it with an interferon response-inducible *Mx1*-Cre mouse showed no defects in hematopoietic stem cells (HSCs). This was also observed by others when using a tissue-specific developmental (*Vav-Cre*) Cre-line, which shows efficient deletion in all hematopoietic cell types, crossed with a floxed *Gcn5* allele (Bararia et al. 2016). Here, HSCs numbers were unchanged, and despite the possibility that other KATs could compensate for the absence of *Gcn5*, the ortholog *Pcaf* seems unlikely, because it is normally absent in HSC and progenitors (Domingues et al. 2020). Despite the observation that GCN5 is necessary during embryogenesis, it was surprising that the HAT function of SAGA is not required in blood stem cells, which was further demonstrated in mESCs (Fischer et al. 2021;

Lin et al. 2007). However, there seems to be a role for the HAT module in cell differentiation, especially in lymphoid blood lineages, such as the maturation of B cells, T cells, and iNKT cells (Gao et al. 2017; Kikuchi et al. 2014; Lin et al. 2007; Wang et al. 2017).

In B cells, the roles of GCN5 and PCAF were assessed using the *Cd19*-Cre line, which leads to deletion of the floxed allele in early B cell development (Oksenych et al. 2022; Rickert et al. 1997). Analysis of the mice showed smaller spleens in the case of conditional *Gcn5* deletion and a relative accumulation of pro-B cells, which the authors interpreted as a developmental block at the pro-B cell stage (Oksenych et al. 2022).

1.4.2 Tada3

Similar to *Gcn5*, germline deletion of *Tada3* in mice is embryonic lethal. The generation of a *Tada3* floxed allele allowed conditional deletion of this allele and analysis in specific tissues. The authors generated MEFs from these mice and infected them with a Cre-expressing plasmid to induce deletion, which led to cell cycle arrest (Mohibi et al. 2012). Cells could not proliferate because of several defects in the cell cycle, and both *Myc* mRNA and protein levels were reduced. As expected, H3K9ac levels were diminished in MEFs upon loss of *Tada3* (Mohibi et al. 2012).

To date, there are no existing floxed alleles of the other HAT-module genes *Tada2b* or *Sgf29*, or the ATAC-complex-specific HAT-gene *Tada2a*.

1.5 SAGA-complex versus ATAC-complex

In metazoans, the HAT module of SAGA is also found in the so-called Ada2a-containing (ATAC) complex (Guelman et al. 2006; Nagy et al. 2010). However, there is a notable difference between SAGA-HAT and ATAC-HAT, which is the different incorporation of TADA2a and TADA2b which are paralogues of the yeast Ada2 protein(Kusch et al. 2003; Muratoglu et al. 2003). These two proteins were first identified in *Drosophila* and were named TADA2a and TADA2b in the hSAGA-complex (Herbst et al. 2021; Muratoglu et al. 2003). Studies in *Drosophila* have shown that, while Tada2a is exclusively found in the ATAC-complex, Tada2b is part of the SAGA-complex (Kusch et al. 2003; Muratoglu et al. 2003). This allows to distinguish between the two HAT modules.

The ATAC-complex shares the HAT module with the SAGA-complex, but it also possesses a second HAT subcomplex, ATAC2, which is preserved in mammals and flies (Guelman et al. 2006; Riss et al. 2015). Further differences comprise proteins that might have structural or regulatory functions in the ATAC complex (Mi et al. 2018). One important question that needs to be addressed is the shared and unique functions of these complexes. Overlapping and unique binding sites have been reported for SAGA and ATAC, whereas SAGA has been primarily found at promoters, and ATAC is also associated with enhancers (Bonnet et al. 2014; Krebs et al. 2011). However, the activators responsible for recruiting SAGA or ATAC to their unique target sites remain to be elucidated (Cheon et al. 2020).

Regarding histone acetyltransferase activity, SAGA primarily acts on H3, whereas ATAC shows H3/H4-activity (Guelman et al. 2006; Orpinell et al. 2010; Spedale et al. 2012) with a preference for H4 in *Drosophila* and H3 in humans (Helmlinger et al. 2017; Suganuma et al. 2008). Interestingly, a growing number of non-histone substrates for ATAC, as well as for the SAGA-complex, have been identified, and are postulated to contribute to protein stability or activity. ATAC non-histone targets include p53 (Liu et al. 1999), autoacetylation of PCAF (Blanco-García et al. 2009) and CDK2 (Mateo et al. 2009). For the SAGA-complex, CDC6 and MYC have been described previously (Paolinelli et al. 2009; Patel et al. 2004).

1.6 Role of SAGA complex in gene regulation

1.6.1 Global and cell-specific role of SAGA-complex

Most components of the SAGA-complex are ubiquitously expressed, indicating a general importance and necessity. However, there is an enrichment of several components in organs, such as the brain and reproductive system, which goes hand in hand with an established role in developmental programs and neurodegenerative diseases (Bu et al. 2007; Maurice et al. 2007). The role of GCN5 has been studied in more depth in early development, where it is very important, and a reduced contribution in terminal differentiation has been suggested (Arede et al. 2021). The opposite is true for PCAF (Xu et al. 2000; Yamauchi et al. 2000).

Despite the different expression patterns in some tissues, some studies have indicated that SAGA functions as a general coactivator which is broadly required for transcription (Baptista et al. 2017; Bonnet et al. 2014). This contrasts with other studies that conversely elucidated specific targeting to gene promoters through interactions with specific

transcription factors (Grau et al. 2008; Nagy et al. 2009; Weake et al. 2012). To date, both possibilities have been discussed, but more recent reports have indicated that the SAGA-complex might regulate and control gene expression at specific genomic locations. It may therefore have unique roles depending on the cellular context being studied (Cheon et al. 2020). Its different roles at different developmental stages and in different cell types could be due to selective interaction with TFs. The belief that a ubiquitous expression pattern indicates general importance often does not hold true when we look closer at expression in all tissues and developmental stages. Over the years, several studies have led people to adjust the number of so-called housekeeping genes that have been identified and also challenge the concept of universally important factors as opposed to tissue-specific factors, with probably some exceptions, such as polymerase and other genes required for DNA, RNA, and protein synthesis (Joshi et al. 2022; Pál et al. 2006; Papp et al. 2004; Rancati et al. 2017; Zhan et al. 2015).

1.6.2 The role of SAGA-complex in cell cycle control

A functional cell cycle is necessary for cell differentiation and/or proliferation. Studies in yeast and mice have suggested that the ATAC-complex, but not the SAGA-complex, is necessary for progression through the G2/M phase (Guelman et al. 2006; Howe et al. 2001; Orpinell et al. 2010). There are different possibilities for the failure of cell cycle progression after the S-phase occurred in these studies. One is that the transcription of cell cycle-specific genes depends on ATAC, or that it serves a non-transcription-related function. The suggested mechanism involves acetylation of non-histone target Cyclin A. At the onset of mitosis, Cyclin A must be degraded to continue mitosis, which is promoted by ATAC-mediated acetylation. When ATAC-components were absent Cyclin A levels remained high, as Cyclin A failed to be efficiently degraded leading to defective chromatin compaction and mitotic failure (Orpinell et al. 2010). Surprisingly, Orpinell et al. (2010) did not find that the SAGA-complex is necessary for G2/M progression.

A different study has suggested that the SAGA-complex plays a role in cell cycle control. GCN5 can regulate the cell division cycle (CDC)-6 protein, an important cell cycle protein. GCN5-mediates the acetylation of CDC6, thereby controlling its relocalization to the cytoplasm and stability. Unlike Cyclin A, CDC6 should remain intact for proper cell cycle

progression and is first found in the nucleus but then relocates to the cytoplasm during the cell cycle. The acetylation function of GCN5 is vital, because catalytically inactive GCN5 mutants do not relocalize CDC6 (Paolinelli et al. 2009; Petersen et al. 1999). Further evidence for the involvement of the SAGA-complex in controlling the cell cycle was provided by generating DT40 (a chicken B cell line) mutants for GCN5, which caused a delayed growth rate and suppressed cell cycle progression (Kikuchi et al. 2005). PCAF deficiency failed to produce similar results, and both PCAF and GCN5 were found to be dispensable for cell viability (Kikuchi et al. 2005). When the authors examined how GCN5 controls cell growth and cell cycle-related genes, they observed deregulated mRNA for *Cyclin-A* and *Myc* and many more genes involved in the G1/S transition phase. This study suggested a role for the SAGA-complex, but failed to differentiate the possibly shared or unique involvement of the ATAC-complex in this process (Kikuchi et al. 2005). However, the authors emphasized that GCN5 and PCAF show tissue (or cell type) specific expression patterns; the former is abundantly expressed in DT40 cells, but the expression of the latter is insignificant (Kikuchi et al. 2005). This further emphasizes the importance of considering possible changes in the expression patterns of so-called ubiquitously expressed factors.

Overall, these studies indicate that ATAC and the SAGA-complex could both be involved in cell cycle control and do so by regulating the expression of several cell cycle-related genes and by acetylation of different non-histone targets.

1.6.3 HAT-independent co-activator role of SAGA-complex

The existence of a coactivator function of the SAGA-complex, which is independent of enzymatic activity, has been suggested by studies in *Drosophila* and yeast (Bhaumik and Green 2001; Weake et al. 2009). In *Drosophila*, SAGA-core mutants that did not lead to complete loss of the complex and had unchanged H3K9ac levels showed defects in gene expression of a set of SAGA-regulated genes (Weake et al. 2009). More recently, a GCN5-independent role for the SAGA-complex during embryonic development in Drosophila has been described (Ciabrelli et al. 2023). The authors showed that despite the global distribution of H3K9ac, the SAGA-complex activated a distinct set of genes during zygotic genome activation and catalytical dead GCN5-mutants were still able to do their job (Ciabrelli et al. 2023).

1.7 Types of modifications by the SAGA-HAT-module

Modifications of histones, such as acetylation, influences how DNA is packaged by histones into nucleosomes and, therefore, affects higher-chromatin structure (Martinez et al. 2001). Acetylation is a covalent modification that affects the interaction between histones and DNA (Bradbury, 1992; Strahl et al. 2000). Reversible acetylation of specific lysine residues within the N-terminal tails of nucleosomal core histones often occurs during transcription when certain chromatin structures are accessible (Bradbury 1992; Struhl 1998). The SAGA-complex is one of many transcriptional cofactors involved in this process (Brown et al. 2000). Acetylation occurs in an AcCoA-dependent manner, where the central HAT domain of GCN5 catalyzes the transfer of an acetyl group from AcCoA to a primary amine of the target lysine, thereby neutralizing the positive charge of the histone tails and decreasing their affinity for DNA (Hong L. et al. 1993). This can alter nucleosome conformation and allow TFs to interact with open chromatin (Lee et al. 1993; Norton et al. 1989; Struhl 1998; Vettese-Dadey et al. 1996).

Originally, H3K9, H3K14, and H3K18 were thought to be the primary acetylation sites for SAGA (Grant et al. 1997; Lee et al. 2007). More recently, this observation was adapted to H3K9ac as the main mode of action, and to H3K14ac to a lesser extent (Li et al. 2016). All these modifications are associated but do not necessarily initiate transcriptional activity in mammalian cells (Jin et al. 2011; Morgan et al. 2020).

1.8 The role of H3K9 acetylation in different cell types

Embryonic stem cells (ESCs) are pluripotent and contain much more open chromatin with less condensed heterochromatin than differentiated cells, suggesting the high plasticity of their genome (Mattout et al. 2010). The open conformation of chromatin is induced and/or maintained by histone modifications. Such modifications include the hyperacetylation of histone H3, which is known as an active marker that is often associated with transcription (Heintzman et al. 2009; Wang et al. 2008). Different lysine residues of H3 can be modified, and H3K9ac has been identified to contribute to gene activation, whereas H3K9 methylation is associated with gene silencing (Jenuwein et al. 2001). Detection of H3K9ac at the

transcription start site (TSS)-flanking nucleosomes, together with other features, is expected to increase chromatin accessibility and enable the assembly of the RNA Pol II pre-initiation complex (Chen et al. 2022). The causal relationship between the chromatin status at the promoter and transcription is although not entirely clear and despite H3K9ac being associated with gene activity, some findings suggest that H3K9ac marks recently transcribed genes rather than driving transcription itself (Chen et al. 2022; Howe et al. 2017; Morgan et al. 2020).

H3K9ac is especially important for ESCs and is correlated with pluripotency and reprogramming capacity (Hezroni et al. 2011). The relative enrichment of activity-associated histone modifications in ESCs makes them an interesting system for studying the roles of these modifications, including H3 acetylation, in gene regulation. Nevertheless, the genome-wide role of H3 acetylation in mouse embryonic stem cells (mESC) is not well understood (Karmodiya et al. 2012).

The role of H3K9ac in terminally differentiated cells, on the other hand, has not been studied to such extent. This might be partially due to the reduction of H3K9ac levels with ES cell differentiation, which is less prevalent in differentiated cells (Bártová et al. 2008; Efroni et al. 2008; Krejči et al. 2009). This phenomenon indicates a different, if not less prominent importance for global H3K9ac levels in differentiated cells and especially for quiescent cells (Mattout et al. 2010; Qiao et al. 2015; Revilla-I-Domingo et al. 2012). Although H3K9ac is still an active chromatin marker in differentiated cells, the cells might depend less on it. However, this does not exclude that H3K9ac is important for differentiated cells or that there are non-global, site-specific effects regulated by this chromatin modification. H3K9ac may have more context-dependent roles in differentiated cells than in ESCs, and other histone modifications may become more prominent in regulating cell-specific gene expression (Bártová et al. 2008; Baxter et al. 2004; Revilla-I-Domingo et al. 2012). In addition, a role in driving cell differentiation and commitment has been described for H3K9ac together with H3 lysine 14 acetylation (H3K14ac), which is also an active chromatin mark (Liu et al. 2023).

The importance of histone acetylation has been partially revealed by studying the writers of these marks. The histone acetyl transferases GCN5/PCAF are mainly responsible for H3K9ac in mammalian cells (Jin et al. 2011; Lee et al. 2007; Nagy et al. 2007). Despite their biochemical similarity, GCN5 and PCAF might serve different functions because the loss of

either of these factors causes different phenotypes in mice (Xu et al. 2000). For metazoans, it is debatable whether both GCN5 and PCAF can be incorporated into the HAT module of the SAGA complex (Chen et al. 2021; Nagy et al. 2007), or if GCN5 is the primary KAT of SAGA and PCAF for the ATAC-complex (Herbst et al. 2021; Nagy et al. 2007). It remains challenging to allocate the role of the SAGA-complex to H3K9ac by targeting only the KAT. Inevitably, a SAGA-specific subunit, such as TADA2b, needs to be included in the equation to address the role of the SAGA-complex. Studies that have suggested a global role for the SAGA-complex in H3K9ac by only targeting *GCN5* fail to differentiate between the possibly different roles of the ATAC-complex and SAGA-complex (Kikuchi et al. 2005). However, a recent publication has observed an overall decrease in H3K9ac levels by deleting *Tada2b*, which leads to the disruption of SAGA-HAT activity (Fischer et al. 2021). This suggests a global disposition of H3K9ac by the SAGA-HAT module (Nagy et al. 2007). Otherwise, these changes would not be detectable in total histone preparations. If the global or rather site-specific loss of H3K9ac at key genes is responsible for the observed phenotypes in these cells cannot be entirely addressed. However, deregulation of a limited number of genes involved in the cell cycle despite decreased global H3K9ac levels suggested the latter (Kikuchi et al. 2005). A role as a co-activator at site-specific loci through interactions with specific TFs (such as MYC) and at different developmental stages or in different cell types is likely (Flinn et al. 2002; Kahata et al. 2004; Lebedeva et al. 2005; Liu et al. 2003; McMahon et al. 1998).

Over the years, an increasing number of studies have examined the role of the SAGA-complex and H3K9ac, and its effects on gene regulation. Considering that the SAGA-complex is mainly responsible for H3K9ac and that this histone modification is associated with active transcription, one would expect detrimental effects on the expression of SAGA target genes in the absence of the SAGA-HAT module and correlated loss of H3K9ac. However, the described observations are more complex with respect to the relevance of H3K9ac to different cell types and developmental stages.

1.8.1 H3K9ac and SAGA-complex in stem cells

The role of SAGA-complex in mESCs has been assessed by several studies with some contradicting findings.

Fischer et al. (2021) studied the role of SAGA-complex and ATAC-complex in mESCs. They found that when *Tada2b* (SAGA-specific), Tada2a (ATAC-specific), or *Tada3* (shared between SAGA and ATAC) were deleted, the HAT-modules could not be formed (Fischer et al. 2021). This led to a reduction in H3K9ac levels, but there was no observation of growth defects or proliferation issues in mESCs. The reduction of H3K9ac was stronger when the SAGA-HAT module was deleted than when the ATAC-HAT module was deleted. In contrast, the remaining SAGA-core was important for mESC proliferation and self-renewal and ATAC-complex also for cell survival, which might be due to HAT-independent activities. The authors showed that the SAGA core protein SUPT7L was essential for the integrity of the entire SAGA-complex and mESCs were still able to survive without SAGA-complex but self-renewal was affected (Fischer et al. 2021). A similar picture was obtained for the ATAC-complex: *Tada2a* mutant cells had an intact core but the HAT module was missing. This showed no defects, whereas core-specific ATAC mutants had affected cell growth and decreased proliferation but also affected survival. Neither SAGA nor ATAC were able to compensate for each other's missing core complexes, which points to unique, non-overlapping functions in mESC. Therefore, both SAGA- and ATAC-complex have unique non-HAT-dependent functions to maintain the mESC state (Fischer et al. 2021). The authors further analyzed newly synthesized mRNA levels in ATAC-complex and SAGA-complex mutant mESCs and found hardly any overlap of controlled genes between the two complexes and a more prominent role for the ATAC-complex (Fischer et al. 2021). TADA3 protein degradation experiments showed no effects on overall Pol II activity suggesting that the associated reduction of H3K9ac levels do not affect global transcription in mESCs.

The unique roles of SAGA-complex and ATAC-complex may be dictated by the recruitment by specific TFs. MYC was identified to be bound to ATAC-regulated genes potentially recruiting the ATAC-complex, which may partially explain the cell-cycle defects observed upon inactivation of ATAC in mouse ESCs (Fischer et al. 2021). This was in contrast to an earlier study that found the SAGA-complex to be important for the regulation of *Myc* and the MYC network in mESCs, which they attributed to its acetylation capacity (Seruggia et al. 2019).

A HAT-dependent function was shown by studies of *Gcn5* mutant ESCs, which showed a requirement for the HAT complex during cell differentiation (Lin et al. 2007; Wang et al. 2018). It was suggested that the histone-modifying activities of both SAGA and ATAC play a more critical role during differentiation than during mESC self-renewal, which is consistent with the requirement for GCN5 activity during mouse embryonic development (Xu et al.

2000; Yamauchi et al. 2000). In line with this, catalytic inactivation of another lysine acetyltransferase, TIP60 (Tat interactive protein 60 kDa; also called KAT5), whose binding sites overlap with MYC (Ravens et al. 2015), did not impair mouse ESC growth or self-renewal, but resulted in defects during mouse embryonic development (Acharya et al. 2017).

1.8.2 H3K9ac and SAGA-complex in differentiated immune cells

The function of the SAGA-complex in specific cell types is unclear. A role for SAGA in regulating differentiation and maturation in innate and adaptive immune cells has been suggested (Bararia et al. 2016; Chen et al. 2022; Gao et al. 2017).

In T cells, it has been shown that Gcn5 is important for T cell activation and naive *Gcn5* -deficient $CD4^+$ T cells showed a significant reduction in proliferation and IL-2 production (Gao et al. 2017). It was further shown that Gcn5 mediated H3K9ac at the interleukin-2 (IL-2) promoter, increased IL-2 production (Gao et al. 2017). Recently, two CRISPR screens identified SAGA components as novel regulators that promote the expression of FOXP3, the master TF of regulatory T cells (Tregs) (Cortez et al. 2020; Loo et al. 2020). The levels of FOXP3 are maintained through both transcriptional and post-translational regulations by USP22 (DUB-module): both the *Foxp3* locus as well as the protein are deubiquitylated thereby maintaining transcription and preventing protein degradation (Chen et al. 2021).

In vitro studies of B cells have indicated that GCN5 is required for their development and activation (Kikuchi et al. 2011; Kikuchi et al. 2008). The *in vitro* studies were performed using the chicken immature B cell line DT40, which has a follicular B-cell gene expression program and is used to study BCR-signalling and differentiation (Alinikula et al. 2011; Koskela et al. 2003). It has been suggested that GCN5 plays a key role in the epigenetic regulation of the PI3K/AKT pathway (Kikuchi et al. 2011). This was demonstrated by treating DT40-B cells with hydrogen peroxide, which activates the phosphatidylinositol 3-kinase (PI3K) signaling cascade but failed to do so in the absence of Gcn5. The expression of SYK and BTK was dysregulated. Phosphorylation of AKT was remarkably suppressed in *Gcn5*-deleted cells, and suppressed AKT activation caused intense apoptotic cell death (Kikuchi et al. 2011). Another important role of Gcn5 in B-cell activation and differentiation has been described (Kikuchi et al. 2014). The authors used DT40-B cells that lacked *Gcn5*. They showed that B-cell differentiation was impaired and identified *Irf4* as a target gene of GCN5. ChIP-PCR analysis showed binding of

GCN5 to the *Irf4* gene, reduced H3K9ac in *Gcn5* null cells and subsequently, reduced *Prdm1* expression (Kikuchi et al. 2014). However, the relevance of this finding to *in vivo* B-cell activation and differentiation remains elusive.

In summary, only the SAGA-enzyme GCN5 has been studied in B and T cell development where it seems necessary for cell activation and differentiation but no *in vivo* experiments have been performed.

1.9 SAGA-complex in cancer

Genome-wide CRISPR-based screens have identified hSAGA genes to play a role in different cancer cell types (Chen et al. 2021; Cortez et al. 2020; Loo et al. 2020). *KAT2A* (encodes GCN5) was found to be a differentially essential gene to some but not all tested acute myeloid leukemia (AML) cell lines. The genetic disruption of *KAT2A* affected the proliferation and reduced the growth of some AML lines and the authors suggested that GCN5 would be an attractive drug target (Tzelepis et al. 2016). It was further implied that GCN5 may not be essential to hematopoietic stem cells, which is very important when identifying targets for rational therapeutics to minimize adverse effects (Bararia et al. 2016; Domingues et al. 2020). In addition, conditional mutagenesis of a *Kat2a* allele crossed with an *Mx1*-Cre allele that drives deletion in an interferon-dependent manner showed normal hematopoiesis but was later corrected to have effects on the erythroid lineage (Arede et al. 2022; Domingues et al. 2020; Kühn et al. 1995).

Studies of *MYCN*-amplified neuroblastoma cell lines have identified a discrete set of genes that are either unique to or only minimally shared with other tumor types. Among these genes, *TADA2b* may be required for the growth and survival of these cells (Durbin et al. 2018).

1.9.1 The transcription factor MYC

The proto-oncogene and basic helix-loop-helix transcription factor MYC belongs to the MYC family (Chen et al., 2018). In B lymphocytes, *Myc* and N-*Myc* are expressed, whereas only *Myc* is initially expressed during the pro-B cell stage in response to cytokines. Both members are induced following pre-BCR expression in large pre-B cells during maturation and expansion

into small pre-B cells. Thereafter, only *Myc* is expressed following mature B-cell activation (DePinho et al. 1987; Habib et al. 2007).

In healthy cells, MYC is activated by several pathways that induce mitosis (Stefan and Bister 2017). Subsequently, MYC controls cellular growth and proliferation by enhancing the transcription of proliferation-associated genes upon growth factor signaling (Dang 2012; Meyer et al. 2008). The ability of MYC to promote cell cycle progression goes hand-in-hand with its blocking effects on cell differentiation (Wasylishen et al. 2010). Here, MYC must be downregulated for cells to exit the cell cycle and undergo differentiation et al. 2010). Otherwise, as an activator, MYC would reinforce the current cell state, which was seen in ES cells, where MYC maintained the undifferentiated state. Once cells have managed to differentiate, the upregulation of MYC can reinforce the new cell state (Nie et al. 2012). These processes are especially important for B cell proliferation, where the gain of MYC is associated with B cell malignancies (De Alboran et al. 2001).

MYC is overexpressed in the majority of human cancers and has been shown to interact with different effector proteins, such as chromatin remodelers (Dang 2012; McMahon et al. 1998). Whether MYC acts only on specific target genes or has a role as a general transcriptional amplifier has been heavily debated in the field. Some studies have reported that it can bind to or be recruited by interaction partners to the promoter regions of active genes and cause global transcriptional amplification, whereas others have suggested gene-specific regulation (Guo et al., 2014; Lin et al. 2012; Lorenzin et al. 2016; Nie et al. 2012). The observed differences in target genes between different cell types were explained by MYC acting as an amplifier of the transcribed genes in a given cell; therefore, the regulated gene profiles varied between different cell types (Lin et al. 2012; Littlewood et al. 2012; Nie et al. 2012). The role of a general amplifier was further supported by the observation that MYC was bound to most active promoters (Lin et al. 2012).

Nevertheless, the perception that MYC acts as a general amplifier of transcription has recently been challenged by conducting acute protein degradation experiments coupled with analysis of immediate gene expression changes in human leukemia cells (Muhar et al. 2018). Muhar et al. (2018) found MYC to be a selective transcriptional activator, controlling metabolic processes such as ribosome biogenesis and nucleotide biosynthesis. Whereas the earlier reported increase in total cellular RNA upon MYC overexpression might be caused by secondary effects (Muhar et al. 2018). The previous observation that MYC is bound to most

active promoters is likely due to low-affinity, non-sequence-specific interactions, a phenomenon termed “invasion” (Lin et al. 2012; Nie et al. 2012; Tesi et al. 2019). In addition, *Myc* deletion in LPS-activated B cells affected only a subset of genes (Tesi et al. 2019).

1.9.2 SAGA-complex and its interaction with MYC

Studies have shown that the SAGA-complex can acetylate and stabilize MYC and, in turn, MYC can induce SAGA-components expression in a positive-feedback loop (Hirsch et al. 2015; Patel et al., 2004). Acetylation of MYC occurs in the C-terminal part of the protein at the nuclear localization sequence (NLS) and leucine zipper motif, which increases its half-life by almost 3-fold (Patel et al. 2004). Moreover, MYC was shown to directly interact with GCN5 and TRRAP to recruit SAGA to MYC’s downstream targets (McMahon et al. 2000). Because MYC has been shown to interact with TBP as well as TRRAP via non-overlapping regions, and cryo-EM analysis of hSAGA failed to show a stable TBP interaction for the complex, MYC might play a role in TBP recruitment to metazoan SAGA (Feris et al. 2019; Herbst et al. 2021; McMahon et al. 1998; Wei et al. 2019).

As the N-terminal transcription activation domain (TAD) of MYC interacts with SAGA but not or only weakly with ATAC components, a role of the SAGA-complex in MYC-driven cancers seems more likely (Zhang et al. 2014). Furthermore, the overexpression of SAGA-components (TADA3) and the associated increased acetylation and stabilization of MYC led to enhanced proliferation of breast cancer cells (Griffin et al. 2016). Significantly, no recurrent *KAT2A* mutations have been described in cancer, pointing to the possibility that GCN5 HAT-ability is needed for establishing and/or maintaining tumorigenic programs but is not causative (Arede et al. 2021). The lack of mutations could reflect conflicting roles of *KAT2A* loss or over-expression at different stages of cancer progression (Arede et al. 2021).

Since the MYC protein itself has often been considered “undruggable,” new therapeutic targets are needed (Ott et al. 2013). Because of the stabilizing and positive effects of the SAGA-complex on MYC and its half-life, it could be an interesting candidate (Mustachio et al. 2020). Nevertheless, the available KAT inhibitors have limited utility in clinical trials owing to issues with selectivity, toxicity, and potency, despite promising initial results in cancer cell lines (Mustachio et al. 2020; Wapenaar et al. 2016; Xia et al. 2017).

1.10 The role of MYC in GC B cells

MYC is needed throughout B cell development and *CD79a*-Cre driven deletion of *Myc* at the pro-B cell stage resulted in the loss of pro-B cells and subsequent B cell populations (Pérez-Olivares et al. 2018; Vallespinós et al. 2011). At later stages, *Myc* expression is induced by mitogenic stimulation and is necessary for B-cell proliferation, differentiation and especially germinal center (GC) formation (Calado et al. 2012; Fernández et al. 2013). The relevance of MYC in GC B cells has been debated, with conflicting studies showing high protein expression in GC B cells, whereas others have reported similar low expression as in quiescent follicular (FO) B cells, where MYC does not play a major role (De Alboran et al. 2001; Cutrona et al. 1997; Cutrona, Dono et al. 1997; Klein et al. 2003; Shaffer et al. 2001). The observation that the master regulator of proliferation was not upregulated in GC B cells, which have one of the fastest proliferation rates in mammals, was puzzling (Klein et al., 2003; Liu et al. 1991; Shaffer et al. 2001). A *Myc*-reporter allele analysis showed that GC B cells expressed MYC to varying degrees with a small subset of MYC positive hyperproliferative cells, which are essential for the formation and maintenance of GCs (Calado et al. 2012). The expression in a defined subset of GC B cells explains why *Myc* could not be detected in bulk GC B cells (Calado et al. 2012; Dominguez-Sola et al. 2012; Ott et al. 2013). In pre-GC B cells, *Myc* deletion led to the complete absence of GC formation, highlighting its necessity for this transitional state (Calado et al. 2012). Nevertheless, MYC protein was not higher expressed in early GCs than in FO B cells which might be due to the fact that *Myc* transcription is an early event in this transition that peaks at ~2 hours after B cell activation (Kelly et al. 1983). In contrast, mature GCs showed a light zone (LZ) MYC-positive subpopulation that had just been activated, marked by higher MYC expression than FO B cells, was proliferative, and poised to re-enter the dark zone (DZ) (Calado et al. 2012). Interestingly, MYC was absent in highly proliferative DZ GC B cells. *Myc* ablation in mature GCs, led to a strong decrease of GC B cells, revealing the necessity for GC maintenance (Calado et al. 2012; Dominguez-Sola et al. 2012).

At the transcriptional level, the proposed model states that in the early stages of GC formation, *Myc* is transiently upregulated before *Bcl-6* expression (Ott et al. 2013). Once BCL6 is expressed, it directly represses MYC, which is later re-expressed in a subset of activated LZ GC B cells that begin to downregulate BCL6 (Basso et al. 2010; Calado et al. 2012; Shaffer et al. 2000). The LZ MYC-positive GC B cells are prepared to re-enter the DZ for further

proliferation, whereas MYC-negative LZ GC B cells may exit the GC by upregulating *Prdm1*, which represses MYC and promotes plasma cell differentiation (Calado et al. 2012; Lin et al.1997).

Intriguingly, the cell cycle program in GC B cells has been suggested to be MYC-independent due to the repressive relationship between MYC and BCL6 (Phan et al. 2005; Shaffer et al. 2000). BCL6 inhibits the expression of the MYC-target gene *Ccnd2,* whereas *Ccnd3*, a non-MYC target gene, plays an essential role in GC B cell proliferation (Bouchard et al. 1999; Cato et al. 2011; Perez-Roger et al. 1999; Shaffer et al. 2000).

In summary, MYC plays a role in GC formation as well as maintenance, and here, its role might involve the decision of a GC B cell to proliferate.

2 Results

2.1 CRISPR-Cas9 screen to identify regulatory factors of plasmablasts

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Comprehensive CRISPR-Cas9 screen identifies factors which are important for plasmablast development

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Plasma cells (PCs) and their progenitors plasmablasts (PBs) are essential for the acute and long-term protection of the host against infections by providing vast levels of highly specific antibodies. Several transcription factors, like Blimp1 and Irf4, are already known to be essential for PC and PB differentiation and survival. We set out to identify additional genes, that are essential for PB development by CRISPR-Cas9 screening of 3,000 genes for the loss of PBs by employing the *in vitro*-inducible germinal center B cell (iGB) culture system and Rosa26$^{Cas9/+}$ mice. Identified hits in the screen were *Mau2* and *Nipbl*, which are known to contribute to the loop extrusion function of the cohesin complex. Other examples of promising hits were *Taf6*, *Stat3*, *Ppp6c* and *Pgs1*. We thus provide a new set of genes, which are important for PB development.

KEYWORDS

plasmablast, CRISPR/Cas9, sgRNA libraries, plasmablast differentiation, NIPBL, MAU2

Introduction

The production and secretion of high-affinity antibodies is an essential part of the immune response and is the main function of plasma cells (PCs). When a mature B cell encounters an antigen, it differentiates into a short-lived, antibody-secreting plasmablast (PB) which can migrate to survival niches in the bone marrow and develop into a quiescent long-lived PC (1).

Several factors are known to be essential for PC differentiation and survival. To name some, the most prominent transcription factors are Blimp1 (2), Irf4 (3), the E-box proteins E2A and E2-2 (4), Aiolos (5) and Ikaros (6). We set out to identify additional genes essential for PB development and probed 3000 genes for their role in PB development in a Local Outlier Factor-based negative selection CRISPR screen in

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primary Cas9$^+$ B cells which we cultured in the iGB system (7). This system allowed us to culture Cas9$^+$ B cells for an extended period of time compared to other *in-vitro* PB culture systems which is necessary to evaluate loss of function effects on PB development. Newly identified targets in this screen will help us to gain a better understanding of which transcription factors directly or indirectly control PB development.

Methods

Mice

The *Rosa26*$^{Cas9/+}$ mice (8) and Blimp1$^{Gfp/+}$ mice (9) were maintained on the C57BL/6 genetic background. All animal experiments were carried out according to valid project licenses which were approved and regularly controlled by the Austrian Veterinary Authorities.

Antibodies for flow cytometry

The following monoclonal antibodies were used for flow cytometric analysis of the cultured cells: CD19-BV785 (1D3, BD), CD19-Fitc (1D3, Biolegend), CD19-BV421 (1D3, BD) CD23-Fitc (B3B4, ebioscience), CD43-PE (S7, Pharmingen), CD90.1-BV421 (OX-7, BD), CD138-APC (281-2, Biolegend), CD138-BV605 (281-2, BD) antibodies.

Definition and flow cytometric sorting

The cells were sorted with a FACS Aria machine (Becton Dickinson) as follows: activated B cells (act B, CD19$^+$CD23$^+$CD138$^-$), pre-plasmablasts (pre-PBs, CD19$^+$CD23$^-$ CD138$^-$), plasmablasts (PBs, CD19$^+$CD23$^-$CD138$^+$).

Selection criteria for genes tested in the screen

For the first screen we selected 998 genes, including *Irf4* and *Prdm1* as controls. 508 genes were at least 5-fold upregulated in RNA-Sequencing (RNA-Seq) data comparing either day-9 PBs (Blimp1$^+$CD138$^+$) and day-6 act B cells (Blimp1$^-$CD138$^-$) or day-9 pre-PBs (Blimp1$^+$CD138$^-$) and day-6 act B cells, cultured in the iGB system (7). 490 additional genes were selected as being at least 8-fold upregulated between mature B cells and PCs (10). We tested 6 single guide RNAs (sgRNAs) per gene except for some small genes where only fewer sgRNAs could be predicted.

For the second screen we targeted 2,000 genes, which were also selected from the RNA-Seq data of the iGB system. We selected genes, which had an expression of higher than 5 TPM in either the act B cells, pre-PBs or PBs. We annotated the genes and selected the categories kinases, ligand-dependent nuclear receptors, phosphatases, translation regulators, signal transducers, transcription factors, or chromatin modifiers. We then subtracted all genes which were included in the first screen or which are known to be essential for cell survival. We further excluded genes which have multiple locations in the genome. SgRNAs against *Prdm1*, *Irf4* and *Sdc1* were added as positive controls, as well as 500 neutral sgRNAs. We used 6 sgRNAs for most genes.

RNA-sequencing

Total RNA was prepared by using the RNeasy Mini kit (QIAGEN). Genomic DNA was eliminated by using a gDNA eliminator spin column (QIAGEN). Approximately 1–5 ng of cDNA were used as starting material for the generation of sequencing libraries. About 1–5 ng of cDNA was used as starting material for the generation of sequencing libraries with the NEBNext Quick Ligation Module and NEBNext Ultra Ligation Module and NEBNext End Repair/dA-Tailing module. DNA fragments of 150–700 bp were selected with AMPure XP beads (Beckman Coulter), followed by PCR amplification with the KAPA Real Time Amplification kit (KAPA Biosystems). Completed libraries were quantified with the Agilent Bioanalyzer dsDNA 1000 assay kit and Agilent QPCR NGS Library Quantification kit. Cluster generation and sequencing was carried out through use of the Illumina HiSeq 2000 system with a read length of 50 nucleotides, according to the manufacturer's guidelines.

Design of sgRNA

The design of the sgRNA was performed as described before (11).

Cloning of the library

The 81 mer oligo pools of the gene libraries were ordered from Twist Bioscience. The cloning was performed as described before (11).

LentiX transfection

LentiX 293T cells were transfected with the library pool, using a transfection mix of the library vector pool, the helper plasmid pCMV-R8.74 and the envelope plasmid pCMV-Eco (4:2:1) by adding PEI (MW 25K, Polyscience, 24µg/ml). After

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24 hours, the medium was changed to the target medium which was harvested after 12 hours and filtered (0.2 µm filters).

Transduction of iGB-cultured B cells

The spleens of 8 week old Rosa26$^{Cas9/+}$ mice were harvested and magnetically enriched using the B cell isolation kit (130-090-862 Miltenyi). For each sample one female and one male mouse were pooled and the cells were plated at 0.5 mio/ml in 2 ml on 6 well plates in iGB medium (RPMI medium supplemented with 10% fetal calf serum, 1 mM glutamine, 50 µM beta-mercaptoethanol, 10 mM Hepes and 1 mM sodium pyruvate), plus LPS (Sigma-Aldrich, 25 µg/ml), IL-4 (produced inhouse, 20 ng/ml), CD40L (R&D system, 100 pg/ml) and Baff (AdipoGen Life science, 100 pg/ml). After one day of culture the cells were transduced with the Lentiviral library using spinfection and Polybrene (Millipore, 5 µg/ml, 45 min, 2,400 rpm, 32°C). On day two the cells were counted and seeded on 75 cm^2 flasks as 3 mio/flask in 25 ml iGB medium + IL-4 (20 ng/ml) + CD40L (100 pg/ml) + Baff (100 pg/ml) + G418 (Gibco, 200 µg/ml) on inactivated 40LB cells. The 40LB cells were cultured in DMEM supplemented with 10% fetal calf serum and either inactivated by 30 minutes irradiation (screen 1) or with mitomycine (Sigma-Aldrich, 10 µg/ml) which was added for 3 hours at 37°C (screen 2). 15 ml iGB medium + IL-4 (20 ng/ml) + CD40L (100 pg/ml) + Baff (100 pg/ml) +G418 (200 µg/ml) was added on day 4. On day 6 all cells were harvested, counted and reseeded or sorted. For reseeding, 3 mio cells were plated per flask in 50 flasks in 24 ml iGB medium + IL-21 (produced inhouse, 10 ng/ml) + CD40L (100 pg/ml) + Baff (100 pg/ml) + G418 (100 µg/ml). The rest of the cells was sorted for CD19$^+$CD90.1 (Thy1.1)$^+$CD23$^+$CD138$^-$ (act B) and the pellet was washed with PBS and frozen at -80°C. On day 9, the cells were harvested and sorted for CD19$^+$CD90.1(Thy1.1)$^+$CD23$^-$ CD138$^-$ (pre-PBs) and CD19$^+$CD90(Thy1.1)$^+$CD23$^-$CD138$^+$ (PBs). The pellets were frozen at -80°C. A representation of at least 500 cells per sgRNA was maintained for all samples.

Library preparation for sequencing

DNA was prepared as previously described (11), as was the labeling for the sequencing (12). The PCR product was cleaned after both reactions by either AMPure XP beads (A63881, Beckman Coulter) (screen 2) or by agarose gel purification (screen 1). Sequencing was performed by the use of the Illumina HiSeq 2000 system with a read length of 50 nucleotides with 8% PhiX added, according to the manufacturer's guidelines.

Bioinformatical analysis

The bioinformatic analysis was done using MAGeCK (13). In the first step we calculated the guide-based DE expression, which was count based, similar to DESeq/EdgeR. We performed pairwise comparisons and calculated the log2-fold change (lfc_{gene} = mean($l2fc_{gene\_sgRNAs}$)) and p-value. In a second step we ranked the sgRNAs based on the *P*-value (robust rank aggregation). We then tested for positive effects (elimination of gene leads to boost of cell growth) and negative effect (elimination of gene leads to cell death).

Results

To identify novel factors required for PC or PB development and survival, we performed two separate CRISPR-Cas9 screens in the iGB system with two distinct PB/PC transcriptome based libraries. This divided approach was used because targeting everything in one approach would not have been experimentally feasible. To enrich the library for potentially relevant genes, we employed the following transcriptomics based strategy: Screen 1 contained sgRNAs targeting 998 genes of which 508 are 5-fold upregulated either between act B and pre-PBs or between act B and PBs in the iGB system (Figure 1A). Another 480 genes were at least 8-fold upregulated between mature B cells and PCs (10). By including genes that are primarily expressed in PBs and their precursors, we expected to target factors that are truly relevant to PB development and not earlier in the B cell lineage. Screen 2 targeted 2000 selected genes, which are expressed at a TPM of at least 5 in either act B, pre-PBs or PBs in the iGB (Figure 1B) and belong to selected gene categories (Figures 1B, C). This approach allowed us to test genes which are expressed in late B cell development regardless of whether they show any difference in their expression. They might, nevertheless, play a more subtle role in PB development. SgRNAs against *Prdm1* (Blimp1), *Irf4* and *Sdc1* (CD138) served as positive controls in both screens.

To enable 'near-physiological' B cell differentiation *in vitro*, we used the iGB system, in which B cells from the spleen are cultured on 40LB feeder cells which express CD40L and Baff for up to 10 days (7). As B cells are rather difficult to transduce, we modified the culture to efficiently achieve high transduction rates in the following way (Figure 1D). First, B cells from *Rosa26*$^{Cas9/+}$ mice (8) were isolated by magnetic cell sorting (Figure 1E) and plated in iGB medium containing IL-4, CD40L, Baff and LPS and were kept in culture for 24 hours. This activation of B cells increases transduction efficiency. Then the cells were transduced using ecotropic envelope packaged lentiviral particles (Figure 1F). After another 24 hours the cells were plated on the 40LB feeder cells in iGB medium containing G418. On day 6 and 9 of the culture the cells were sorted for day-6 act B cells (CD19$^+$ Thy1.1$^+$ CD23$^+$ CD138$^-$),

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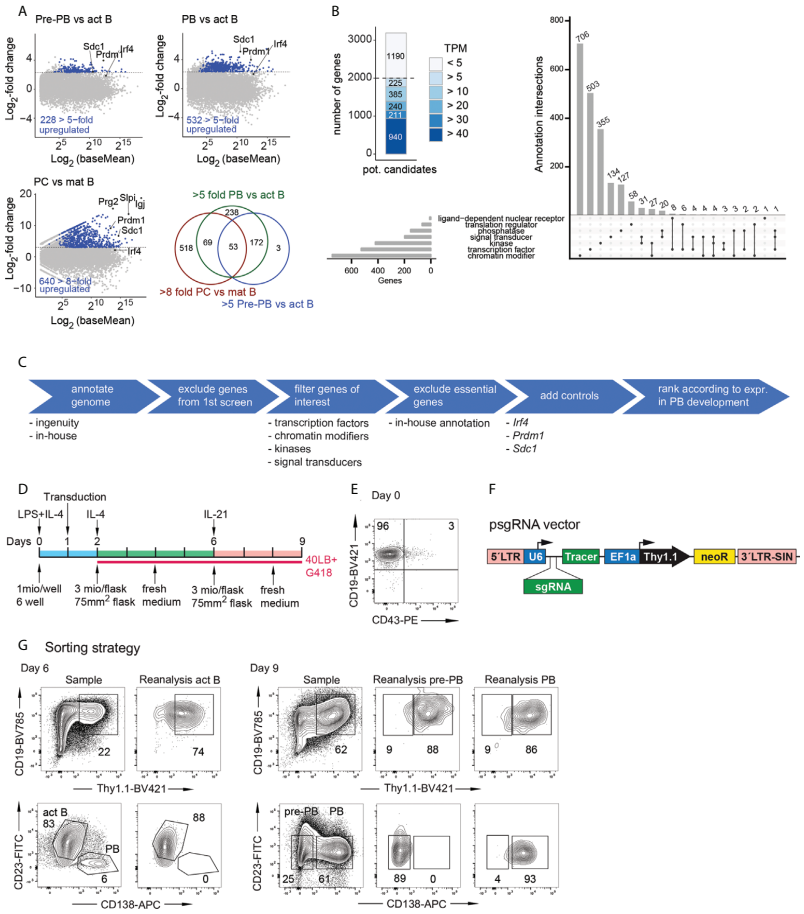

FIGURE 1
Experimental setup of the screens. **(A)** M/A blots depicting the overview of the genes which were chosen for screen 1. Data from RNA-Seq of iGB derived act B cells ($CD19^+Blimp1^-CD138^-$), pre-PB ($CD19^+Blimp1^+CD138^-$), or PB ($CD19^+Blimp1^+CD138^+$) from $Blimp1^{Gfp/+}$ mice. Chosen genes had to be at least 5-fold upregulated in the act B vs pre-PBs or act B vs PBs comparison (top row). RNA-Seq of PCs and mature B cells, depicted are genes which are 8-fold upregulated (10) (bottom row left). Overlap of the genes of the 3 M/A blots (bottom row right). **(B)** Overview of the genes of screen 2. Genes were selected by the criteria in **(C)** and grouped by their expression. Genes which did not reach an expression of at least 5 transcripts per million (TPM) in the act B, pre-PBs or PBs were discarded (left blot). The remaining genes fall into the depicted categories (Upset blot). **(D)** Timeline of the iGB culture for the screens. The cells were transduced on day 1, seeded on 40LB cells on day 2 und 6 and G418 was added from day 2 on. **(E)** Flow cytometric analysis of the splenic B cells after magnetic cell enrichment. **(F)** Structure of the psgRNA-U6-Thy1.1 vector. LTR= long terminal repeat, U6 = U6 promotor, sgRNA = single guide RNA, Tracer = tracer sequence, used for the pooled sequencing of the screen, EF1a = EF1a promoter, neoR = neomycin resistance. **(G)** Flow cytometric sorting of the cells of screen 2 on day 6 (left) or day 9 (right). The unsorted sample is shown and the reanalysis of the different populations after the cell sort.

day-9 pre-PBs ($CD19^+Thy1.1^+CD23^-CD138^-$) and day-9 PBs ($CD19^+Thy1.1^+CD23^-CD138^+$) (Figure 1G). Considering the timeframe of 9 days, we were not only able to efficiently delete a given gene of interest in act B cells but also to look at subsequent effects on PB development.

For screen 1 we obtained data from four replicates of the act B cells and pre-PBs and three replicates of the PBs. When we compared the act B cells with the PBs, we found sgRNAs targeting 5 genes which were significantly depleted in the PB population with a false discovery rate (fdr) of at least 1%

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(Figure 2A; Supplementary Table 1). Besides our positive controls *Prdm1* and *Sdc1*, those were *Rexo2, Dnajc3* and *Cd44*. Another 5 genes had a fdr of at least 10% (*Slc7a5, Pim1, Ern1, Tmed10* and *Irf4)* and 1 gene, *BC003331*, had a fdr of less than 25%. Besides the positive controls *Prdm1* and *Sdc1*, sgRNAs targeting *Ern1* and *Dnajc3* resulted in a depletion of PBs compared to pre-PB (Figure 2B; Supplementary Table 1). Based on the effect size of the screen we selected 19 genes for single knockout validations in the iGB system. To score the effect of each knockout on PB differentiation capacity, we compared Thy1^{+} with Thy1^{-} PBs. To correct for PB formation capacity of infected versus non-infected Rosa26$^{Cas9/+}$ cells, this ratio was normalized to the neutral control knockouts empty (empty vector) and Chr1 (a sgRNA targeting a gene desert region on chromosome 1). As a positive control, samples were transduced with sgRNAs targeting *Prdm1*. We could confirm *Slc7a5,*

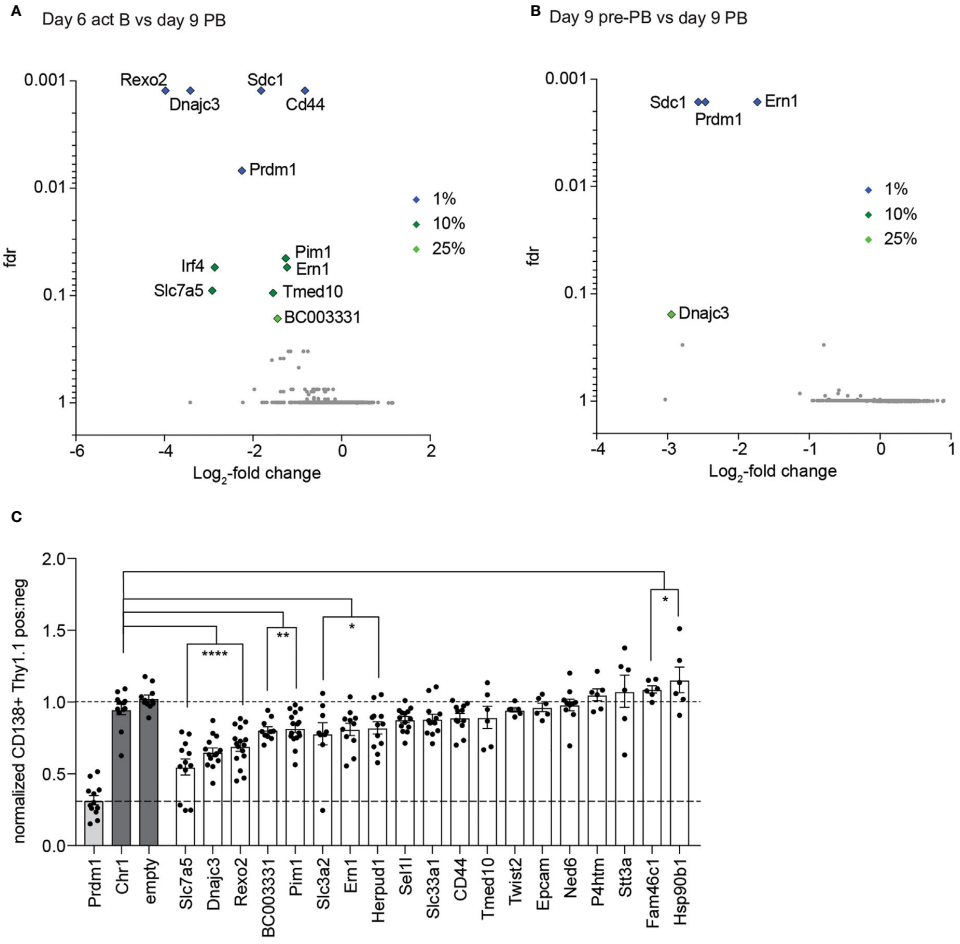

FIGURE 2
Results of screen 1. **(A, B)** Results of screen 1. Volcano blots, depicting the negative enrichment of sgRNAs, from the comparison act B vs PBs **(A)** and pre-PBs vs PBs **(B)**. Fdr= false discovery rate. **(C)** Single guide validation of first screen. The ratio of the amount of PBs in the Thy1.1 negative and positive population was calculated and normalized to the average of the value of the negative controls Chr1 (targeting a gene free region on Chromosome 1) and empty vector (the plasmid without sgRNAs). Top two performing sgRNAs from the screen were used for each gene. Each dot represents a replicate. All statistical data show as mean values with SEM; *P < 0.05; **P < 0.01; ****P < 0.0001 (Student's *t* test).

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Dnajc3, Rexo2, BC003331, Pim1, Slc3a2, Ern1 and *Herpud1* as being important for PB development or PB survival (Figure 2C).

Screen 2, which contained sgRNAs targeting 2000 genes that are expressed in act B cells, pre-PBs and PBs, was performed and analyzed in a similar manner as screen 1. We obtained 2 replicates of independent iGB cultures each of the 6 days act B cells, 9 days pre-PBs and PBs with a representation of at least 500 cells per sgRNA. We also sequenced the sgRNA pool used for transduction of the cells. To remove genes which were essential for all tested cell populations, we first compared the initial pool of sgRNAs with the act B cells and defined genes which scored in this comparison with a log-fold change (lfc) of smaller than -1 and a fdr of less than 1% as essential. The remaining genes were selected for a lfc < -1 and sorted by their fdr of the comparison act B versus PBs to identify the most promising hits. For screen 2, this revealed 26 genes with a fdr < 0.1%, 30 genes with a fdr < 1%, 48 genes with a fdr < 10% and 15 genes with a fdr < 25% (Figure 3A; Supplementary Table 2). Our controls *Prdm1* and *Irf4* scored in this analysis, as did *Ikzf1* (Ikaros) and *Tcf3* (E2A), which have also been shown to be essential for PCs (4, 6). Similarly, we compared the initial pool of sgRNAs with the pre-PBs and defined genes which scored in this comparison with a lfc < -1 and a fdr of less than 1% as essential. We identified the most promising hits in the comparison pre-PBs with PBs and found 20 genes with a fdr < 0.1%, 2 genes with a fdr < 1%, 9 genes with a fdr < 10% and 4 genes with a fdr < 25% (Figure 3B; Supplementary Table 2). We validated 53 of the hits from both analyses in single validations using the 2 best performing sgRNAs from the screen (Figures 3C–E). We looked at 2 criteria. First, whether the cell survival of the transduced cells was impaired, by comparing the amount of transduced cells on day 6 and day 9 (Figures 3C, D) and second whether the PBs in the transduced cell population were reduced (Figures 3C, E). 39 genes showed a reduction in PBs without impairing the survival of the cells. The high confidence hits with the biggest impact specifically on PBs were *Mau2, Nipbl, Stat3, Ppp6c, Pgam1, Pgs1, Kdm1a, Ippk, Eef1g, Jak3, Hbs1l, Ppp2r4, Dnttip1, Tada3, Brd2, Hira, Dpy30, Pgk1, Pik3c3, Dnajc2, Gtf3c6, Ddx1* and *Elmsan1*.

Discussion

In this study, we screened 3,000 genes for their impact on PB development and survival. We expected to find new promising genes in the first screen where 1000 genes, which are upregulated in the PBs or PCs, were targeted. Strongly upregulated genes are often expected to be important for the respective cell type, but surprisingly the number of genes of which the depletion impacted the PBs was rather small. One of the top hits from this screen were the genes *Slc7a5* and *Slc3a2*, which together form CD98. It has been shown that the loss of CD98 leads to strongly reduced PC numbers (14), consistent with our finding. The second screen in which 2000 genes of different important categories which are at least moderately expressed in the PBs were screened, revealed a vaster number of interesting genes. 24 of those genes can be related to biosynthetic pathways (15), which is in line with the massive metabolic changes PBs undergo in order to maintain a high antibody secretion (16) and are thus sensitive to the loss of genes involved in metabolism. However, the genes in the second screen were preselected for certain categories, making a functional enrichment analysis rather biased. The hits in this screen also included the genes *Nipbl* and *Mau2*, which are known to contribute to the loop extrusion function of the cohesin complex (17, 18). Those factors have never been shown to be specifically important for PBs. Another top scoring candidate gene, *Kdm1a* (Histone demethylase LSD1), has been shown to regulate B cell proliferation and PB differentiation which further validates the validity of our approach (19). In total we could confirm 8 hits from the screen 1 and 39 hits from the screen 2 to be essential for PBs without affecting the overall cell survival in the iGB system, which means that we have identified 47 hits to be exclusively relevant to PB development. An important step for the future would be an *in vivo* validation of those hits.

One advantage of our culture system over other common B cell cultures, like LPS stimulation, was the culture time. Act B cells were cultured for 6 days before we induced PB development. As the cells were infected on day 1 of the culture with the sgRNA library to induce gene inactivation, loss of the respective proteins from the cells should be complete at day 5 after infection. Hence by the time PB development was induced, the cells were full knockouts of the targeted genes.

A similar screen was recently published (20). While our approach followed the same principle, there are numerous differences. The screen of Newman et al. (2021) largely focused on the difference between $IgG1^+$ versus IgE^+ PBs, while we compared different developmental stages (act B cells, pre-PBs versus PBs). Our screen thus focuses on the development that leads to the PB formation while theirs looks at differences when the cell are already PBs. There are further differences, like the screened sgRNA library itself. Newman et al. used an untailored sgRNA library, which allows them on one hand to find unexpected targets, but on the other hand, includes a lot of sgRNAs targeted against genes which are not expressed in PBs at all. Our sgRNA library was tailored for the iGB cells and contained all genes, which are upregulated between the act B cells and the PBs or between mature B cells and PCs and another 2,000 genes which are expressed in those cell types and fall into categories of interest.

With our approach we managed to target 3,000 genes and thus supply a new and extensive list of genes that are important for the development or survival of plasmablasts.

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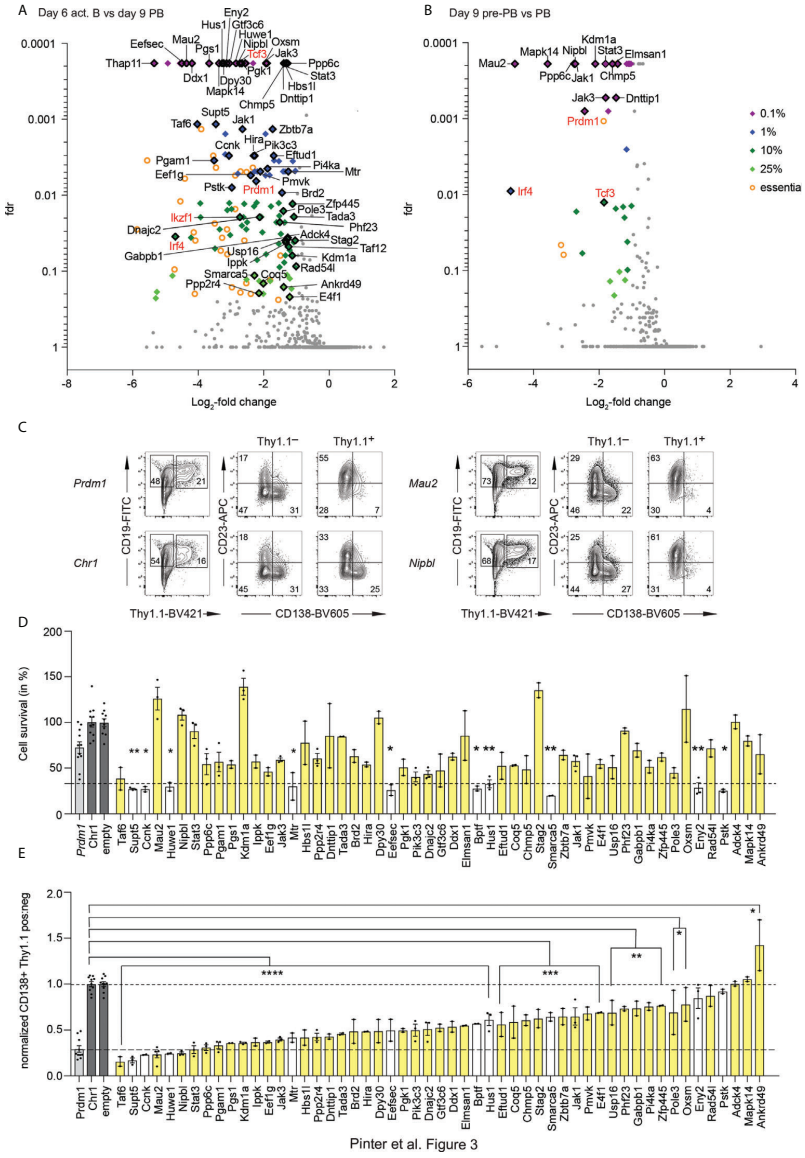

FIGURE 3
Results of screen 2. **(A, B)** Volcano blot of the screen 2, depicting interesting hits in the comparison of the act B from day 6 versus the PBs from day 9 **(A)** and the pre-PBs versus the PBs from day 9 **(B)**, as in Figure 1G. Fdr = false discovery rate. Factors which are known to be essential for PCs are highlighted in red. **(C)** Example of single guide validation. Control Chr1 is targeting a gene free region on Chromosome 1. **(D, E)** Single guide validation. Cell survival was determined by the ratio of the amount of Thy1.1$^+$ cells on day 6 versus day 9, normalized to Chr1 and the empty vector (the plasmid without any sgRNAs). Stars indicate significant difference compared to Chr1 **(D)**. The ratio of the amount of PBs in the Thy1.1 negative and positive population was calculated and normalized to the average of the value of the negative controls Chr1 and empty vector to determine the impact of the sgRNA on the PBs **(E)**. One or two best performing single sgRNAs from the screen were used. Each dot represents one replicate. Genes colored in white showed reduced survival in **(D)**. All statistical data are shown as mean values with SEM; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001 (Student's *t* test).

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/, GSE203436.

Ethics statement

The animal study was reviewed and approved by Austrian Veterinary Authorities. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

TP performed the single guide validation of the second screen, MaF performed the bioinformatic analysis, MiF cloned the sgRNA library, JJ and MS provided assistance and knowledge for the design of the project and the preparation of the DNA for the sequencing, JZ provided the tools for the sgRNA prediction and the library plasmid, MB and MW planned the project and MW performed the screens, the single guide validation of the first screen and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.979606/full#supplementary-material

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2.2 Role of the SAGA-HAT in B cell development

2.2.1 The SAGA-HAT module was identified in a CRISPR-Cas9 PB screen

The SAGA-complex comprises several functional modules, including a histone acetyltransferase (HAT)-module (**Fig. 1A**). Several members of the HAT are shared with the related ATAC-complex, except for TADA2b, which is only found in SAGA, whereas TADA2a is unique for ATAC (**Fig. 1A**). These two proteins allow differentiation between the two HAT modules, while TADA3 is found in both HAT modules (**Fig. 1A**).

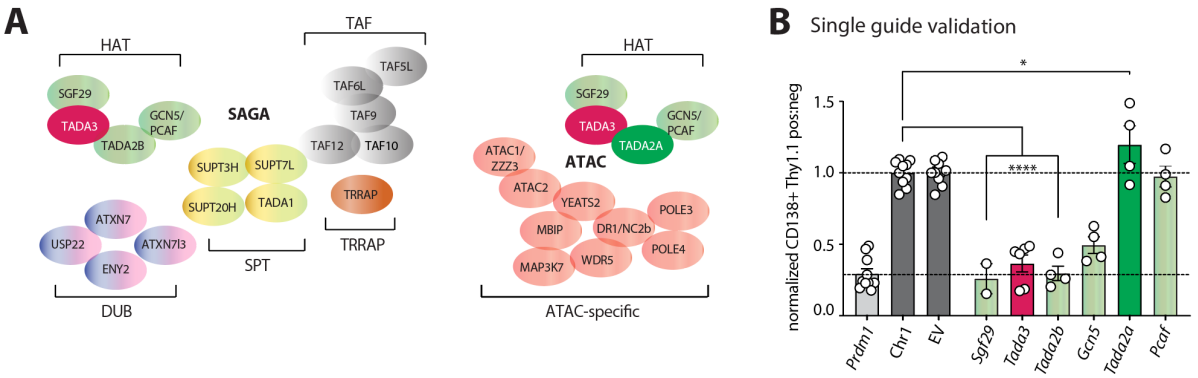

Fig. 1. | Components of the SAGA-complex and their effect on PB-formation.
a, Schematic of proteins and modules of the SAGA-complex and the ATAC-complex in mice. Four of five modules are shown for SAGA-complex and all for ATAC-complex. HAT, Histone-acetyltransferase module; DUB, Deubiquitinilation module; TAF, TBP-associated factors (core module); SPT, Suppressor of Ty (core module); TRRAP, Transformation/Transcription domain Associated Protein module. **b**, Single guide validation of SAGA-HAT and ATAC-HAT genes. The ratio of the amount of PBs in the Thy1.1 negative and positive population was calculated and normalized to the average of the value of the negative controls Chr1 (targeting a gene free region on Chromosome 1) and empty vector (EV, the plasmid without sgRNAs) to determine the impact of the sgRNA on PB formation. Top two performing sgRNAs from the screen were used for each gene. Each dot represents a replicate. All statistical data are shown as mean values with SEM; *$P < 0.05$; ****$P < 0.0001$ (Student's *t*-test).

We conducted a sgRNA-CRISPR/Cas9 dropout screen targeting genes that may be important for PB development (Pinter et al. 2022). Briefly, Cas9-expressing mature B cells were cultured in the iGB system (Nojima et al. 2011), stimulated to initiate PB formation, and infected with sgRNAs to promote gene deletion. Successful infection was indicated by the expression of Thy1.1$^+$. Subsequently, the cells were analyzed, and their DNA was sequenced on day 6 (act B) and day 9 (PB) of the culture. The sequences of the remaining cells were compared to the initial pool of sgRNAs, and the absence of sgRNA indicated the necessity of this gene for the development of the respective cell stage. Genes that were already important for B cell activation (day 6 compared to the initial pool) were excluded, as we wanted to focus

on genes that merely play a role in PB formation. Therefore, we only considered sgRNAs that led to cell loss between B-cell activation and PB formation (**Fig. 2A, left**). Here, *Tada3*, a member of the HAT module of the SAGA-complex, was identified to be important for PB formation, as well as other SAGA-complex genes (**Fig. 2A, left**). When we compared PBs to pre-PBs (day 9), more members of the SAGA HAT-module showed relevance to this developmental step, and *Tada3* and *Sgf29* were among the top hits with *Tada2b* just below our cutoff of 2-fold change (**Fig. 2A, right**). To verify the importance of SAGA-complex genes in PB formation, we single validated the sgRNAs targeting these genes, analyzed the cells by flow cytometry, and quantified the effect by comparing sgRNA-infected Thy1.1-expressing PBs to unedited Thy1.1$^-$ PBs (day 9), and normalized this to an empty vector control, which had no effect on PB formation (**Fig. 1B; Fig. 2C**). All SAGA-HAT genes were relevant for PB formation and had an effect of similar magnitude to the known PB gene *Prdm1* (**Fig. 1B**, **Fig. 2C**). To ensure that the genes were not generally lethal to the cells, we assessed the viability of edited cells by comparing the Thy1.1-infection rate between days 6 and 9 of the culture (**Fig. 2B**). The loss of SAGA-HAT genes did not affect the survival of all sgRNA-infected cells but was PB-specific (**Fig. 1B, Fig 2B**). This was in contrast to the ATAC-specific gene *Tada2a* and *Pcaf*, which had no effect on PB formation (**Fig. 1B**). The loss of other SAGA-subunit genes (*Taf6l, Atxn7l, and Eny2*) reduced cell viability, indicating an earlier function in B cell development (**Fig. 2B**). Next, we examined the expression of SAGA-HAT genes during B-cell development (**Fig. 2D**). All SAGA-HAT genes were ubiquitously expressed to similar degrees, whereas *Tada2a* and *Pcaf* were barely detectable in B cells (**Fig. 2D**). Hence, the SAGA-complex, and specifically its HAT-module, might play a role in PB development, and this is not the case for the related ATAC-complex.

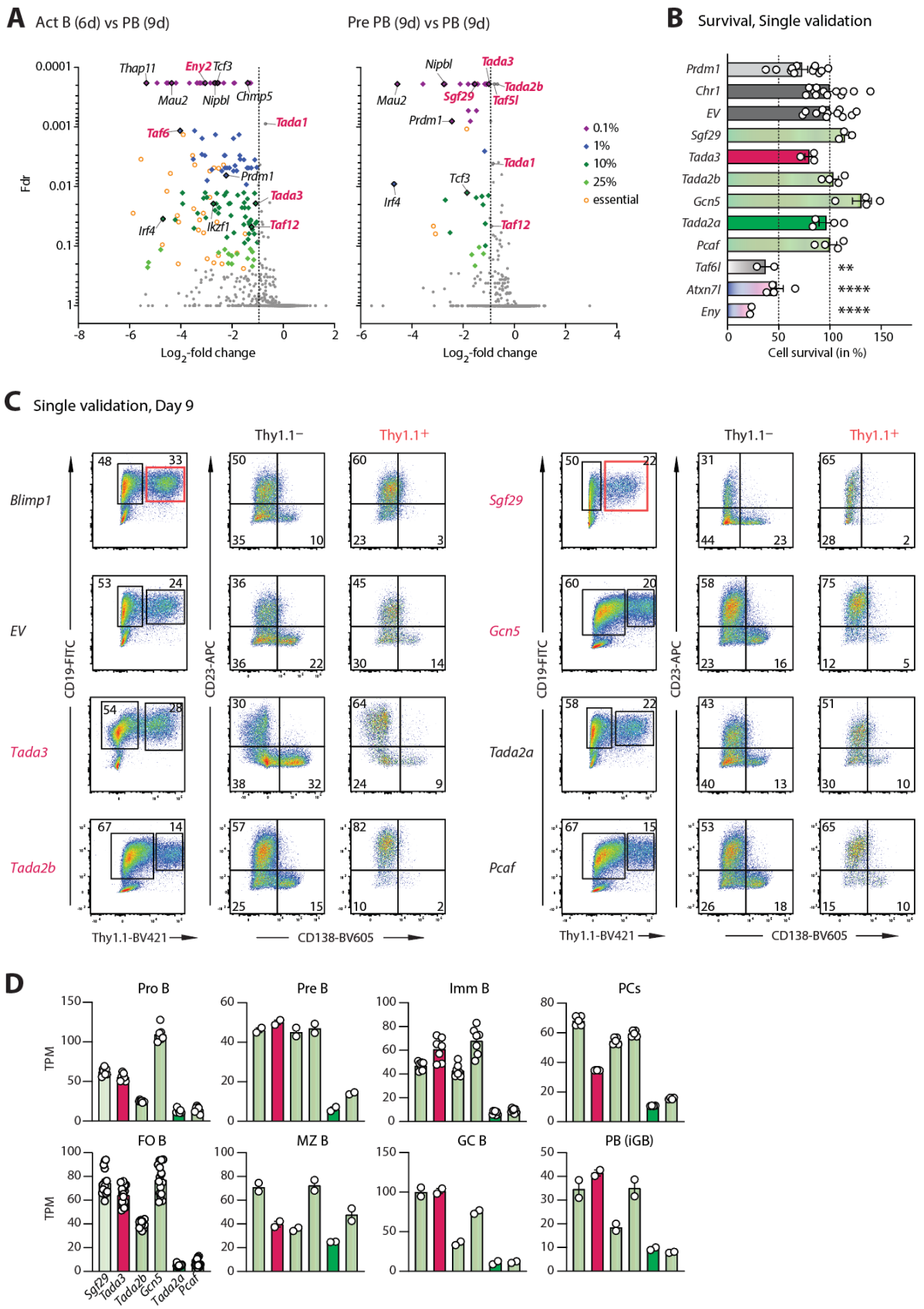

Fig. 2 | SAGA-HAT proteins affect PB formation and are expressed in B cells.

a, Differentially expressed genes of the screen 2, depicting interesting hits in the comparison of activated B cells (Act B, day 6) versus plasmablasts (PBs, day 9) (left) and the pre-PBs versus the PBs from day 9 (right). Fdr = false discovery rate. SAGA-complex genes are highlighted in pink. **b**, Survival of sgRNA-infected cells. The cell survival

was determined by the ratio of the amount of Thy1.1^{+} cells on day 6 versus day 9, normalized to Chr1 and the empty vector (the plasmid without any sgRNAs). Stars indicate significant difference compared to Chr1. Each dot represents one replicate. Only HAT genes showed no survival defect in bulk B cell populations. All statistical data are shown as mean values with SEM and are analyzed with one-way ANOVA with Holm-Šídák multiple comparison test; **P < 0.01; and ****P < 0.0001. **c**, Flow cytometric analysis of single guide validation of SAGA-HAT and ATAC-HAT genes on day 9. Control Chr1 is targeting a gene free region on Chromosome 1. Thy1.1-expression indicates sgRNA-infected cells. Act B (CD23^{+} CD138^{-}), pre-PB (CD23^{-} CD138^{-}), PB (CD23^{-} CD138^{+}). One of at least two experimenst is shown. **d**, The expression values (TPM) of SAGA-HAT and ATAC-HAT genes in B cell development. TPM values are shown for early B cells in the bone marrow (top) and B cells in the spleen (bottom). Two genes are highlighted (*Tada3,* pink; *Tada2a*, dark green).

2.2.2 Absence of *Tada3* in early B cell development affects pro-B to pre-B cell differentiation

Because of the ubiquitous expression profile of the SAGA-complex, we decided to analyze the role of the HAT module in B cell development *in vivo*. We focused on *Tada3* since a floxed allele was available (Mohibi et al. 2012), and despite being shared with the ATAC-complex, possible phenotypes would represent exclusively the role of SAGA-HAT due to the lack of expression of *Tada2a* in B cells (**Fig. 2D)**. *Tada3*$^{fl/fl}$ mice were crossed with different Cre-recombinase-expressing mouse lines, which allowed the deletion of *Tada3* at different developmental stages of B cells. *Cd79a*-Cre initiates gene deletion during the transition from pre-pro-B cells to fully committed pro-B cells (Kwon et al. 2008) and was used to delete the floxed *Tada3* allele. Flow cytometry analysis showed that pre-B cells and all subsequent B cell populations were lost in the bone marrow of *Cd79a*-Cre *Tada3*$^{fl/fl}$ mice compared with *Tada3*$^{fl/fl}$ littermate controls (**Fig. 3A, 3B**). Cell counts suggested that pro-B cells were not significantly reduced in the absence of *Tada3*, and PCR amplification of sorted pro-B cells revealed the efficient deletion of the floxed allele (**Fig. 3A, 3C**). These observations suggest a developmental block at the pro-B cell stage. We did not find an accumulation of pro-B cells and hypothesize that there is a possible dysfunction in pre-BCR expression and transition from pro-B to pre-B cells. Overall, the observed loss of cells between the pro-B and pre-B cell stages in the absence of *Tada3* suggests a role for SAGA-HAT in the cell transition, which precedes our previously suggested role in PB development.

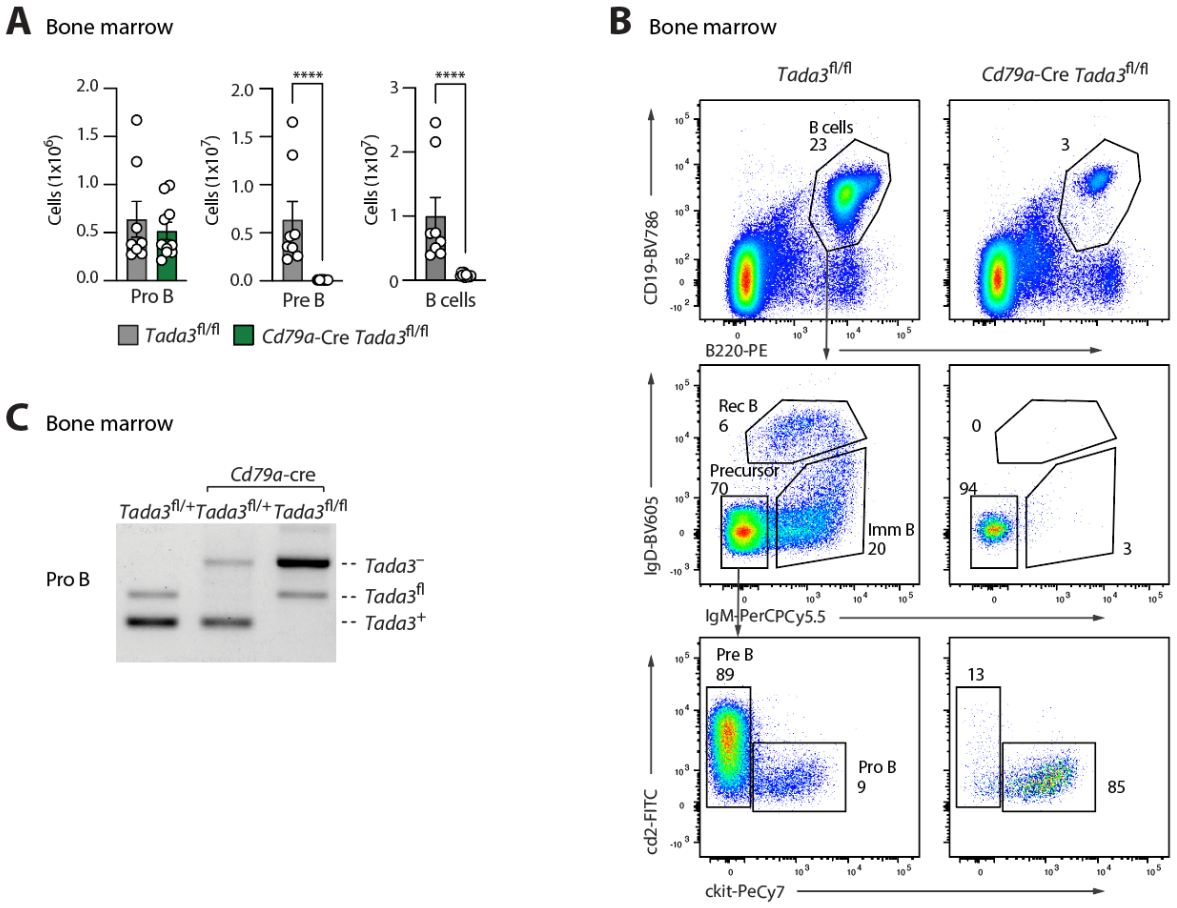

Fig. 3 | Early B cell development in *Tada3*-deficient animals.
a and **b**, Flow cytometric analysis of B cells in the bone marrow of *Cd79a*-Cre *Tada3*$^{fl/fl}$ and *Tada3*$^{fl/fl}$ control mice at the age of 5 weeks. The cell numbers (a) refer to the percentage of cells in the indicated gate (b). (a) Two independent experiments are shown as mean value with SEM and the data are analyzed with Mann-Whitney test; ****$P < 0.0001$. (b) One of two experiments is shown. Imm, immature; Rec, recirculating. The flow cytometric definition of the different B cell types is also shown in Methods. **c**, PCR analysis of the deletion efficiency of the floxed *Tada3* allele in sorted pro-B cells of *Cd79a*-Cre *Tada3*$^{fl/fl}$, *Cd79a*-Cre *Tada3*$^{fl/+}$ and *Tada3*$^{fl/+}$ mice.

2.2.3 *Tada3*-deletion in mature B cells

Once B cells leave the bone marrow, they migrate to secondary lymphoid organs, where they reside as mature but quiescent FO B and MZ B cells (Loder et al. 1999). These cells can get activated upon encountering antigens, which can eventually give rise to PBs. To investigate whether *Tada3* also plays a role in *in vivo* PB formation or at an earlier cell stage, we used the *Cd23*-Cre mouse line, which initiates deletion in immature B cells and shows complete deletion in mature B cells of the spleen (Kwon et al. 2008). *Cd23*-Cre *Tada3*$^{fl/fl}$ mice were analyzed using flow cytometry and compared to *Tada3*$^{fl/fl}$ littermate controls (**Fig. 4A, 4B**). Both MZ B and FO B cells were detected to a similar extent as in the controls (**Fig. 4A**).

Deletion of the floxed allele by *Cd23*-Cre driven recombinase was assessed in both cell types and displayed efficient deletion (**Fig. 4C**). This suggests that SAGA-HAT does not play a vital role in the maturation of B cells.

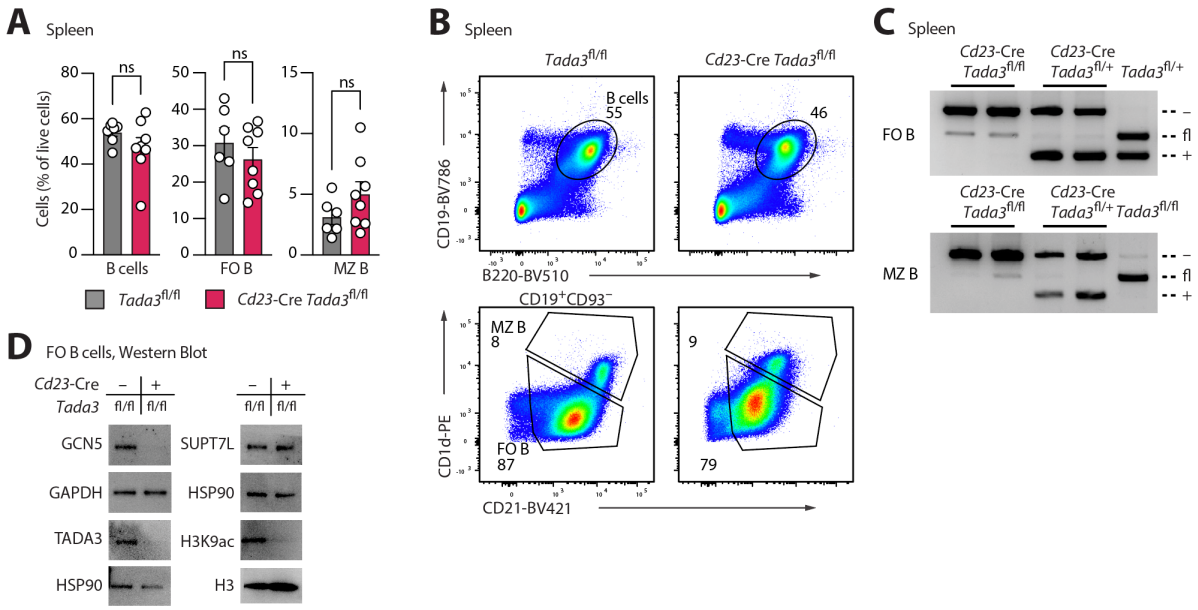

Fig. 4| Late B cell development in *Cd23*-Cre *Tada3*$^{fl/fl}$ mice and loss of the SAGA-HAT.
a and **b**, Late B cell development in *Cd23*-Cre *Tada3*$^{fl/fl}$ and *Tada3*$^{fl/fl}$ mice, as determined by flow cytometry of splenocytes from 10-13-week old mice. The frequencies of total B, FO B and MZ B are shown as mean values with SEM and were tested with Student's *t*-test. (**b**) Corresponding flow cytometry plots of the indicated genotypes. *Cd23*-Cre *Tada3*$^{fl/fl}$ and *Tada3*$^{fl/fl}$ mice were analysed on the same day and one of two experiments is shown. The percentage of cells is indicated next to the gate of the different B cell types. **c**, FO B and MZ B cells from the spleen of *Cd23*-Cre *Tada3*$^{fl/fl}$ *Cd23*-Cre *Tada3*$^{fl/+}$ and *Tada3*$^{fl/fl}$ mice were sorted and DNA from the cells was PCR-amplified to detect deletion efficiency of the *Tada3*$^{fl/fl}$ allele. **d**, Immunoblot analysis of SAGA-complex proteins. Whole cell extracts from *Cd23*-Cre *Tada3*$^{fl/fl}$ and *Tada3*$^{fl/fl}$ FO B cells were used. For the HAT-module: GCN5 and TADA3 were detected and GAPDH and HSP90 served as loading controls, respectively. For the SAGA-core, SUPT7L was detected and anti-HSP90 antibody was used as a loading control. Anti-H3K9ac antibody was used to detect SAGA-acetylation activity and anti-H3 antibody was used as a loading control.

To understand whether the deletion of *Tada3* in mature B cells led to the exclusive loss of the HAT-module or the entire SAGA-complex, we examined the protein expression of HAT components as well as the core module in *Cd23*-Cre *Tada3*$^{fl/fl}$ FO B cells (**Fig. 4D**). The absence of TADA3 was confirmed, and the enzyme GCN5 was not detectable, indicating that the HAT module was lost (**Fig. 4D**). By contrast, a member of the core module, SUPT7L, which is vital for complex integrity, was still present (**Fig. 4D**). This is in line with previous observations in mESCs, where the deletion of *Tada2b* led to disassembly of the HAT-module but did not affect the remaining complex (Fischer et al. 2021). As H3K9ac is the main acetylation mark of the

SAGA-complex, we probed for this in *Cd23*-Cre *Tada3*$^{fl/fl}$ FO B cells and, surprisingly, observed an almost complete loss (**Fig. 4D**), suggesting that resting FO B cells did not depend on H3K9ac.

To assess the capacity of *Cd23*-Cre *Tada3*$^{fl/fl}$ mice to mount an immune response *in vivo,* we immunized *Cd23*-Cre *Tada3*$^{fl/fl}$ mice and littermate controls with the T cell-dependent antigen NP-KLH, which induces a robust GC reaction and PB formation in control animals (**Fig. 5A, 5B**). In contrast, animals with B cell-specific deletion of *Tada3* not only showed a significant reduction in PBs as well as NP-specific PBs, but more strikingly failed to form GC B cells (**Fig. 5A, 5B**). In line with this finding, no NP-specific ASCs were detected (**Fig. 5C**). PCR amplification of the floxed allele from the remaining PBs of immunized mice showed insufficient deletion, indicating that there might have been counter selection (**Fig. 5D**).

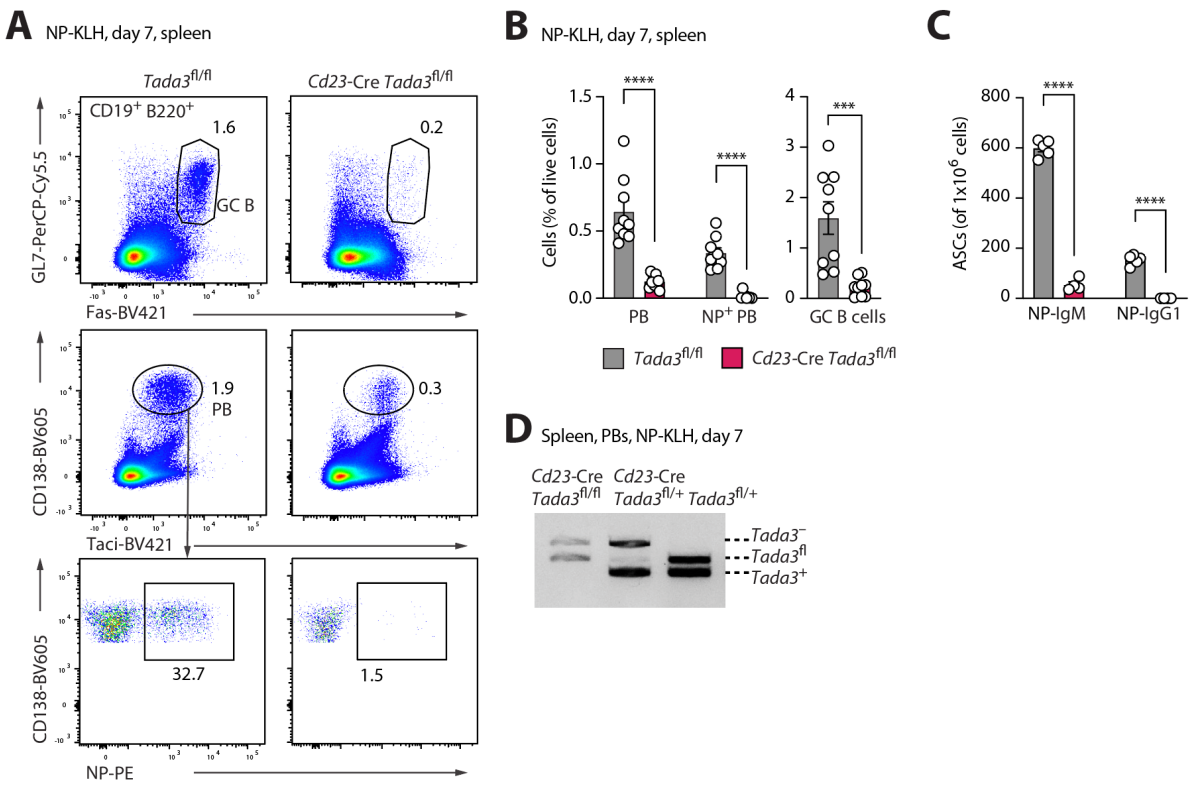

Fig. 5 | No germinal center formation and affected plasma cell formation in the absence of *Tada3*. **a–d**, *Tada3* is required for GC B cells and PBs. Flow cytometric analysis of splenic B cells from mice of the indicated genotypes at the age of 8-12 weeks at day 7 after immunization with NP-KLH (**a**). (a) Two independet experiments are shown as mean value with SEM (**b**) and the data were analyzed with *t*-test (PBs) or Mann-Whitney test (NP$^+$-PBs); ***$P < 0.001$, ****$P < 0.0001$. (**c**) ELISPOT analysis of splenocytes of NP-KLH immunized *Cd23*-Cre *Tada3*$^{fl/fl}$ and *Tada3*$^{fl/fl}$ mice on day 7 after immunization. NP-specific IgM or IgG1 secreting ASCs were detected and data are shown as mean values with SEM. Statistical testing was done with *t*-test; ****$P < 0.0001$.

(**d**) PCR detection of *Tada3* deletion in sorted plasmblasts (spleen) of NP-KLH immunized mice of indicated genotypes on day 7 after immunization.

In non-immunized adult animals, PBs in the spleen and PCs in the bone marrow were significantly reduced in *Cd23*-Cre *Tada3*$^{fl/fl}$ mice (**Fig. 6A, 6B**). The remaining PBs and PCs contained a residual-floxed allele (**Fig. 6C**).

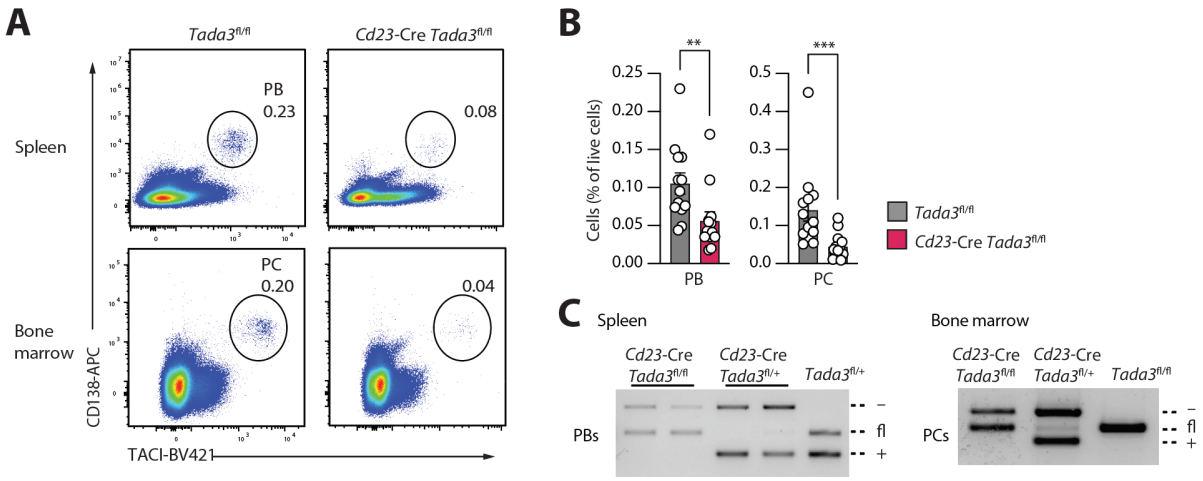

Fig. 6 | Reduced PB and PC development in *Cd23*-Cre *Tada3*$^{fl/fl}$ mice.
a and **b**, PBs and PCs in unimmunised *Tada3*-deficient mice. Plasmablasts in the spleen and plasma cells in the bone marrow of the indicated genotypes were identified as TACI$^+$CD138$^+$ cells (**a**) and the amount of cells is shown as mean value with SEM and anlyzed with Mann-Whitney test; $^{**}P < 0.01$, $^{***}P < 0.001$. Each symbol represents one mouse, and the data are pooled from four experiments. **c**, PCR detection of *Tada3* deletion in sorted plasmblasts (spleen) and plasma cells (bone marrow) of indicated genotypes. The residual floxed (fl) allele, deletion band (–) or wildtype band (+) are shown.

When *Cd23*-Cre *Tada3*$^{fl/fl}$ mice were immunized with the T-cell independent antigen NP-Ficoll, animals failed to form PBs and NP$^+$-PBs at similar amounts as littermate controls (**Fig. 7A, 7B**). As expected, no NP-specific ASCs were detected by ELISPOT analysis (**Fig. 7C**), and the remaining PBs displayed insufficient allele deletion (**Fig. 7D**).

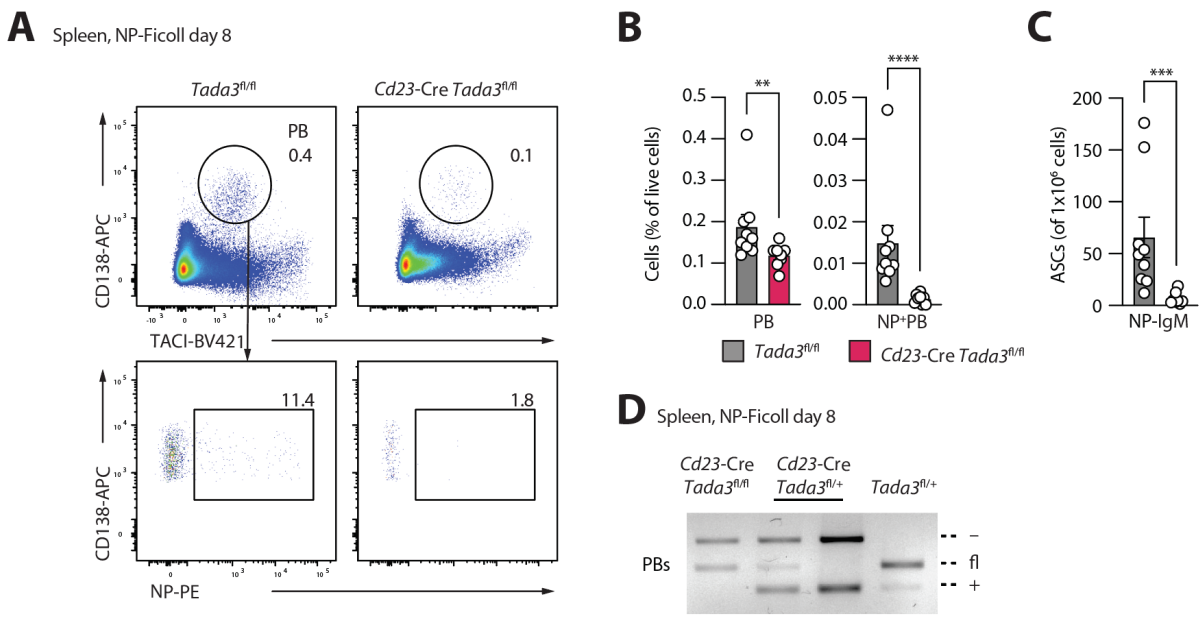

Fig. 7 | Affected PB development in *Cd23*-Cre *Tada3*$^{fl/fl}$ mice upon NP-Ficoll immunization.
a and **b**, PBs and PCs in mice immunized with the T-cell-independent antigen NP-Ficoll. Splenic plasmablasts and NP^{+}-plasmablasts from 9-10-week old *Cd23*-Cre *Tada3*$^{fl/fl}$ and *Tada3*$^{fl/fl}$ mice were analyzed by flow cytometry at day 8 after immunization. One of two experiments is shown (**a**). (**b**) Data are shown as mean values with SEM and were analyzed with Mann-Whitney test; $^{**}P < 0.01$, $^{****}P < 0.0001$. Each dot represents one mouse. **c**, ELISPOT analysis of splenocytes of NP-Ficoll immunized *Cd23*-Cre *Tada3*$^{fl/fl}$ and *Tada3*$^{fl/fl}$ mice on day 8 after immunization. NP-specific IgM secreting ASCs were detected and data are shown as mean values with SEM. The data were pooled from two independent experiments and analyzed with Mann-Whitney test; $^{***}P < 0.001$. **d**, PCR detection of *Tada3* deletion in sorted plasmblasts (spleen) of NP-Ficoll immunized *Cd23*-Cre *Tada3*$^{fl/fl}$, *Cd23*-Cre *Tada3*$^{fl/+}$ and *Tada3*$^{fl/fl}$ mice on day 8 after immunization.

Together, these data show that animals with a specific deletion of *Tada3* in mature B cells have FO and MZ B cells with similar amounts of littermate controls but cannot mount a robust GC reaction. This could be due to dysfunctional B-cell activation, and along these lines, significantly lower amounts of PBs and PCs.

To further understand whether the deletion of *Tada3* affects the formation and survival of PBs and PCs *in vivo*, we crossed the *Tada3* floxed allele with a *Bhlha15*-Cre mouse line that expresses GFP and initiates deletion in forming PBs in the spleen but is only fully active in the bone marrow (internal Busslinger lab data). When we analyzed *Bhlha15*-Cre *Tada3*$^{fl/fl}$ mice by flow cytometry, we observed a slight decrease in Cre-GFP-expressing PBs in the spleen, which was aggravated for PCs in the bone marrow (**Fig. 8A, 8B**). In the bone marrow, we also observed a substantial reduction in total IgM- and IgG1-ASCs despite the presence of non-Cre-expressing cells that could mitigate the phenotype (**Fig. 8C**). Cre-GFP^{+} PCs and PBs were

FACS-sorted for PCR-analysis of the floxed *Tada3* allele which showed that GFP-expression was indicative of deletion but Cre-expression was a gradual process (**Fig. 8D, 8E**). Overall, these experiments showed that *Tada3* also plays a role in late B cell development, and that upon deletion, PCs in the bone marrow were reduced, which could be due to affected cell formation and/or maintenance.

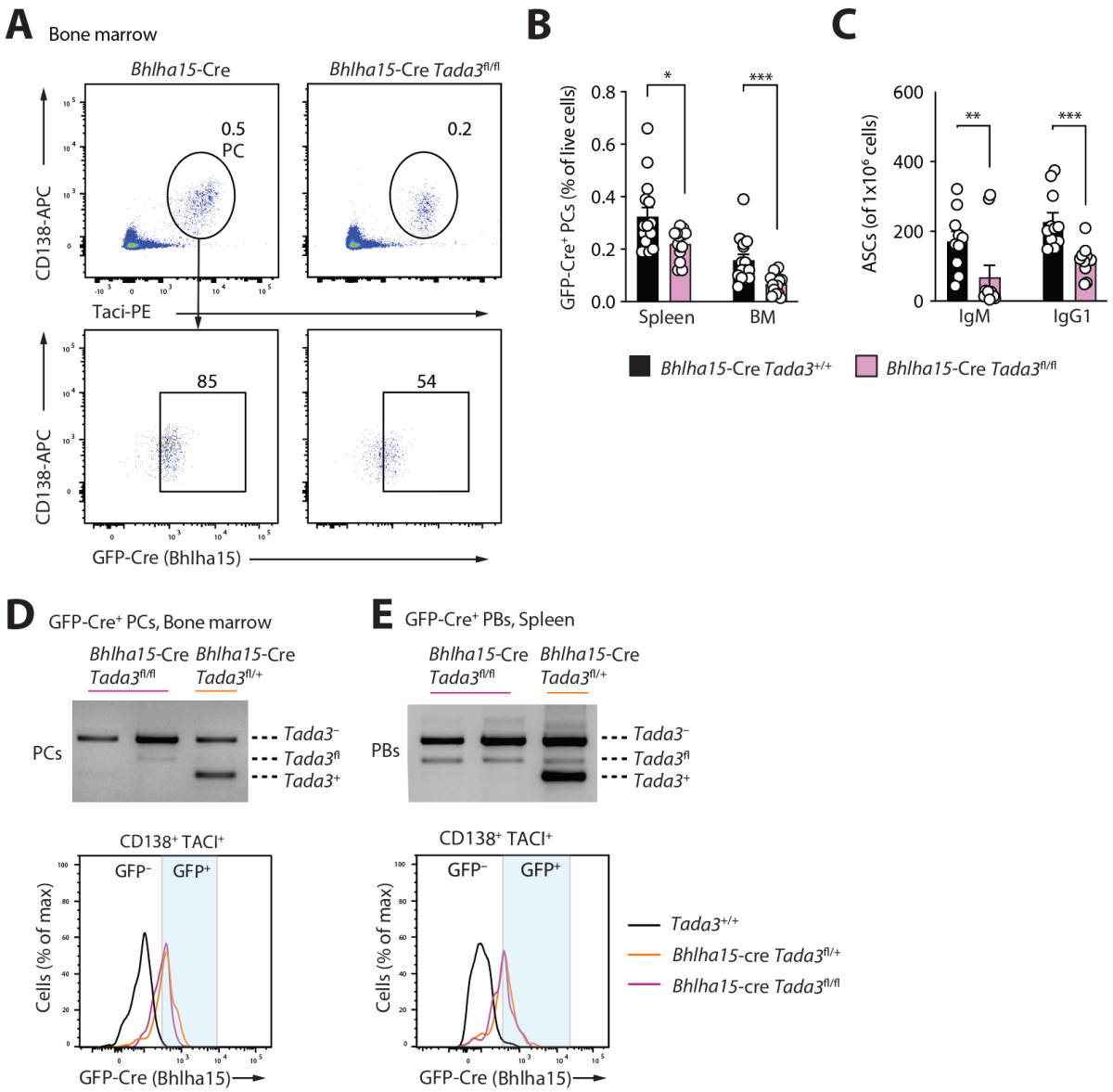

Fig. 8 | PC-specific deletion of *Tada3* and effects on PB and PC formation.
a–c, Absence of *Tada3* affects percentage of plasma cells (PCs) in the bone marrow. (**a**) Flow cytometric analysis of unimmunzed bone marrow of 13-15-week-old *Bhlha15-Gfp*-Cre *Tada3*$^{fl/fl}$ and *Bhlha15-Gfp*-Cre control mice. The percentage of cells is indicated next to the gate of the PCs and Cre-expressing PCs. One of three experiments is shown. (**b**) The percentages of GFP-Cre-expressing plasma cells in the bone marrow and plasmablasts in the spleen are shown as mean value with SEM. The data are pooled from three different experiments and analyzed with Mann-Whitney test; **P* < 0.05, ****P* < 0.001. (**c**) ELISPOT analysis of bone marrow of indicated genotypes detecting NP-specific IgM and total IgG ASCs. The data are shown as mean value

with SEM and are analyzed with Mann-Whitney test (IgM) or *t*-test (IgG); $**P < 0.01$, $***P < 0.001$. **d** and **e**, Flow cytometric analysis and sorting strategy for GFP-Cre expressing PCs from the bone marrow (**d**) and PBs from the spleen (**e**) of unimmunized 13-15-week-old mice of indicated genotypes. Cells were sorted (blue shade) and *Tada3* deletion was detected from isolated DNA by PCR analysis. The deletion band (–), the residual floxed allele (fl) and the wildtype band (+) are shown.

2.2.4 *Tada3*-deficiency affects B cell proliferation upon TLR and BCR stimulation

The observation that *Cd23*-Cre *Tada3*$^{fl/fl}$ mice could not form GCs led us to question whether *Tada3* was important for B cell activation. Despite their ability to generate similar amounts of FO and MZ B cells (**Fig. 4A**), cells displayed dysregulated surface markers (**Fig. 9A, 9B**). This could be indicative of dysfunctional B cell activation. Hence, we stimulated FO B cells from LNs with LPS and IL-4, which leads to B cell activation mainly via TLR4 signaling, resulting in the formation of PBs. On day 4, wells with *Tada3*-deficient B cells showed decreased viability and significantly fewer PBs were formed (**Fig. 9C, 9D**). Viable *Tada3*-deficient cells were able to blast and had similar cell size as the controls (**Fig. 9E, left**); however, the capacity of the cells to proliferate over 4 days of activation was significantly impaired in the absence of *Tada3* (**Fig. 9E, right**). The few remaining PBs proliferated as expected, and PCR analysis of these cells revealed insufficient deletion of the *Tada3* allele, highlighting the necessity of *Tada3* (**Fig. 9E right, 9F**).

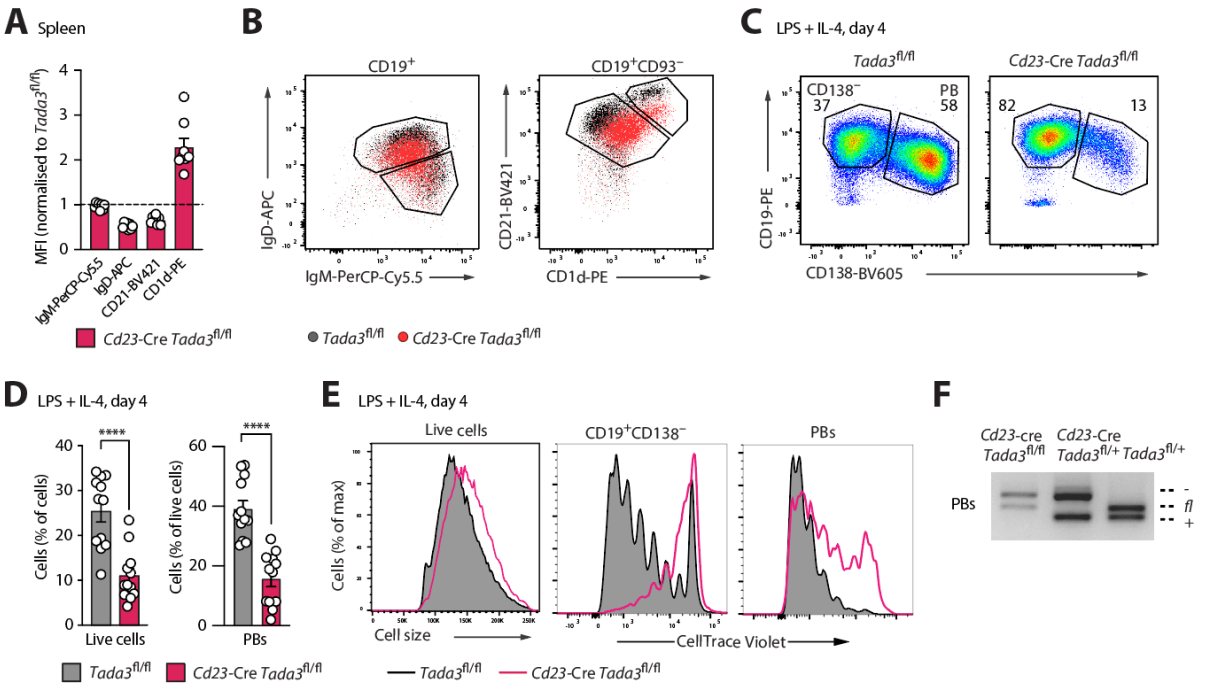

Fig. 9 | *Tada3*-deleted mature B cells express deregulated surface markers and show proliferation defects. **a** and **b**, Surface marker analysis of mature B cells. FO B and MZ B cells from *Cd23*-Cre *Tada3*$^{fl/fl}$ and control *Tada3*$^{fl/fl}$ mice were analyzed by flow cytometry. The median fluorescence intensity (MFI) of the indicated mature B cell surface markers is shown relative to control *Tada3*$^{fl/fl}$ mice. Two different experiments are shown and each dot represents one mouse. (**b**) Overlay of flow cytometric data of Imm B, Mature B, FO B and MZ B cells of *Cd23*-Cre *Tada3*$^{fl/fl}$ and control *Tada3*$^{fl/fl}$ mice. The flow cytometric definition of the different B cell types is also shown in Methods. **c–f**, Proliferation and PB formation following LPS plus IL-4 stimulation. FO B cells from lymph nodes of *Tada3*$^{fl/fl}$ and *Cd23*-Cre *Tada3*$^{fl/fl}$ mice were stimulated with LPS plus IL-4 for 4 days and PB formation was analysed by flow cytometry (**c**). (**d**) The cells were stained with the Viability Dye eFluor 780 to determine cell viability and the data are displayed as mean value with SEM and were analyzed by *t*-test; ****$P < 0.0001$. Each dot represents one mouse. (**e**) Flow cytometry was used to determine the cell size (FSC) of live cells and cell proliferation (for CellTrace Violet treated cells) of the indicated cell types. (**f**) On day 4 of LPS and IL-4 treatment, remaining PBs (CD19^{-}CD22^{-}CD138+) were sorted for PCR analysis of a residual *Tada3*$^{fl/fl}$ allele.

To understand whether the lack of proliferation as well as the reduced viability occurred at the start of the culture, we assessed these parameters over time. Both defects were observed from the beginning and accumulated over time (**Fig. 10A, 10B**).

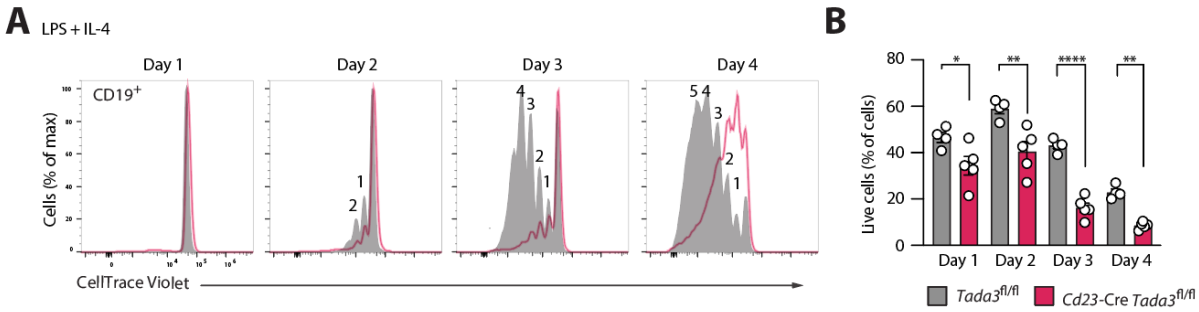

Fig. 10 | TADA3-deficient FO B cells respond to TLR4 stimulation.
a and b, Proliferation to LPS plus IL-4 stimulation. CellTrace Violet-labelled FO B cells from lymph nodes of *Cd23*-Cre *Tada3*$^{+/+}$ and *Cd23*-Cre *Tada3*$^{fl/fl}$ mice were stimulated with LPS and IL-4 for 4 days and analyzed by flow cytometry at the indicated time points (**a**) and stained with the Viability Dye eFluor 780 to determine viability of cells as % of all cells (**b**). (**b**) Statistical data are indicated as mean value with SEM and were analyzed by two-way ANOVA with Šídák multiple comparison test; **P* < 0.05, ***P* < 0.01, and *****P* < 0.0001. Each dot represents one mouse.

When we examined early activation markers, such as CD69 and CD86, we saw that the cells were initially activated and TLR signaling occurred in *Tada3*-deficient FO B cells (**Fig. 11A, 11B**). Therefore, the signaling cascade must be affected further downstream.

Together, these data show that *Tada3*-deficient B cells can be initially activated via TLR4 signaling and blast but fail to proliferate and cannot differentiate into PBs.

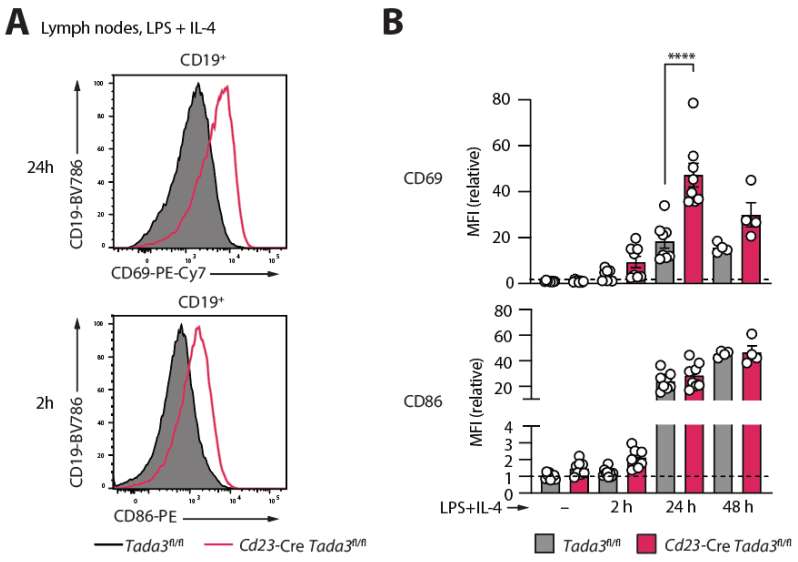

Fig. 11 | TADA3-deficient mature B cells express activation markers.
a and **b**, Expression of immediate early markers following LPS plus IL-4 stimulation. FO B cells from indicated genotypes were stimulated with LPS and IL-4 for 2, 24 and 48 hours and expression of immediate early genes CD69 and CD86 were analyzed by flow cytometry. The time point with the highest expression for each respective surface marker is shown (**a**). (**b**) The data are displayed as mean value with SEM relative to untreated wildtype cells and were analyzed by one-way ANOVA with Tukey's multiple comparison test (CD86) or with Holm-Šídák

multiple comparison test (CD69); ****$P < 0.0001$. Each dot represents one mouse and two idenpendent experiments are shown.

It has been shown that TLR signaling also engages the PI3K pathway (Rawlings et al. 2012) and in B cells additional BCR signaling is necessary for TLR4-mediated proliferation (Otipoby et al. 2015; Schweighoffer et al. 2017). We analyzed BCR signaling in a different culture setting by stimulating the cells with anti-IgM antibody and IL-4 for 4 days. Here, the viability of *Tada3*-deficient B cells was greatly reduced relative to that of the control B cells (**Fig. 12A**). Although the viable cells were only slightly smaller (**Fig. 12B, left**), when they were treated with CellTrace Violet, they showed a strong proliferation phenotype upon BCR stimulation (**Fig. 12B, right**).

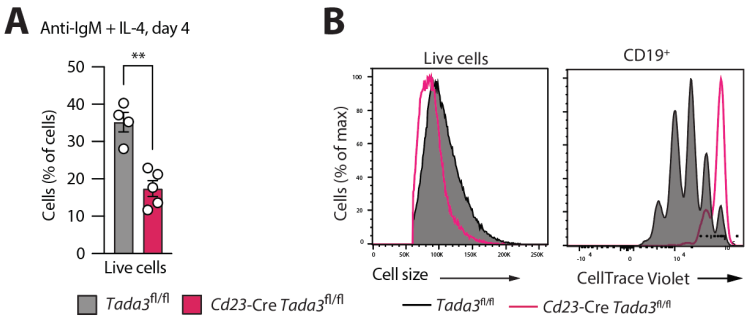

Fig. 12 | TADA3-deficient mature B cells show proliferation defects upon BCR stimulation.
a and **b**, Proliferation to anti-IgM stimulation. CellTrace Violet-labelled FO B cells from lymph nodes of *Tada3*$^{fl/fl}$ and *Cd23*-Cre *Tada3*$^{fl/fl}$ mice were stimulated with anti-IgM plus IL-4 for 4 days and analyzed by flow cytometry and stained with the Viability Dye eFluor 780 to determine viability of cells (**a**). (**b**) The cell viability is displayed as % of all cells and statistical data are indicated as mean value with SEM and were analyzed by *t*-test; **$P < 0.01$. Each dot represents one mouse.

BCR initiates PI3K signaling and induces phosphorylation of the PI3K target AKT. Surprisingly, flow cytometry analyses of the initial BCR signaling cascade showed that there was no significant difference in the phosphorylation of AKT (Ser473 and Thr308) between *Tada3*-deficient B cells and control B cells upon activation with anti-IgM antibody (**Fig. 13A, 13B**).

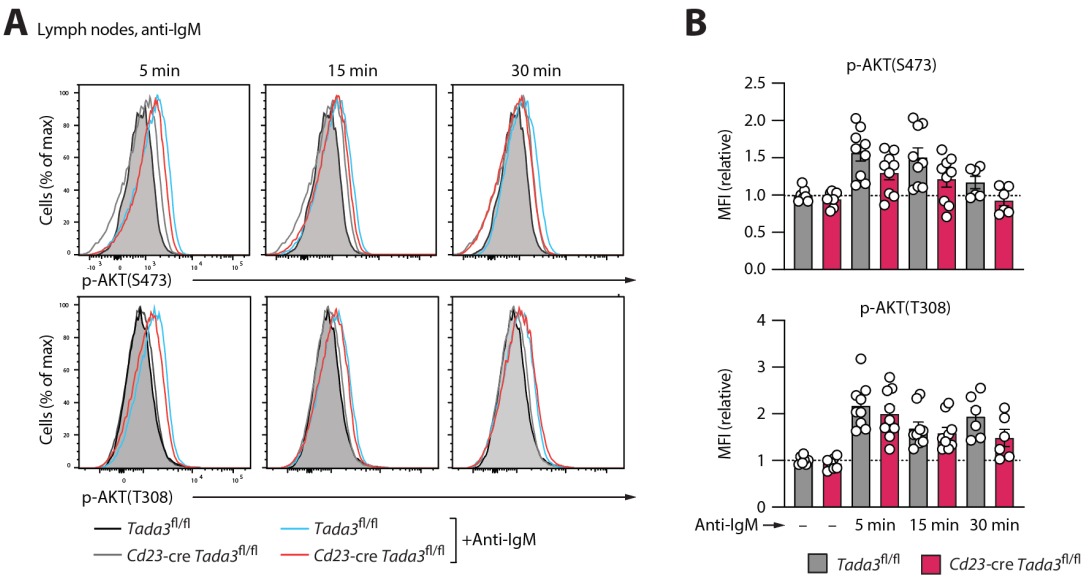

Fig. 13 | TADA3-deficient FO B cells respond to BCR stimulation.
a and b, Intracellular BCR signaling. The phosphorylation (p-) status of signal transducers of the PI3K signaling pathway was determined in lymph node FO B cells of the indicated genotypes before or after stimulation with anti-IgM for 5, 15 and 30 min. (**a**) Flow cytometry was performed with antibodies specific for p-AKT (p-Thr308) and p-AKT (p-Ser473). (**b**) The median fluorescence intensity (MFI) for untreated and stimulated FO B cells of the indicated genotypes is shown relative to the average MFI of untreated wildtype FO B cells. Statistical data are indicated as mean value with SEM and were analyzed by one-way ANOVA with Tukey's multiple comparison test. Each dot represents one mouse. Three different experiments are shown.

In summary, these data indicate that despite initial functional BCR signaling, TLR signaling, and blasting of the cells, *Tada3*-deficient B cells failed to proliferate and subsequently could not differentiate into PBs.

Another downstream signaling cascade that can be induced by TLR and BCR signaling is the activation of the transcription factor NF-κB via the degradation of the NF-κB inhibitor IκBα. We investigated whether this signaling pathway was impaired in *Tada3*-deficient FO B cells. Since TLR9 is expressed at high levels in FO B cells, we treated *Tada3*-deficient and control B cells with CpG oligodeoxynucleotide for 1.5 hours and intracellularly stained for IκBα degradation. IκBα was efficiently degraded in both genotypes, although less efficiently in *Tada3*-deficient B cells, indicating NF-κB activation in the absence of *Tada3* (**Fig. 14A, 14B**).

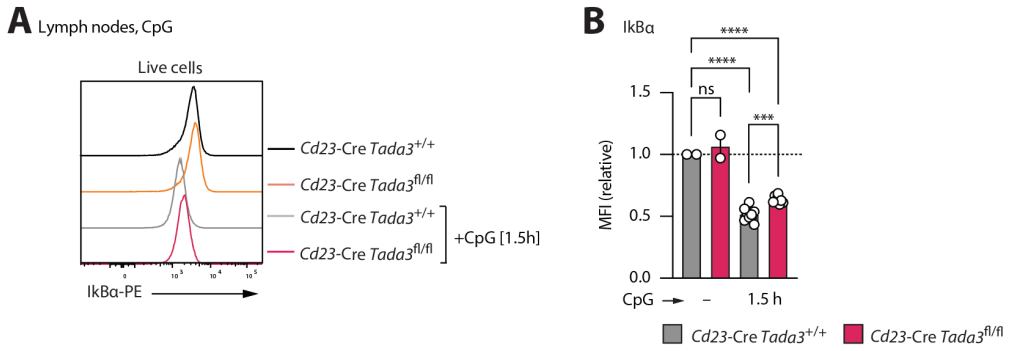

Fig. 14 | TADA3-deficient FO B cells are receptive to TLR9 stimulation.
a and b, IκBα degradation upon TLR signaling. The IκBα protein amount was determined by intracellular staining of CD43⁻ FO B cells from lymph nodes of *Cd23*-Cre *Tada3*$^{fl/fl}$ or control *Cd23*-Cre *Tada3*$^{+/+}$ mice before (black; orange) and after stimulation (gray; pink) for 1.5 hours with CpG oligodeoxynucleotides (**a**). (**b**) Statistical data of IκBα degradation are indicated as median MFI value with SEM relative to the MFI of the untreated control (set to 1) and were analyzed by one-way ANOVA with Holm-Šídák multiple comparison test; ***$P < 0.001$, and ****$P < 0.0001$. Each dot represents one mouse and two different experiments are shown.

Myc is an immediate-early gene that is essential for antigen-induced B-cell proliferation and is associated with cell cycle entry and progression (De Alboran et al. 2001). Since it has been shown that SAGA-complex stabilizes MYC protein by acetylation and in turn MYC reinforces SAGA expression (Hirsch et al., 2015; Patel et al. 2004), we investigated whether MYC expression was affected once *the Cd23*-Cre *Tada3*$^{fl/fl}$ cells were activated. When we stimulated the BCR with anti-IgM and analyzed MYC protein levels by flow cytometry, we observed no MYC upregulation in *Tada3*-deficient cells (**Fig. 15A, 15B**). Further analysis by real-time PCR with primer pairs amplifying *Myc* revealed no increase in transcripts in activated *Cd23*-Cre *Tada3*$^{fl/fl}$ cells compared to *that in Tada3*-sufficient control cells (**Fig. 15C**), suggesting that SAGA-HAT is involved in the regulation of *Myc* expression.

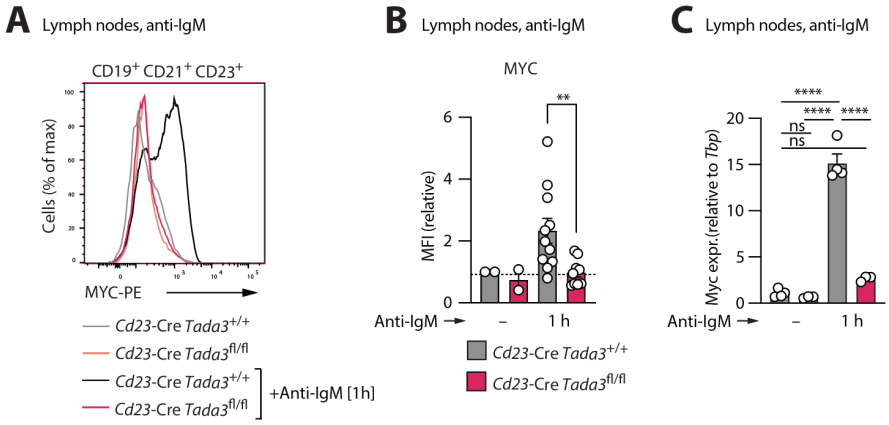

Fig. 15 | TADA3-deficient FO B cells do not upregulate *Myc* upon stimulation.
a and b, Intracellular MYC staining of *Cd23*-Cre *Tada3*$^{+/+}$ and *Cd23*-Cre *Tada3*$^{fl/fl}$ FO B cells before (gray; orange) and after (black; pink) stimulation for 1 hour with anti-IgM (**a**). MYC expression (**b**) is shown as median MFI value with SEM relative to the MFI of the untreated control (set to 1). Differences in stimulated cells were analyzed by *t*-test; $^{**}P < 0.01$. The data is based on two different experiments and each dot represents one mouse. **c**, RT-qPCR amplification of *Myc* mRNA in CD43^{-} FO B cells of of *Cd23*-Cre *Tada3*$^{+/+}$ (gray) and *Cd23*-Cre *Tada3*$^{fl/fl}$ (pink) mice after stimulation for 1 hour with anti-IgM. The *Myc* mRNA levels are shown rleative to *Tbp* and were analyzed by one-way ANOVA with Tukey's multiple comparison test; $^{****}P < 0.0001$. Each dot represents one mouse.

In summary, we showed that mature *Tada3*-deficient B cells, despite expressing deregulated surface markers, were still capable of undergoing initial activation via TLR and BCR stimulation but could not proliferate or differentiate to the next developmental cell stage. While some immediate early genes were successfully expressed following activation, neither *Myc* transcripts nor the protein were induced in *Cd23*-Cre *Tada3*$^{fl/fl}$ cells. Disturbed MYC expression might explain the observed phenotypes, since *Myc*-deficient B cells lack the ability to undergo full activation and proliferate normally (De Alboran et al. 2001).

2.2.5 Generation of a TADA3-AID-tagged allele for acute protein degradation

Conditional mutagenesis of genes of interest using Cre-recombinase-expressing mouse lines has been vital for understanding the roles of these genes and their genetic targets in several fields of biology. However, this approach is not only limited by the availability of stage-specific Cre lines, but also does not allow a defined temporal resolution to differentiate between directly or indirectly regulated targets. The latter could be a secondary consequence of deregulated expression of direct target genes. Moreover, certain cell types cannot be analyzed because they do not tolerate the loss of important transcription factors or chromatin modifiers. These limitations also apply to the attempt to identify targets of SAGA-HAT in B cell

activation underlying our reported phenotypes. GC B cells were absent from *Cd23*-Cre *Tada3*$^{fl/fl}$ NP-KLH-immunized animals (**Fig. 5A, 5B**), making it impossible to investigate the genetic targets in this cell type. Immediate gene expression changes following Cre-recombinase-driven deletion of *Tada3* in activated mature B cells cannot be assessed because of the likely discrepancy between the deletion of the gene, resulting in expression changes and delayed time of analysis.

The auxin-inducible degron (AID) system is a form of acute protein degradation that was originally described in plants and adapted for use in mammalian cells (Nishimura et al. 2009; Teale et a 2006). Acute protein degradation allows for high temporal resolution of gene expression changes and circumvents many limitations. This system requires the expression of an AID-tagged protein of interest and the plant E3 ligase adaptor protein Tir1, which, as part of the cell-inherent Skip-Cul1-F-box (SCF) E3 ubiquitin ligase complex, interacts with the AID sequence only in the presence of the plant hormone auxin (IAA) (Nishimura et al. 2009). This interaction leads to the rapid ubiquitination and proteasomal degradation of AID-containing proteins. More recently, the AID system was improved by introducing the F74G mutation in the auxin-binding pocket of Tir1, which increases the affinity of Tir1-F74G for the auxin analog 5-phenyl-indole-3-acetic acid (5-Ph-IAA) by approximately 500-fold relative to the affinity of Tir1 for auxin (Yesbolatova et al. 2020). As a result, spontaneous degradation of the tagged protein in the absence of auxin is prevented, and *in vivo* protein degradation in mice has become possible (Phanindhar and Mishra 2023; Yesbolatova et al. 2020).

To study the direct effects of the SAGA-HAT module on B cell development, we established an *in vivo* system for rapid degradation of TADA3, based on the AID methodology. We generated a *Tada3*^{AID} allele by in-frame fusion of the AID and V5 epitope sequences at the C-terminus of TADA3 using CRISPR/Cas9-mediated genome editing in mouse zygotes (**Fig. 16A**, **Fig. 17A** and **Methods**) (Yang et al. 2013). Homozygous *Tada3*^{AID} pups were viable, indicating a functional allele, because germline deletion of *Tada3* results in early embryonic lethality (Mohibi et al. 2012). Recent *in vivo* rapid degradation experiments of mice that express different AID-tagged B cell-specific transcription factors and Tir1 from the ubiquitously expressed *Rosa26* locus (*Rosa26*^{Tir1}) (Baker et al. 2016) exhibited defects in B cell development in the absence of auxin (Fedl et al. 2024 manuscript in preparation). This is likely caused by spontaneous degradation by Tir1 and can be bypassed by the insertion of the F74G mutation in the auxin-binding pocket of Tir1 (Yesbolatova et al. 2020). The necessary

Rosa26$^{Tir1(F74G)}$ allele was generated in the Busslinger laboratory, and the F74G mutation abrogated the spontaneous degradation activity of Tir1 *in vivo* (Fedl et al. 2024 manuscript in preparation). Because of the chromosomal proximity and concomitant gene linkage of the *Rosa26* locus and *Tada3*, we generated *Rosa26*$^{Tir1(F74G)/Tir1(F74G)}$ *Tada3*$^{AID/AID}$ mice with the same targeting strategy at the C-terminus of *Tada3*, but this time in mouse *Rosa26*$^{Tir1(F74G)/Tir1(F74G)}$ zygotes (**Fig. 16A**, **Fig. 17A** and **Methods**). Flow cytometry analysis revealed normal frequencies of pro-B, pre-B, immature B, and plasma cells in the bone marrow of *Tada3*$^{AID/AID}$ and *Rosa26*$^{Tir1(F74G)/Tir1(F74G)}$ *Tada3*$^{AID/AID}$ mice compared with wildtype mice (**Fig. 16B, Fig. 17B**). Similarly, animals that had been immunized with the T-cell-dependent antigen sheep-red blood cells (SRBC) displayed similar frequencies of GC B cells, FO B, MZ B cells, and plasmablasts (**Fig. 16C, Fig. 17C**). Hence, neither the AID-tag nor the presence of Tir1-F74G caused a detectable phenotype, and *Rosa26*$^{Tir1(F74G)/Tir1(F74G)}$ *Tada3*$^{AID/AID}$ mice can be used to study the acute loss of TADA3 and the resulting gene expression changes *in vivo*.

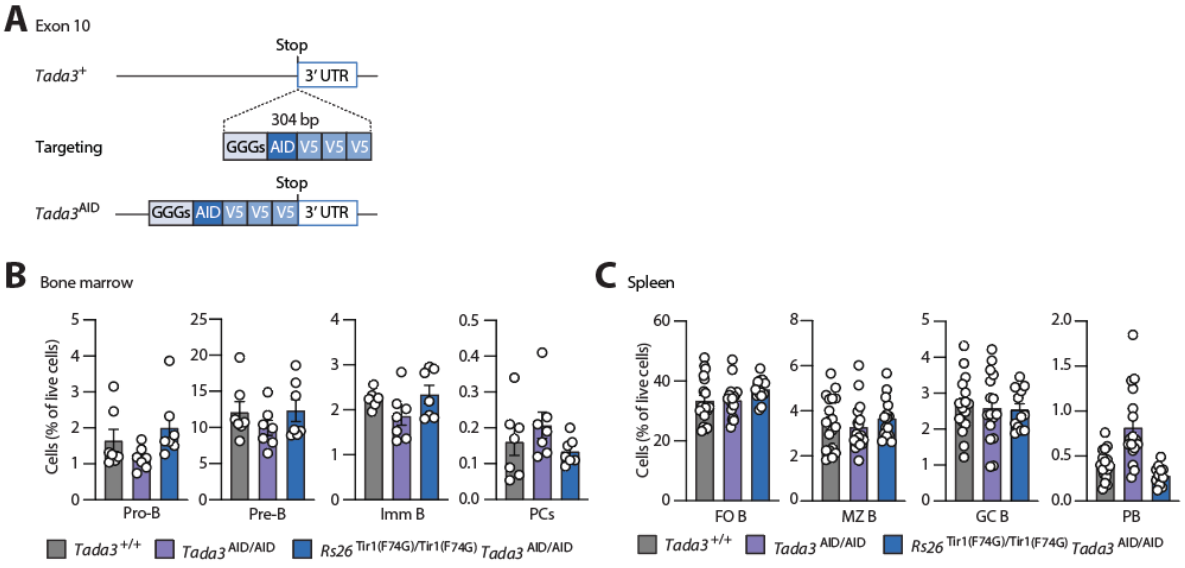

Fig. 16 | Generating a functional Tada3-AID-tagged allele.
a, Schematic diagram of the AID-tagged *Tada3* allele. The indicated C-terminal tag sequence, consisting of a glycine linker, a minimal auxin-inducible degron (AID) sequence and three times the V5 epitope were added in-frame to the last amino acid of TADA3. **b**, Early B cell development in wildtype controls, *Tada3*$^{AID/AID}$ and *Rosa26*$^{Tir1(F74G)/ Tir1(F74G)}$ *Tada3*$^{AID/AID}$ mice, as determined by flow-cytometric analysis of the bone marrow from 9-12-week-old mice (Fig. S4B). The frequencies of pro-B, pre-B, immature (Imm) B, and plasma cells are shown as mean values with SEM. **c**, Late B cell development in wildtype controls, *Tada3*$^{AID/AID}$ and *Rosa26*$^{Tir1(F74G)/ Tir1(F74G)}$ *Tada3*$^{AID/AID}$ mice, as determined by flow-cytometric analysis of the spleen from SRBC immunized 9-17-week-old mice (Fig. 17C). The frequencies of FO B, MZ B, GC B cells and plasmablasts (PB) at 8-10 days p.i. are shown as mean values with SEM. Statistical data (**b, c**) were analyzed by one-way ANOVA. Each dot (**b, c**) corresponds to one mouse.

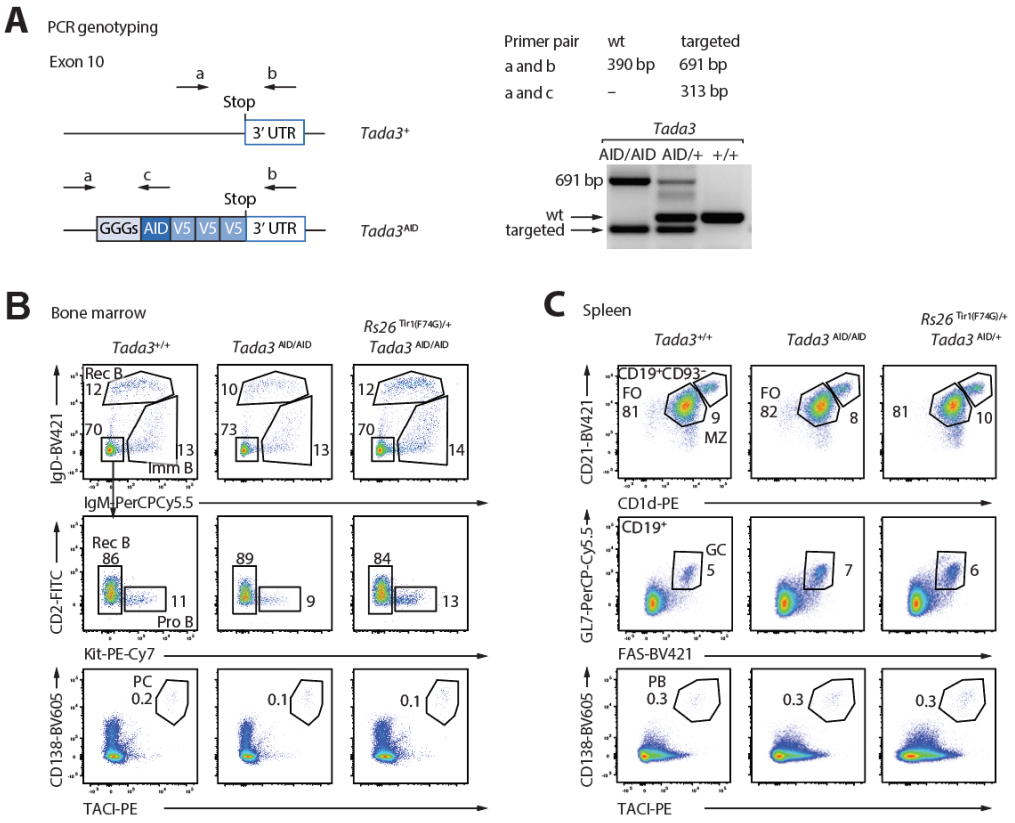

Fig. 17 | Characterization of the Tada3-AID-tagged allele.
a, PCR genotyping strategy for the generated *Tada3*$^{AID/AID}$ allele. PCR genotyping results of tail DNA from *Tada3*$^{AID/AID}$, *Tada3*$^{AID/+}$ and *Tada3*$^{+/+}$ mice are shown. The PCR fragments indicative of the *Tada3*^{AID} (313-bp and 691-bp) and WT *Tada3* (390-bp) alleles are shown. **b**, Early B cell development in wildtype controls, *Tada3*$^{AID/AID}$ and *Rosa26*$^{Tir1(F74G)/\ Tir1(F74G)}$ *Tada3*$^{AID/AID}$ mice, as determined by flow-cytometric analysis of the bone marrow from 9-12-week-old mice. The flow-cytometric analysis of the different mice was performed on the same day. The percentage of cells is indicated next to the gate of the different B cell types. One of three experiments is shown. Imm, immature; Rec, recirculating; PC, plasma cells. The flow cytometric definition of the different B cell types is also shown in Methods. **c**, Late B cell development in wildtype controls, *Tada3*$^{AID/AID}$ and *Rosa26*$^{Tir1(F74G)/\ Tir1(F74G)}$ *Tada3*$^{AID/AID}$ mice, as determined by flow-cytometric analysis of the spleen from SRBC immunized 9-17-week-old mice. The flow-cytometric analysis of the different mice was performed on the same day. The percentage of cells is indicated next to the gate of the different B cell types. One of five experiments is shown. FO, follicular; MZ, marginal zone; GC, germinal center; PB, plasmablast. The flow cytometric definition of the different B cell types is also shown in Methods.

We further validated the functionality of the *Tada3*^{AID} allele *in vitro* under different stimulation conditions that lead to the formation of PBs. The cultures were analyzed on day 4, and the tagged protein did not cause any defects in PB formation in the presence or absence of Tir1-F74G compared with the wildtype allele (**Fig. 18A**).

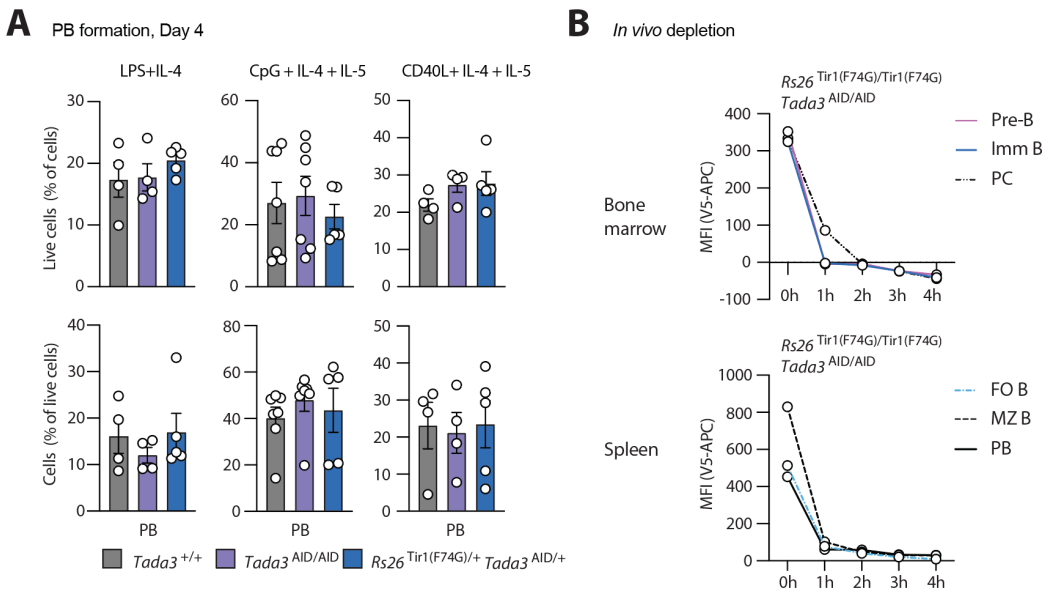

Fig. 18 | Characterization and acute protein degradation time course of the Tada3-AID allele. **a**, PB formation of CD43$^-$ FO B cells upon different stimulating conditions. *Rosa26*$^{Tir1(F74G)/+}$ *Tada3*$^{AID/+}$ FO B cells were stimulated with LPS and IL-4; CpG and IL-4 and IL-5; CD40L and Il-4 and IL-5 for 4 days. Viability of cells and PB formation was analyzed on day 4 by flow cyometry. Live cells are displayed as % of total cells and PBs as % of live cells. Each dot corresponds to one mouse. **b**, Time course analysis of TADA3 degradation upon intraperitoneal injection of 5-Ph-IAA into *Rosa26*$^{Tir1(F74G)/ Tir1(F74G)}$ *Tada3*$^{AID/AID}$ mice. At 1, 2, 3 and 4 hours after injection, the mice were analyzed together with untreated *Tada3*$^{AID/AID}$ mice (0 h, positive control) and wildtype mice (0h, negative control) by intracellular staining with an anti-V5 antibody and flow-cytometric analysis of pre-B, Imm B, PCs in the bone marrow and FO B, MZ B and PBs in the spleen. The median fluorescence intensity (MFI) of the anti-V5 antibody staining is shown for one mouse analyzed per timepoint and is displayed minus the MFI value of the fluorescent background (wildtype, 0h). One of 2 experiments is shown.

The introduced V5 epitope allowed us to detect and compare TADA3 expression throughout B cell development by intracellular staining, which had not been possible before due to the lack of a flow cytometry-compatible anti-TADA3 antibody. We analyzed TADA3$^{AID/AID}$ expression in the bone marrow and spleen and noted a uniform expression pattern, except for GC B cells where TADA3^{AID} peaked, which was in line with the mRNA expression data (**Fig. 19A, 19B, Fig. 2D**).

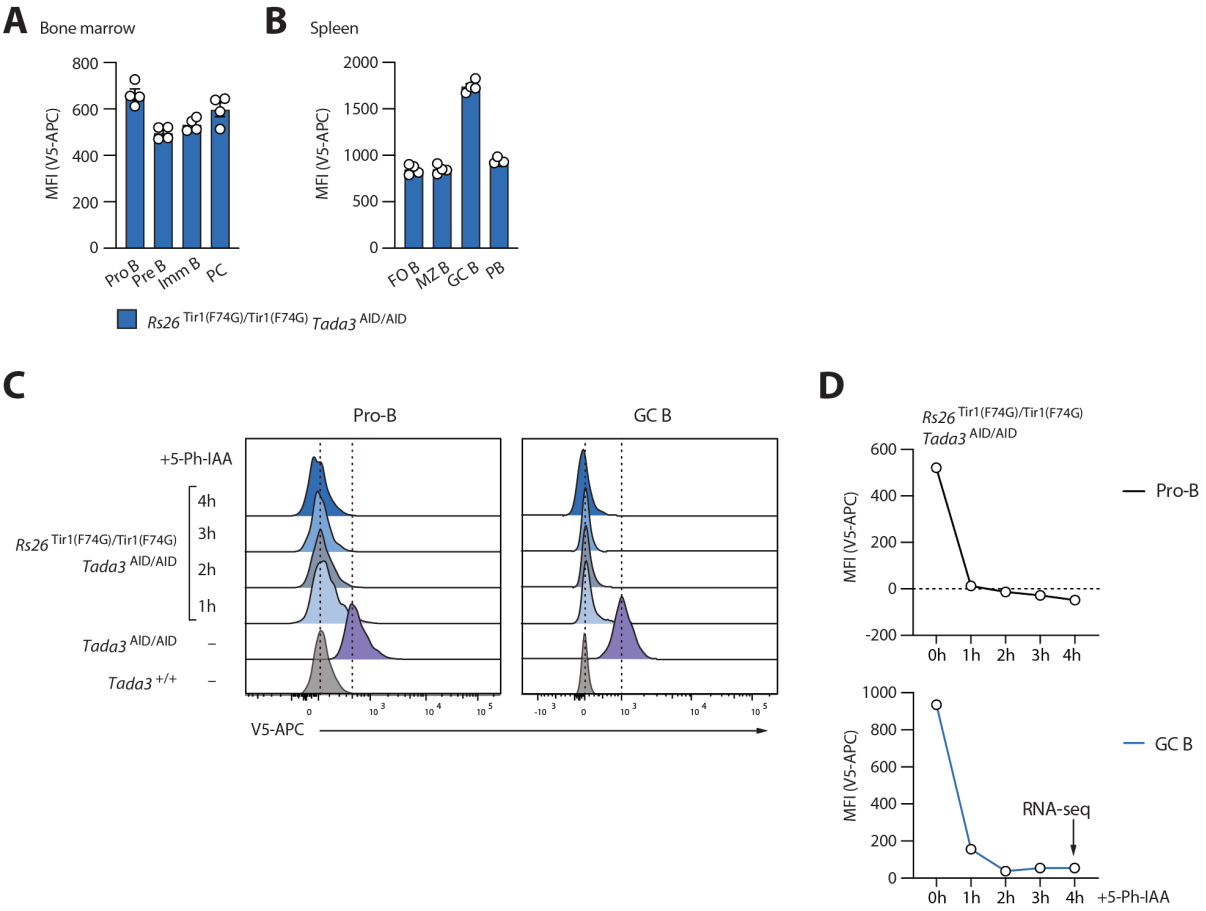

Fig. 19 | Expression of the Tada3-AID-tagged allele in B cells and acute protein degradation time course. **a and b**, Expression of the TADA3 protein during early B cell development (**a**) and late B cell development (**b**). The protein levels of TADA3 were determined by intracellular staining of bone marrow cells (**a**) or splenocytes (**b**) from *Rosa26*$^{Tir1(F74G)/ Tir1(F74G)}$ *Tada3*$^{AID/AID}$ mice with an anti-V5 antibody and gating on pro-B, pre-B, immature B and plasma cells (**a**) or FO B, MZ B, GC B and PBs (**b**). For GC B cells, mice were immunized with SRBCs and analyzed on day 10 p.i. The median fluorescence intensity (MFI) of the anti-V5 antibody staining is shown as mean value with SEM, based on two different experiments (**a**), or five different experiments (**b**). **c**, Time course analysis of TADA3 degradation upon intraperitoneal injection of 5-Ph-IAA into *Rosa26*$^{Tir1(F74G)/ Tir1(F74G)}$ *Tada3*$^{AID/AID}$ mice (SRBC-immunized, day 10 p.i. for GC B cells). At 1, 2, 3 and 4 hours after injection, the mice were analyzed together with untreated *Tada3*$^{AID/AID}$ mice (0 h, positive control) and wildtype mice (0h, negative control) by intracellular staining with an anti-V5 antibody and flow-cytometric analysis of pro-B and GC B cells. **d**, The median fluorescence intensity (MFI) of the anti-V5 antibody staining is shown for one mouse analyzed per timepoint and is displayed minus the MFI value of the fluorescent background (wildtype, 0h). One of 2 experiments is shown. Arrow indicates the selected timepoint used for acute degradation of TADA3 in GC B cells. Statistical data (**a, b**) were analyzed by one-way ANOVA. Each dot (**a, b**) corresponds to one mouse.

Next, we studied if we could degrade TADA3 *in vivo* by administering 5-Ph-IAA and assessed its kinetics. We administered 5-Ph-IAA by intraperitoneal injection and assessed protein degradation by flow cytometry. TADA3 was rapidly degraded within three hours in all analyzed cell types but in GC B cells where TADA3 reached very low levels four hours after 5-

Ph-IAA injection (**Fig. 19C, 19D**, **Fig. 18B**). Therefore, four hours of 5-Ph-IAA treatment was chosen as the optimal time point for TADA3 depletion in GC B cells.

Together, we show that our newly generated *Rosa26*$^{Tir1(F74G)/Tir1(F74G)}$ *Tada3*$^{AID/AID}$ mice display normal B cell frequencies and that TADA3 can be rapidly degraded *in vivo*, which allows us for the first time to identify the direct targets of SAGA-HAT in B cell development.

2.2.6 Experimental design for identifying direct targets of SAGA-HAT in B cell activation

Since we have shown that *Cd23*-Cre *Tada3*$^{fl/fl}$ mature B cells can be initially activated and express some primary response genes, but fail to express *Myc,* we were interested in dissecting the role of SAGA-HAT in B cell activation further. By employing acute TADA3 protein degradation coupled with nascent transcript analysis, we hope to identify the direct targets of SAGA-HAT without studying secondary effects. We addressed this in two ways: first, by degrading TADA3 in resting *ex vivo* mature B cells prior to their activation, and second, by degrading TADA3 *in vivo* in activated B cells such as GC B cells, which are highly active and proliferative cells (Victora and Nussenzweig 2022). These two approaches will allow us to dissect the role of SAGA-HAT in B cell activation from two perspectives.

Initially, we determined the time required for *in vitro* 5-Ph-IAA treatment to deplete TADA3. Both *Rosa26*$^{Tir1(F74G)/Tir1(F74G)}$ *Tada3*$^{AID/AID}$ MZ B and FO B cells displayed sufficient TADA3 degradation after 1h of 5-Ph-IAA treatment (**Fig. 20A**). Based on this observation, we equilibrated FO B and MZ B cells from *Rosa26*$^{Tir1(F74G)/Tir1(F74G)}$ *Tada3*$^{AID/AID}$ mice in B cell medium to ensure a resting state of the cells before 5-Ph-IAA was added for one hour to induce degradation of TADA3 while control wells were left untreated (Harvest I: degraded vs. non-degraded) (**Fig. 20B**). Protein degradation was followed by activation of the BCR for one hour (Harvest II: 1h activated, degraded vs. 1h activated, non-degraded) or for four hours (Harvest III: 4h activated, degraded vs. 4h activated, non-degraded) (**Fig. 20B**). The degradation of TADA3 as well as efficient activation of the cells were verified by flow cytometry, and the cells at all indicated time points were subjected to total RNA sequencing to quantify nascent transcript changes (**Fig. 20B**, **20C, 20D**). By analyzing changes in nascent transcripts one hour after BCR activation, we hope to establish whether the presence of SAGA-HAT is required for the expression of immediate early genes, such as *Myc*, *Egr2*, and *Egr3* (De Alboran et al. 2001; Calderón et al. 2021; Li et al. 2012; Tullai et al. 2007), which are

increased compared to the resting state as early as 30 minutes after stimulation (Fowler et al. 2015). The analysis at four hours after BCR stimulation will allow us to study the potential necessity of SAGA-HAT in the regulation of delayed primary response genes, as well as some secondary response genes (Tullai et al. 2007).

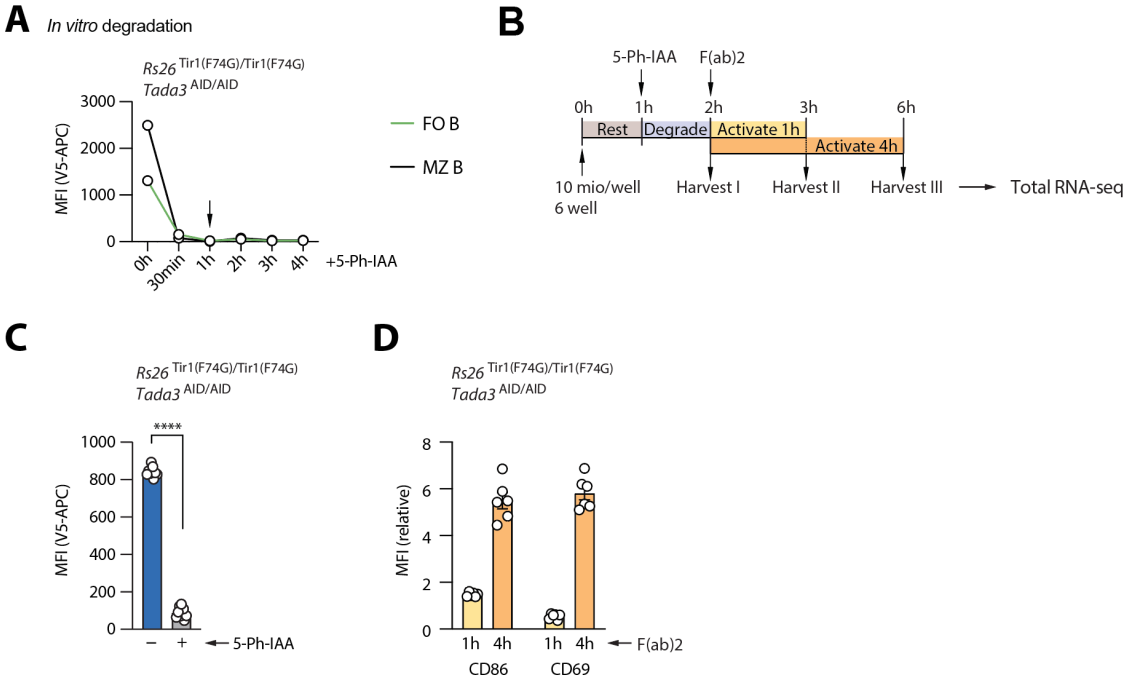

Fig. 20 | Strategy for *ex vivo* acute protein degradation of TADA3^{AID}.
a, Time course analysis of TADA3 degradation in *ex vivo* cultured CD43^{-} FO and MZ B cells of *Rosa26*$^{Tir1(F74G)/Tir1(F74G)}$ *Tada3*$^{AID/AID}$ mice. The cells were treated with 5-Ph-IAA for indicated time points and analyzed by intracellular staining with an anti-V5 antibody and flow-cytometric analysis of FO B and MZ B cells. The median fluorescence intensity (MFI) of the anti-V5 antibody staining is shown as mean value of three mice analyzed per timepoint and is displayed minus the MFI value of the fluorescent background (wildtype, 0h). The arrow indicates the selected time point for acute protein degradation for *ex vivo* experiments (1h). **b**, Experimental outline of acute TADA3-AID-tagged protein degradation in cultured FO B and MZ B cells from spleen and lymph nodes of *Rosa26*$^{Tir1(F74G)/Tir1(F74G)}$ *Tada3*$^{AID/AID}$ mice. The indicated amount of cells was seeded and equilibrated in medium for 1 hour prior to 1 hour of 5-Ph-IAA treatment. This was followed by BCR activation for 1 or 4 hours. The cells were harvested at indicated time points and will be analyzed by total RNA-seq. The protein degradation (**c**) and cell activation (**d**) were confirmed by flow cytometry. (**c**) The fluorescent background (wildtype, 0h) of the anti-V5 antibody staining is deducted from the MFI of FO B and MZ B cells and the value is shown as mean value with SEM. (**d**) The MFI of the activation markers CD86 and CD69 are shown relative to the unstimulated timepoint (0h) at 1 and 4 hours of anti-IgM stimulation for FO B and MZ B cells. (**c, d**) Each dot represents one sample.

The second approach to elucidate the role of SAGA-HAT in B cell activation focuses on TADA3 depletion in highly active cells, such as GC B cells. To address the role of SAGA-HAT in GC B cells, we immunized *Rosa26*$^{Tir1(F74G)/Tir1(F74G)}$ *Tada3*$^{AID/AID}$ mice with SRBC, which leads to a

peak in GC formation by day ten. Mice were treated with 5-Ph-IAA and TADA3-depleted (4h) GC B cells, and non-depleted cells (0h) from untreated control animals were sorted for total RNA sequencing to quantify changes in nascent transcripts. The degradation of TADA3 in GC B cells after four hours of 5-Ph-IAA treatment was verified using flow cytometry. Analysis of changes in nascent transcripts upon acute degradation of TADA3 will be crucial for understanding the role of SAGA-HAT in GC B cells and possible dependent B cell-specific transcription factors. Considering the vital role of *Myc* in the formation and maintenance of GCs (Calado et al. 2012; Dominguez-Sola et al. 2012), we hope to establish a clear understanding of the direct or indirect role of *Myc* regulation by SAGA-HAT.

Together, these experimental approaches will shed light on the immediate genetic targets of TADA3 and the SAGA-HAT module in B-cell activation from two different angles.

3 Discussion

Several transcription factors are essential to PB and PC development and survival such as BLIMP1, IRF4, Aiolos and Ikaros, the E-box proteins E2A and E2-2 (Cortés and Georgopoulos 2004; Mittrücker et al. 2017; Schwickert et al. 2019; Shaffer et al. 2002; Wöhner et al. 2016). The network of transcription factors and potential co-factors is still not fully understood, as illustrated by the example of IRF4 and its transcriptional target, *Prdm1* (encodes BLIMP1) (Kurosaki et al. 2010). IRF4 is required for both the differentiation and survival of PCs; however, analysis of the underlying regulatory network in these cells is constrained by the loss of PCs upon *Irf4* deletion. Only a few downstream targets of IRF4 have been identified, and it is unclear how *Irf4* deletion leads to plasma cell death. While *Prdm1* is also essential for PC differentiation, retroviral reconstitution of *Prdm1* in *Irf4*$^{-/-}$ B cells did not rescue PC formation, suggesting that there are other yet unknown regulators (Kwonet al. 2009; Sciammas et al. 2006; Sze et al. 2019). In line with this, although the E-proteins E2A and E2-2 activate *Prdm1* and *Xbp1* and have been shown to be essential regulators of PCs, as their absence leads to stringent arrest at the onset of PB formation, restoration of BLIMP1 expression in E2A-and E2-2-deficient B cells failed to rescue PB formation (Wöhner et al. 2016). This further suggests the presence of unknown target genes. More recently, it has also been shown that the three important PC regulatory factors, BLIMP1, XBP1, and IRF4, regulate RNA signatures with only minimal overlap, suggesting a possibly bigger and more different pool of interaction partners (Sze et al.2019).

3.1 sgRNA Screen 1

We conducted two different sgRNA CRISPR-Cas9 screens and screened in total 3,000 genes for relevance to PB development (Pinter et al. 2022). To mimic ‘near-physiological’ B cell differentiation *in vitro*, we made use of the *in vitro*-inducible germinal center B cell (iGB) system (Nojima et al. 2011) which allowed us to culture B cells for an extended period of time which had not been possible before. This system mimics a T cell-dependent antigen response and generates GC-phenotype B cells with subsequent PB formation.

In the first screen, we focused on genes that were upregulated in bone marrow PCs compared to mature B cells (Minnich et al. 2016), as well as genes that were upregulated in PBs generated in the iGB system. We assumed that these genes might be relevant to PB development, which allowed us to develop a focused screening approach. However, we could only find significant effects on PB development for 8 genes that affected PB development or survival. The sgRNAs of the positive controls *Prdm1* and *Irf4* showed significant effects on PB development, validating our approach. Genes that were identified included *Slc7a5* and *Slc3a2* that together form CD98, which has been shown to be essential for PC formation (Cantor et al.2009).

Since we used stringent cutoffs for target gene selection (≥ 5-fold upregulated in iGB-cultured PBs versus activated B cells; ≥ 8-fold upregulated in bone marrow PCs versus mature B cells (Minnich et al. 2016)), and used 6 sgRNAs per gene and 2 sgRNAs for single knockout validations, we are confident that our observations are accurate. Half of the included target genes were selected based on their upregulation in bone marrow PCs compared to mature B cells, which could be a reason why their function could not be efficiently examined in the iGB system. The perhaps surprisingly low number of genes that showed an effect on PB development does not exclude the possibility that some other selected genes have a more subtle role in PB development *in vivo* which we cannot detect in our *in vitro* system. The artificial stimulation environment of an *in vitro* culture system could provide pro-survival conditions that compensate for the loss of certain genes. In the iGB culture system, the cells were exposed to high levels of BAFF, CD40L, and IL-4, which provided a stimulating environment that may influence cell survival and overshadow some otherwise observable effects. Additionally, co-dependencies of the tested genes could not be accounted for.

3.2 sgRNA Screen 2

Since we tailored the first screen to PB-upregulated genes, assuming that the upregulation is indicative of relevance to the cell, we analyzed more candidate genes in the second screen (Pinter et al. 2022). This included genes that were selected only for expression (TPM>5) in activated B cells (act B), or in pre-PBs, or in PBs in the iGB system, and could be assigned to certain functional groups, such as transcription factors or chromatin modifiers. In this setup, we retrieved more potential hits whose function for PB development was also validated in the

iGB system by sgRNA knockout validation (53 genes selected, two sgRNAs per gene). Since the genes in the second screen were different from those in the first screen and some affected PB development, we conclude that the target selection in screen 1 based on upregulation alone might be a too stringent criterion. On the other hand, the prerequisite that target genes of the second screen had to be members of certain functional groups and the expression data used for target selection came from cells that were cultured in the iGB system added a selection bias. However, the selection of functional groups was necessary to limit the otherwise unfeasibly large number of genes for target selection.

The high-confidence hits in the second screen again included our positive controls *Irf4* and *Prdm1*, whereas the control sgRNAs (*Chr1*, targeting a gene-free region on chromosome 1, and the empty vector control) had no effect on PB formation. To ensure that the identified hits did not affect overall cell survival in the culture, we assessed the cell viability of the sgRNA-infected cells over the culture period and excluded targets that compromised the survival of all B cell populations. In addition, we wanted to ensure that these genes did not affect earlier B cell development, which we analyzed by comparing the sgRNA abundance in activated B cells with the initial sgRNA pool. If a sgRNA was absent in this comparison, this indicated that the targeted gene was already important prior to or for B cell activation.

We identified several genes as important to PB formation, including *Kdm1a* (histone demethylase LSD1), which has been shown to regulate PB differentiation, thus further validating our approach (Haines et al.2018). Other top hits included *Tada3*, *Tada2b*, and other SAGA-HAT genes that have not been previously described as PB-relevant genes. Since *Tada3* and other SAGA-HAT genes were identified in the aforementioned extensive analysis, we assumed that deletion of *Tada3* would not affect general cell survival and that it would be important for PB development, despite its ubiquitous expression profile. However, this was not entirely reflected in our *in vivo* experiments, where we deleted *Tada3* throughout B cell development.

3.3 B cell developmental phenotypes upon *Tada3*-inactivation

Once we had identified *Tada3* and other SAGA-HAT genes in a sgRNA-PB screen, we set out to study the role of *Tada3* in B cell development by conditional mutagenesis. We discovered a role for *Tada3* in early B cell development by analyzing *Cd79a*-Cre *Tada3*$^{fl/fl}$ mice, in which the

transition of pro-B to pre-B cells was efficiently blocked (**Fig. 3A, 3B**). There was no accumulation of pro-B cells but possibly dysfunctional pre-BCR signaling that led to the loss of all subsequent B cell populations. We cannot exclude an even earlier role of TADA3 in B cell development, since *Cd79a*-Cre initiates gene deletion at the transition from pre-pro-B cells to fully committed pro-B cells (Kwon et al. 2008), which might be too late to observe an effect on pro-B cells. Additionally, we did not use a Cre-recombinase-expressing mouse line that induces *Tada3* deletion at earlier B cell stages. Nevertheless, our data point to a role for TADA3 and SAGA-HAT in B cell proliferation and differentiation, rather than in a quiescent and resting cell state. This was supported by the notion that mature, resting FO, and MZ B cells were not affected in frequencies in the absence of *Tada3*, although surface markers were slightly changed. Although these cells were still able to get initially activated via BCR and TLR stimulation, they did not give rise to GC B cells (**Fig. 5A, 5B**) and could not proliferate (for example, **Fig. 9E, right**) or differentiate into subsequent B cell populations. This suggests that TADA3 and SAGA-HAT play an important role in the process of B cell activation, which is an important step towards PBs and PC formation.

Although our *in vivo* analysis of *Tada3* in B cell development points to earlier roles in B cell development, we also observed exclusive defects in the formation of PCs in *Bhlha15*-Cre *Tada3*$^{fl/fl}$ mice. When we focused on GFP-expressing PCs, which indicated Cre-recombinase expression, we observed a significant reduction in PCs in the bone marrow (**Fig. 8F**). Analysis of the spleen showed a slight but significant reduction in PBs (**Fig. 8F**). This can be explained by the delayed expression of this Cre recombinase, which seems to be only fully active in PCs.

Considering that *Cd23*-Cre *Tada3*$^{fl/fl}$ mice could not generate GC B cells upon immunization, it is possible that our previously described screening system and analysis identified genes relevant for GC B cell formation and activation. While the extended length of the culture system should allow for efficient deletion prior to PB formation, we cannot control the exact onset of deletion, which could affect already a B cell stage prior to PBs. In addition, as B cells are very difficult to transduce, we had to activate the B cells prior to infection with a mix of IL-4, CD40L, BAFF and LPS on day 1 of the culture. This enhanced the transduction efficiency but already led to B cell activation, which was followed by sgRNA deletion that should be completed within 5 days of the culture before PB differentiation was induced. Additionally, the cells were maintained in stimulating conditions throughout the experiment. This could further explain why the loss of *Tada3* showed earlier effects on B cell development

in vivo and highlights the necessity to *in vivo* validate the CRISPR/Cas9 screening hits obtained under *in vitro* culture conditions.

Altogether, while *Tada3* and SAGA-HAT genes were originally identified in a PB-specific sgRNA screen, we could show that they have earlier functions in B cell development *in vivo*. However, this does not rule out a role of the SAGA-HAT module in PB and PC development.

3.4 Limitations of existing Cre lines and acute TADA3 protein degradation approach

Consisten with our data, a recent study also demonstrated that SAGA-HAT may be required for early B cell development. Here, the authors induced deletion of the HAT-enzyme GCN5 by B cell-specific *Cd19*-Cre expression (Oksenych et al. 2022; Rickert et al. 1997). Although they observed an accumulation of pro-B cells in the bone marrow, they did not detect a complete loss of all subsequent B cell populations, as was the case in our experiments. This discrepancy can be explained by the limitations of the *Cd19*-Cre allele. The Cre-mediated deletion is induced at a gradually increasing efficiency in the bone marrow, resulting in full deletion only in mature B cells. This is in marked contrast to the *Cd79a*-Cre allele which we used in our experiments (Hobeika et al. 2006; Tabor and Gould 2017). Also, the authors analyzed 8-12-week-old mice and did not assess cell numbers of bone marrow B cells but displayed percentages of cell populations, which could explain their observation of a pro-B cell accumulation. In contrast, we analyzed mice that were appropriate for the study of early B cell development (5-week-old mice) and quantified the total number of B cells in the bone marrow, which revealed no accumulation of pro-B cells.

To study whether the deletion of *Tada3* primarily affected the activation and formation of GC B cells or whether GC B cell maintenance was the reason for our observed lack of GC B cells upon NP-KLH immunization of *Cd23*-Cre *Tada3*$^{fl/fl}$ animals, a different Cre-recombinase would be helpful. Unlike *Cd23*-Cre, which induces full deletion in mature B cells of the spleen, *Cγ1-Cre* or *Aicda(Aid)-Cre* have been used to study the effects in GC B cells. However, both Cre-lines are induced at an activated B cell stage undergoing class switch recombination (CSR), which precedes GC formation and are therefore not ideal for studying the effects on GC maintenance (Casola et al. 2006; Kwon et al. 2008; Roco et al. 2019). However, the onset of

Aid-Cre-expression is most likely delayed and may only be fully expressed after the formation of GC B cells (Osma-Garcia et al. 2021).

Using a tamoxifen-inducible Cre allele could be another way to restrict Cre-expression to GC B cells. Such Cre-lines have been generated in the case of AID-CreERT2 and the GC gene sphingosine-1-phosphate receptor 2, *S1pr2*-CreERT (Dogan et al. 2009; Shinnakasu et al. 2016).

Although conditional mutagenesis of a floxed allele is a powerful tool for unveiling potential phenotypes at specific cell stages, it is only possible to study the underlying transcriptional landscape if a Cre line is available, and the targeted gene is not essential to the cell to be studied. For many transcription factors (TFs), such as IRF4 and BLIMP1, gene deletion led to loss of the cells of interest, and the target genes could not be studied. Additionally, it is difficult to distinguish by mRNA analysis whether a gene is directly regulated by the TF or indirectly as a secondary effect of the deregulated primary targets. Acute protein degradation is an alternative approach that circumvents these limitations. The auxin-inducible degron (AID) system was originally described in plants and has since been adapted for use in mammalian cells (Nishimura et al.2009; Teale et al. 2006). This system allows rapid protein degradation and the identification of direct target genes, when coupled with the analysis of nascent transcripts. We generated an auxin-inducible-degron-tagged *Tada3* allele in a modified *Rosa26*$^{Tir1(F74G)}$ background which prevented the known leakiness of the E3 ligase adaptor protein Tir1. It also increases the affinity for the auxin analog 5-Ph-IAA, which makes *in vivo* degradation in the mouse possible (Yesbolatova et al. 2020). The *Rosa26*$^{Tir1(F74G)/Tir1(F74G)}$ *Tada3*$^{AID/AID}$ mouse allows degradation of TADA3 in any cell of interest owing to the ubiquitous expression of *Tada3* and *Rosa26*. In the B cell lineage, we could use it to identify direct targets of TADA3 in pro-B cells or in any other B cell types that are lost upon conditional mutagenesis.

Since we observed a striking phenotype upon the activation of mature B cells in the absence of *Tada3*, we focused our analysis on late B cell activation and used the generated *Tada3*^{AID} *Rosa26*$^{Tir1(F74G)}$ mice to identify genes that depend on TADA3 and the SAGA-HAT module. We can address the identification of SAGA-HAT dependent genes in B cell activation by *ex vivo* culture of mature B cells and rapid protein degradation of TADA3, followed by activation of the cells. In this ongoing analysis, we aim to identify direct targets at an early and

at a later activation time point by total RNA sequencing and identifying nascent intronic transcripts compared to non-degraded control cells. Additionally, we examined the changes in nascent transcripts in acutely TADA3-degraded GC B cells, which are highly proliferative and active B cells. This analysis is also ongoing and will likely identify SAGA-HAT dependent genes in *in vivo* activated cells.

In contrast to the aforementioned GC-specific Cre lines, the *Tada3*^{AID} allele offers better temporal resolution. This analysis can be combined with ChIP-seq for H3K9ac and ATAC-seq to investigate changes in chromatin modifications and chromatin accessibility at the identified target sites.

3.5 Reasons for studying the role of the SAGA-HAT-complex in B cells

Although the identification of *Tada3* and other SAGA-HAT genes in a CRISPR-Cas9 dropout screen led us to investigate the role of the SAGA-complex *in vivo*, it also raises several questions due to the ubiquitous expression profile of the SAGA-complex. Based on the assumption that the expression of a gene indicates an essential function, the SAGA-HAT module could be considered to be generally important across all cell types. However, the SAGA-Hat components exhibit a more complex expression pattern with higher expression in some specific tissues. For instance, the SAGA-HAT enzyme GCN5 has been suggested to be important for hematopoiesis as opposed to its ortholog PCAF (Arede and Pina 2021). Despite this notion, over the last few years several studies have claimed that the SAGA-complex is a general co-activator that is broadly required for transcription, while more recent publications point towards a more cell-stage and context-specific role. These seemingly contradictory findings, often based on ChIP-seq experiments, might be partially due to the technical limitations of the method for detecting transiently binding coactivators and the use of different antibodies, but also due to the study of different model organisms, such as yeast and mammalian cells (Baptista et al. 2017; Bonnet et al. 2014; Cheon et al. 2020; Grau et al. 2008; Nagy et al. 2009; Weake et al. 2012).

The observation that broadly expressed factors can still have tissue- and context-specific roles is neither new nor unique to co-factors, such as the SAGA-complex. Also transcription factors, which are sometimes similarly expressed across tissues and would not be identified as tissue-specific based on their mere expression, can mediate tissue-specific regulation.

Considering that out of approximately 30,000 genes in the human genome, less than 2,000 encode TFs that need to regulate many tissue-specific functions, it is unlikely that the activation or repression by individual TFs is solely responsible for tissue-specific gene expression changes. For instance, individual TFs, like IRF4 and BLIMP1, can be very important for certain cell types but they rely on many other factors, including co-regulators, which together with TFs can form transcription complexes (Sonawane et al. 2017; Talukdar et al. 2023; Vaquerizas et al. 2009; Wang et al. 2021).

When we analyzed the role of the SAGA-HAT gene *Tada3* throughout B cell development using conditional mutagenesis, we observed different phenotypes as discussed earlier. In brief, we noted that the B cell transition, which depends on cell proliferation and differentiation from the pro-B to the pre-B cell stage and from the mature B cell stage to the activated B cell stage, was affected in the absence of *Tada3*. In contrast, resting and quiescent MZ and FO B cells were largely unaffected. This indicates that the coactivator SAGA has different functions at different B cell stages and is important for B cell activation.

Since TADA3 is shared between the SAGA-HAT and the HAT of the related ATAC-complex, the SAGA-HAT specific protein TADA2b would have been the better candidate to study the unique role of SAGA-HAT in B cell development. *Tada2b* was also shown to be important for PB development in our CRISPR-Cas9 screening approach, whereas the ATAC-HAT gene *Tada2a* showed no effect (**Fig. 1B**). However, due to the lack of an existing *Tada2b* floxed allele, we focused our efforts on *Tada3*. When we analyzed RNA-sequencing data for all SAGA-HAT genes, we noted that they were expressed throughout B cell development, while the ATAC-specific gene *Tada2a* was not expressed as well as the enzyme *Pcaf* (**Fig. 2D**), which is more often mentioned in the context of ATAC-HAT than SAGA-HAT. Moreover, the SAGA-HAT enzyme *Kat2a* (encodes for GCN5) is expressed in B cells, whereas *Kat2b* (encodes for PCAF) is expressed at only low levels (our observation and Kikuchi et al. 2005). Therefore, we assigned all observed phenotypes following the deletion of *Tada3* to the SAGA-HAT complex and propose that SAGA-HAT, but not ATAC-HAT, plays a role in B cell development.

While most studies, that have investigated the role of GCN5 and other SAGA components in mammalian cells, have focused on the early stages of development by using pluripotent cells such as mouse embryonic stem cells (mESCs), few studies have examined its role in

differentiated cells such as B cells. Some lines of evidence that SAGA-HAT could be a relevant co-activator to B cells were provided by studies in the chicken FO B cell line (DT40). Here, the authors activated PI3K-signaling pathway in the absence of *Gcn5* and described defects in AKT phosphorylation (Kikuchi et al. 2011). For us, the deletion of *Tada3* in FO B cells and their activation by different means, including BCR stimulation and TLR stimulation, did not lead to significant differences in the phosphorylation of the serine-threonine kinase AKT at Thr308 or Ser473. In a later study, the same authors showed binding of GCN5 to the *Irf4* gene by ChIP-PCR and reduced *Prdm1* expression, which impaired B cell differentiation (Kikuchi et al. 2014). It would be interesting to verify this finding in *in vivo* B cell activation and differentiation. We hope that our ongoing analysis of rapidly degraded TADA3-AID-tagged protein in GC B cells will shed light on the direct targets of SAGA-HAT that could not be detected before. Since *Irf4* expression is absent in GC B cells but is required for GC B cell formation, due to its functions in B cell activation and early GC B cell formation (Ochiai et al. 2013; Sciammas et al. 2006; Shaffer et al. 2001; Willis et al. 2014), we might not identify *Irf4* as a direct target of SAGA-HAT in GC B cells. This possible regulatory axis could be better investigated when analyzing our *ex vivo* rapid degradation experiment, where we induced TADA3 degradation in FO B cells, followed by BCR activation.

To date, experiments analyzing the SAGA-components in the context of B cell biology have only been performed in cell lines. They have further focused on the enzyme GCN5, which might also be part of other HAT-complexes; therefore, unique claims for the SAGA-HAT complex cannot be made by these data.

Altogether, co-activators, such as the SAGA-complex, have not been the focus of studies on gene regulation in hematopoiesis. Despite a broad expression pattern, the SAGA-complex may have tissue-specific roles and interact with different TFs to regulate gene expression. Our experiments are the first to extensively analyze the role of the SAGA-HAT in B cell development.

3.6 Role of H3K9ac in B cell development

The acetylation of H3K9 is a reversible activating chromatin modification that is the main mode of action of the SAGA-HAT. To date, mainly SAGA is thought to be responsible for generating the H3K9ac modification in mammalian cells (Helmlinger et al. 2017; Jin et al.

2011; Lee et al. 2007; Nagy et al. 2007). Together with other post-translational modifications (PTM) at the transcription start site, it increases chromatin accessibility and is thought to facilitate the assembly of the RNA Pol II pre-initiation complex. Although H3K9ac is considered an activating PTM, it is unclear whether H3K9ac marks recently transcribed genes rather than driving transcription itself (Chen et al. 2022; Howe et al. 2017; Morgan et al. 2020). We and others have shown that the loss of the SAGA-HAT module leads to a global decrease in H3K9ac levels (Fischer et al. 2021). When we analyzed H3K9ac in *Cd23*-Cre *Tada3*$^{fl/fl}$ FO B cells by Western blot, we noted a complete loss of this modification, whereas the FO B cell population showed no defect in viability (**Fig. 4A, 4D**). The FO B cells exhibited cells altered expression of surface markers but did not show any severe defects despite the absence of H3K9ac, suggesting no or little function for H3K9ac in these cells. This leads to the hypothesis that H3K9ac might not be essential for resting FO B cells. At this quiescent cell stage, not many genes need to be expressed compared to the activated and proliferative later B cell stages. This could explain why we observed defects in the absence of *Tada3* once we stimulated the cells. Stimulated B cells showed a striking defect in proliferation and could not form PBs *in vitro* (**Fig. 9D, 9E**). Consistent with this observation, *in vivo* activation with NP-KLH did not result in any GC B cells and consequently, hardly any PBs and PCs were detected (**Fig. 8B, 8C**). The remaining PBs escaped *Tada3* deletion further highlighting the essential function of this gene in PB formation.

H3K9ac is not the only activating chromatin modification but one of many (H3K4me1, H3K4me3, H3K27ac, to name a few) and thus it loss could be compensated by other modifications. H3K4me1 was one of the first methylation marks linked to active transcription but its direct involvement in gene regulation versus being a by-product of activation is under debate. Similar to the SAGA-complex, some studies have suggested that this modification is dispensable for transcriptional regulation in some contexts (Reviewed in Wang et al. 2024). The interplay between several histone modifications could determine transcription, explaining why SAGA-HAT has context-dependent roles. This highlights the necessity of studying the possible lineage-specific functions of histone modifiers.

We did not analyze the levels of H3K9ac in pro-B cells of *Cd79a*-Cre *Tada3*$^{fl/fl}$ mice, but expect a similar loss of H3K9ac, which could be as important at the next proliferative large pre-B cell stage as in GC B cells. Flow cytometric analysis revealed loss of all subsequent B cell

populations, indicating that the activity of SAGA-HAT might be needed for stages of B cell development when cells become active and proliferative.

While other modifications and co-factors may be sufficient to compensate for the loss of SAGA-HAT and H3K9ac in resting FO B cells, this may not be the case for late B cell activation. We speculate that B cell-specific TFs such as IRF4, E2A, E2-2, and PAX5, which are important for late B cell activation (Calderón et al. 2021; Willis et al. 2014; Wöhner et al. 2016), could recruit the SAGA-complex to specific sites in the chromatin to enable transcription or that SAGA-HAT is recruited to the gene loci of these essential TFs that can subsequently be transcribed. Our proposed *ex vivo* rapid degradation experiment of TADA3-AID tagged protein followed by BCR stimulation will help assess the direct targets of SAGA-HAT in B cell activation by total RNA-seq. This could be coupled with ChIP-seq with a H3K9ac antibody to assess possible PTM changes at direct targets, as well as ATAC-seq to assess changes in chromatin accessibility.

3.7 H3K9ac in pluripotent mESCs versus differentiated B cells

Since transient recruitment of the SAGA-complex can hardly be detected by conventional ChIP (Baptista et al. 2017; Bonnet et al. 2014), changes in H3K9ac levels in SAGA-mutants have been used as a proxy for SAGA-localization. A study in MEFs suggested that H3K9ac marks correlate with but are dispensable for Pol II recruitment, which was still recruited to nuclear receptor promoters in the absence of GCN5/PCAF, with hardly any effects on expression levels (Jin et al. 2011). In mESCs, deletion of SAGA-HAT reduced H3K9ac levels but had no effect on mESC growth or survival, and global Pol II activity was not impaired (Fischer et al. 2021). While the authors suggested global binding of the SAGA-complex, loss of the HAT module did not result in significant phenotypic or transcriptional defects (Fischer et al. 2021, 2022). We also observed global H3K9ac reduction in *Cd23*-Cre *Tada3*$^{fl/fl}$ FO B cells and no striking phenotype at this quiescent cell stage but activation of the cells led to defects that were very different from those observed in mESCs.

mESC are rapidly proliferating pluripotent cells that can self-renew. H3K9ac is however thought to be especially important for mESCs but its genome-wide role is unclear (Hezroni et al. 2011; Nagy and Vintersten 2006). Considering this, it seems surprising that loss of the SAGA-HAT module and the accompanying loss of H3K9ac did not affect mESC proliferation or

self-renewal, while loss of SAGA-core module did, pointing to a non-HAT-related role (Fischer et al. 2021). While mESCs and differentiated B cells are very different cell types, B cell activation also leads to a highly proliferative cell stage, a feature shared with mESCs. In our experiments, the loss of SAGA-HAT led to no B cell proliferation, suggesting that SAGA-HAT is very important for B cell proliferation and/or differentiation, in contrast to mESCs. Although the authors did not analyze differentiation defects and focused on self-renewal in mESC, our experiments showed defects in the proliferation and differentiation of B cells, where SAGA-HAT seems to be required.

Fischer et al. (2021) generated an auxin-inducible degron tagged *Tada3* mutant mESC line and performed nascent transcript analysis after depleting TADA3 for 24 hours. They observed a strong global reduction of H3K9ac but no phenotypic defects or changes in Pol II transcription, with only nine genes significantly downregulated upon auxin treatment (Fischer et al. 2021). Our analysis of *ex vivo Rosa26*$^{Tir1(F74G)/Tir1(F74G)}$ *Tada3*$^{AID/AID}$ FO B cells showed that TADA3 was rapidly degraded *in vitro* following 5-Ph-IAA treatment and was depleted after 1 hour (**Fig. 20A**). The chosen time point of Fischer et al. (2021) does not represent rapid degradation and nascent transcript analysis would detect a mixture of direct and indirect targets. Based on this, we would expect many deregulated genes at such a delayed point of analysis unless the SAGA-HAT played no role in the cells, which seems to be the case for mESCs. In addition, Fischer et al. (2021) transfected mESCs with a *Tir1*-expressing plasmid and observed in the absence of auxin already a reduction of H3K9ac which they assigned to interference of the tag with the enzymatic activities of GCN5. This explanation is likely based on a misconception as the lower H3K9ac levels may be caused by the leaky degradation of TADA3 by TIR1 activity. We bypassed this in our experiments by using the modified *Rosa26*$^{Tir1(F74G)}$ allele, which prevents spontaneous degradation in the absence of auxin.

Since we show detrimental defects of activated B cells *in vivo* and *in vitro* in the absence of SAGA-HAT, we expect to find different results in nascent transcript analysis compared with these mESC findings.

A different study (Seruggia et al. 2019) also examined the role of the SAGA-complex in mESCs. The authors identified the TAF proteins TAF5L and TAF6L in a CRISPR-Cas9-mediated loss-of-function screen and suggested transcriptional activation of *Myc* and MYC target genes by TAF5L/TAF6L, which they attributed to H3K9ac deposition. The TAF5L and TAF6L proteins

are part of SAGA, whereas TAF5 and TAF6 are part of TFIID (Cheon et al. 2020; Ogryzko et al. 1998). *Taf5l*, *Taf6l,* and *Tada3* mRNA levels declined as the cells differentiated, suggesting a possible role in mESCs self-renewal (Seruggia et al. 2019). When we analyzed the expression of SAGA-complex components in B cell development, we observed a ubiquitous but relatively low expression of these components. This agrees with the observed decline in transcripts in differentiating mESCs and could add to a different role in mESCs than in B cells.

While Seruggia et al. (2019) focused on the SAGA-core components *Taf5l* and *Taf6l* in mESCs they attributed the control of target genes to H3K9ac deposition. Therefore, the SAGA-HAT module should be important for mESC gene expression control contrary to the suggested HAT-independent role by Fischer et al. (2021). Although Seruggia et al. (2019) observed genome-wide binding of TAF5L and TAF6L, they reported transcriptional control of MYC and MYC-associated genes via H3K9ac and that this interaction regulates the self-renewal of mESC. It was suggested that the SAGA-HAT might play a more critical role during differentiation which was not assessed in these studies (Fischer et al. 2021; Seruggia et al. 2019).

Considering our own observations in B cells, we report a role for SAGA-HAT in B cell activation and observed a reduction in *Myc* gene expression and MYC protein in the absence of *Tada3*. Both publications (Fischer et al. 2021; Seruggia et al. 2019) provide an interesting basis to reflect about our own data. Since the phenotypes observed in the absence of SAGA-HAT in B cells are quite different from those in mESCs, it will be crucial to analyze the direct targets of SAGA-HAT. We hope to shed further light on the proposed SAGA-MYC axis.

3.8 SAGA-HAT and immediate early genes like *Myc*

SAGA has been shown to interact with TFs via its TRRAP-unit and is then recruited to specific promoters to enhance histone acetylation at recruitment sites (Brown et al. 2001; McMahon et al. 2000). An interaction of TRRAP and the TF MYC has been suggested by several studies in human cell lines where SAGA acts as a co-activator of MYC at specific MYC-activated genes but can also stabilize MYC protein via acetylation at its NLS (Hirsch et al. 2015; Liu et al. 2003; McMahon et al. 1998, 2000; Patel et al. 2004). We also observed a significant defect in MYC-protein expression in *Cd23*-Cre *Tada3*$^{fl/fl}$ FO B cells following BCR activation (**Fig. 15A, 15B**). To our knowledge, we are the first to observe transcriptional control of *Myc* by SAGA-HAT in B

cell activation as shown by q-RT-PCR (**Fig. 15C**), although it has been reported that the core proteins TAF5L/TAF6L are involved in activating *Myc* expression (Seruggia et al. 2019). There are numerous reports of a SAGA-MYC-axis and it might be that SAGA-HAT also controls the expression of *Myc* mRNA.

In yeast, it has been shown that the SAGA-complex can interact with Tbp to deliver it to TATA-box containing promoters via a subunit that is not present in the human SAGA-complex. Cryo-EM analysis of hSAGA has failed to detect a stable TBP-hSAGA interaction but there is evidence that MYC can interact with TBP which could be the missing link between TBP and SAGA-complex (Hahn et al. 2011; Han et al. 2014; Helmlinger et al. 2017; Sterner et al. 1999; Wei et al. 2019; Zhang et al. 2022). While TATA-box containing promoters have been studied in great depth, they are not very prevalent in mammalian promoters (between 3-16% of mouse promoters) and often associated with tissue-specific gene expression (Jin et al. 2006; Ponjavic et al. 2006; Schug et al. 2005; Yella and Bansal 2017). This could mean that SAGA-complex acts on a restricted number of promoters and might require MYC for its recruitment.

Some genes that are especially interesting to us in the context of B cell activation are immediate early genes (IEGs) that are quickly expressed following stimulation, like *Egr2*, *Myc* and *Irf4*. While most IEGs, like *Myc* and *Egr2*, have fewer exons and their promoters have a high prevalence of TATA box motifs (Bahrami et al. 2016; Tullai et al. 2007), *Irf4* is more a delayed early response gene.

It has been reported that several IEGs exist as “poised” mRNA which allows the cell to quickly translate them upon activation but IEGs are also quickly transcribed (Fowler et al. 2015; Salerno et al. 2023). Because there is no need for *de novo* synthesis of proteins that can induce IEG expression and their rapid expression, it is challenging to understand their regulation (Bahrami et al. 2016). We observed that the initial activation cascade was largely functional in *the Tada3*-deficient B cells. AKT-signaling reached similar levels as in control cells, and IEGs such as CD69 were expressed (**Fig. 11B, 13B**). However, downstream effects were dysfunctional because the stimulated cells could not proliferate or differentiate (**Fig. 9D-E, 12B**). We hypothesize that the initial IEG transcripts that are present in the cell are translated but the further transcription of IEGs and secondary response genes cannot occur in the absence of SAGA-HAT. This mechanism could depend significantly on the SAGA-MYC axis. A previous study has shown that MYC-deficient B cells can still express early activation

markers which resembles our phenotype; however, while *Myc* deficiency limits increase of cell size, *Tada3*-deficient B cells are still able to blast (De Alboran et al. 2001).

Other co-activators, such as TIP60 and CBP/p300, have also been shown to interact with MYC and could substitute the role of the SAGA-complex in this context (Faiola et al. 2005; Frank et al. 2003; Vervoorts et al. 2003). Considering the striking phenotype that we observed in TADA3-deficient activated B cells, it could be that SAGA-HAT is primarily required for *Myc* and MYC target genes in B cell activation. To identify SAGA-HAT dependent IEGs, we designed an experiment where we degraded the AID-tagged TADA3 protein *ex vivo* by administering 5-Ph-IAA (**Fig. 20B**). Subsequently the BCR was stimulated with anti-IgM for 1 hour to capture IEGs and for 4 hours for secondary response genes. We will examine the abundance of nascent intronic transcripts by total RNA-sequencing to identify direct targets. Several outcomes seem possible: SAGA-HAT could be important for the transcriptional control of many IEGs; SAGA-HAT could be important for the transcription of a defined amount of IEGs and B cell-specific factors; SAGA-HAT is primarily important for *Myc* regulation and genes from the *Myc* network. The identified list of direct targets can be compared to previously identified IEGs in BCR activation, as well as to a subset of genes that were found to be affected in experiments that stimulated *Myc*-deficient B cells with LPS (Fowler et al. 2015; Salerno et al. 2023; Tesi et al. 2019).

Auxin-inducible acute protein degradation offers more temporal resolution than conditional mutagenesis, and we determined TADA3 degradation *in vivo* and ex vivo by performing time series. The chosen time point ensures efficient depletion, while minimizing the risk of secondary effects. Further, we suggest mass spectrometry experiments of acutely degraded cells to determine changes in protein abundance. We assume that secondary effects would go hand in hand with changes in protein levels, which could be another way to validate our approach.

3.9 SAGA-HAT and *Myc* in B cell development

MYC has been studied in the context of B cell development because it is important for B cell proliferation and is often associated with B cell malignancies. A study has reported a block at the pro-B cell stage upon deletion of *Myc*, driven by *Cd19*-Cre expression, and its importance downstream of the BCR for pro-B and pre-B cell proliferation and possibly also for cell

differentiation (Habib et al. 2007). While *Myc* needs to be expressed for proliferation, its repression is required for post-mitotic cells to exit the cell cycle and differentiate, which means that MYC levels need to be tightly controlled (Habib et al. 2007; Ma et al. 2010; Vallespinós et al. 2011). When we compare our flow cytometry results from *Cd79a*-Cre *Tada3*$^{fl/fl}$ mice to the described pro-B cell block in the absence of *Myc* we see similarities. We initially observed similar numbers of pro-B cells but noted a complete loss of all subsequent B cell populations which is a more striking phenotype than the described reduction of pre-B cells as well as later B cell populations upon *Myc*-deficiency (Habib et al. 2007). In line with Habib et al. (2007), we think that pre-BCR signaling might be affected which is important for cell proliferation as well as differentiation. The generated *Rosa26*$^{Tir1(F74G)/Tir1(F74G)}$ *Tada3*$^{AID/AID}$ mice could help to analyze this further.

In contrast to quiescent FO B cells, where no major function was described for MYC, GC B cells require MYC for GC formation, maintenance, and cyclic re-entry (De Alboran et al. 2001; Calado et al. 2012; Dominguez-Sola et al. 2012). The GC does not contain a homogenous B cell population and only a small subset of GC B cells express MYC. By day 10 following SRBC immunization, when GCs reached their peak, only 10% of GC B cells expressed MYC, which were part of the LZ and showed an active MYC-driven gene expression profile. These cells had elevated transcripts of the NF-κB pathway and *Myc* itself (Calado et al. 2012). Therefore, analysis of bulk GC B cells does not show a higher expression of *Myc* compared to FO B cells which could affect the analysis of our acute degradation experiment of TADA3 in GC B cells, where we sorted bulk GC B cells. However, due to the rarity of GC B cells, analyzing an exclusive subset of LZ GC B cells is not feasible.

Whether MYC acts as a general activator or only at specific target genes has long been debated. Several studies showed that the absence of MYC only affects a subset of genes and a recent study performed acute protein degradation experiments followed by nascent transcript analysis to identify a set of direct targets in a leukemia cell line (K562 cells). The authors identified ribosome biogenesis genes and genes that are important for de-novo purine synthesis as direct target of MYC (Muhar et al. 2018). The previous observed effects on global transcription were likely secondary and the reported broad binding of MYC might be due to low-affinity, non-specific interactions which is termed “invasion” (Lin et al. 2012; Lorenzin et al. 2016; Muhar et al. 2018; Nie et al. 2012; Sabò et al. 2014; Tesi et al. 2019; Walz et al. 2014). A similar scenario can be imagined for the SAGA-complex.

Hence, understanding the interaction between SAGA-HAT and MYC in B cell activation is very important with regard to their tissue-specific roles and direct targets in B cells.

4 Material and methods

4.1 Mice

The mice were maintained on a C57BL/6 background: Transgenic *Cd79a*-Cre (Hobeika et al. 2006), *Cd23*-Cre Click or tap here to enter text.Kwon et al., 2008), *Bhlha15-Gfp*-Cre (Stephen F Konieczny lab, unpublished), *Tada3*$^{fl/fl}$ (Mohibi et al. 2012), and *Rosa26*$^{Tir1(F74G)}$ (Fedl et al. 2024, manuscript in preparation). All newly generated mice were backcrossed onto the C57BL/6 background for several generations prior to analysis. Female and male mice were used in the present study. All animal experiments were performed according to valid project licenses, which were approved and regularly controlled by the Austrian Veterinary Authorities.

4.2 Generation of a *Tada3*-AID-tagged allele

To generate the *Tada3*^{AID} allele, we used the *Easi*-CRISPR method combined with CRISPR/Cas9-mediated genome editing in mouse zygotes (Miura et al. 2017; Yang et al. 2013). For C-terminal tagging of TADA3, we generated the building vector pBV-Cterm-AID-3xV5 by inserting codon-optimized sequences encoding a glycine linker, the minimal AID polypeptide, and three V5 epitopes between the BamHI and EcoRI sites in the middle of the polylinker of pBlueScript II SK(+). We first cloned an 804-bp 5’ homology sequence containing the respective exon sequences up to the last amino acid codon, in frame with the AID sequence at the BamHI site of pBV-Cterm-AID-3xV5, and then inserted the 963-bp 3’ homology sequence of the 5’ region of the 3’UTR of *Tada3* into the EcoRI site of the same plasmid. A 691-bp part of the insert DNA of the plasmid was PCR-amplified and turned into single-stranded (ss) DNA using the Guide-it^{TM} Long ssDNA Strandase Kit (Takara Bio). The *Tada3*^{AID} allele was generated by CRISPR/Cas9-mediated genome editing by pronuclear mouse zygote injection (C57BL/6J) with a mixture of Cas9 protein, *Tada3* ssDNA sequence, and sgRNA (obtained from Integrated DNA Technologies, IDT) targeting the genomic sequence at the stop codon of *Tada3*. Once the pups were born, the inserted DNA sequences were verified by sequencing a PCR fragment that was amplified from the *Tada3-Aid*-tagged allele with primers bridging the 5’ and 3’ homology regions.

To generate a *Rosa26*$^{Tir1(F74G)}$ *Tada3*^{AID} allele, we applied a similar approach but injected a mixture of *the Tada3* ssDNA sequence, Cas9 protein, and sgRNA targeting the genomic sequence at the stop codon of *Tada3* into homozygous *Rosa26*$^{Tir1(F74G)}$ mouse zygotes (C57BL/6J).

4.3 PCR genotyping

The wildtype and transgenic alleles can be distinguished by PCR genotyping and oligonucleotide sequences are listed in "sequences." To detect the *Tada3*^{AID} allele, three primers were used that resulted in a 390-bp DNA fragment for the wildtype allele and a 313-bp and 691-bp DNA fragment for the tagged allele. The primer mix for detecting the *Rosa26*$^{Tir1(F74G)}$ allele or the wildtype allele contained four oligonucleotides, resulting in a 509-bp wildtype DNA fragment or a 370-bp transgenic DNA band. For *Bhlha15-Gfp*-Cre, we used three primers that resulted in a 750-bp wildtype DNA band or a transgenic 871-bp DNA fragment. PCR genotyping of *Cd23*-Cre, *Cd79a*-Cre, and *Tada3*$^{fl/fl}$ alleles has been previously described (Hobeika et al. 2006; Kwon et al. 2008; Mohibi et al. 2012).

4.4 Antibodies used in flow cytometric analyses

The following monoclonal antibodies were used for flow cytometric analysis of lymphoid organs of 3-15 week-old mice: CD19-BV786 or-PE (1D3, BD), B220/CD45R-PE or-BV510 (RA3-6B2, BD), CD2-FITC (RM2-5, BD), CD25/IL-2Rα-FITC (PC61, BD), CD117/c-Kit-PE-Cy7 (2B8, eBioscience), IgD-APC (11.26c, eBioscience), IgD-BV421 or-BV605 (11.26c.2a, Biolegend), IgM-PerCP-Cy5.5 (R6-60.2, BD), CD93/AA4.1-PE-Cy7 (PB493, Biolegend), CD138-APC or-BV605 (281-2, BD), CD21/CD35-BV421 (7G6, BD), CD95/FAS-BV421 (Jo2, BD), GL7-PerCP-Cy5.5 (GL7, Biolegend), CD267/TACI-BV421 (8F10, BD), CD267/TACI-PE (8F10, Biolegend), CD1d-PE (1B1, BD), Fixable Viability Dye-eFluor 780 (eBioscience), CD23-FITC (B3B4,Pharmingen), CD22-FITC (Cy34.1, Pharmingen), CD86-PE (GL1, Pharmingen), CD69-PE-Cy7 (H1.2F3, Biolegend), NP(29)-PE (BioSearch Technologies).

The following monoclonal antibodies were used for intracellular staining and phospho-specific flow cytometry: NP(29)-PE (BioSearch Technologies), pAkt (Ser473, D9E, Cell Signaling), pAkt

(Thr308, 244F9, Cell Signaling), IκBα (rabbit polyclonal; Cell Signaling Technology), MYC-PE (D3N8F, Cell Signaling), anti-V5-AlexaFluor647 (SV5-Pk1, Invitrogen).
For the materials and methods of the plasmablast CRISPR-Cas9 screen, see Pinter et al.(2022).

4.5 FACS sorting and definition of hematopoietic cell types

The different hematopoietic cell types were identified using flow cytometry as follows: pro-B ($B220^+CD19^+Kit^+CD25/CD2^-IgM^-IgD^-$), pre-B ($B220^+CD19^+Kit^-CD2/CD25^+IgM^-IgD^-$), immature B ($B220^+CD19^+IgM^{hi}IgD^-$), recirculating B ($B220^+CD19^+IgM^+IgD^{hi}$), total B cells ($B220^+CD19^+$), FO B ($CD19^+CD93^-CD23^+CD21^{lo}CD1d^-$), MZ B ($CD19^+$ $CD93^-CD21^+CD23^{lo}$ $CD1d^+$), GC B cells ($CD19^+B220^+GL7^+Fas^+$), plasmablasts, and plasma cells ($CD138^+$ $TACI^+$).
Flow cytometric analysis and sorting were performed on Aurora (Cytek), LSRFortessa (BD Biosciences), and FACSAria III (BD Biosciences) machines, respectively. The FlowJo Software (Treestar) was used for data analysis.

4.6 Intracellular flow staining of tagged TADA3 and signaling molecules

Intracellular staining of B cells was performed after surface staining by fixation-permeabilization of the cells with the Foxp3 Staining Buffer Set (eBioscience), followed by intracellular staining with an anti-V5 antibody (Sv5-PK1, Invitrogen).
Analysis of phosphorylated BCR signaling components was performed with mature $CD43^-$ B cells from lymph nodes, which were enriched by immunomagnetic depletion with CD43-MicroBeads (Miltenyi Biotec), followed by resuspension in complete RPMI-1640 medium (Gibco), and equilibration in medium for 1 hour at 37 °C. Cells were stimulated with 10 µg/mL anti-mouse IgM antibody (goat anti-mouse IgM $F(ab')_2$ fragment, Jackson ImmunoResearch) for the indicated times at 37 °C and fixed/permeabilized by adding Fixation Buffer (Perm/Wash Buffer BD with 8% paraformaldehyde (PFA, Sigma)) for 15 min at RT and then for 30 min on ice in the dark. Cells were washed twice with Perm/Wash Buffer (BD Biosciences) before they were stained with pAKT-antibodies diluted in Perm/Wash Buffer (BD Biosciences). The samples were incubated for 30 min at RT in the dark and washed twice with Perm/Wash Buffer (BD) before measurement.

For the analysis of intracellular IκBα degradation, mature CD43⁻ B cells from lymph nodes were enriched by immunomagnetic depletion with CD43-MicroBeads (Miltenyi Biotec), followed by resuspension in complete RPMI-1640 medium (Gibco). The cells were stimulated with 1 µg/ml CpG oligodeoxynucleotides (ODN1826, InvivoGen) for 1.5 hours at 37 °C. Cells were harvested and activation was stopped by placing the samples on ice. After surface staining, the cells were fixed with Phosflow Fix Buffer I (BD) for 15 min at 37 °C and stained with IκBα antibody, diluted in Perm/Wash Buffer I (BD Phosflow) for 1 hour at RT in the dark.
The intracellular levels of MYC were analyzed using mature CD43⁻ B cells from lymph nodes that were enriched by immunomagnetic depletion with CD43-MicroBeads (Miltenyi Biotec), followed by resuspension in complete RPMI-1640 medium (Gibco). The cells were stimulated with 10 µg/mL anti-mouse IgM antibody (goat anti-mouse IgM $F(ab')_2$ fragment, Jackson ImmunoResearch) for 1 hour at 37 °C. The stimulation was stopped by placing samples on ice and after surface staining, cells were fixed/permeabilized with the Foxp3 Staining Buffer Set (eBioscience), followed by intracellular staining.
All antibodies used are described in “Antibodies used in flow cytometric analyses.”

4.7 B cell culture

Mature CD43⁻ B cells from lymph nodes were enriched by immunomagnetic depletion using CD43-MicroBeads (Miltenyi Biotec), and the cells were resuspended in IMDM medium containing 10% fetal calf serum, 1 mM glutamine, and 50 µM β-mercaptoethanol. For stimulation, the medium was supplemented with 25 µg/ml LPS and IL-4 (produced in-house, 20 ng/ml), or with 1 µg/ml CpG oligodeoxynucleotides (ODN1826, InvivoGen) and IL-4 (produced in-house, 20 ng/ml) and recombinant mouse IL-5 (R&D Systems, 10 ng/mL), or with CD40L (R&D Systems, 100 pg/ml) and IL-4 and IL-5 for up to 4 days, or the indicated time points.
Alternatively, the cells were resuspended in complete RPMI-1640 medium (Gibco) and stimulated with 1 µg/mL anti-mouse IgM antibody (goat anti-mouse IgM $F(ab')_2$ fragment, Jackson ImmunoResearch) and IL-4 (produced in-house, 20 ng/ml) for up to 4 days.
On day 4, the proliferation of stimulated B cells was assessed by flow cytometry. Live/dead separation of in vitro-cultured cells was achieved by staining with the fixable viability dye eFluor780 (Thermo Fisher Scientific).

For cell proliferation analysis, purified B cells were first stained with CellTrace Violet reagent (5 µM; Invitrogen) before stimulation as described above.

4.8 Immunizations

The immune response of *Cd23*-Cre *Tada3*$^{fl/fl}$ animals to a T cell-dependent antigen was studied by intraperitoneal injection of 100 µg of 4-hydroxy-3-nitrophenylacetyl-conjugated keyhole limpet hemocyanin (NP-KLH; Biosearch Technologies) in alum. We analyzed GC and PB formation in the spleen on day 7 using flow cytometry. The T cell-independent immune response was assessed by intraperitoneal injection of 10 µg of NP (2,4,6-trinitrophenyl)-Ficoll, and PB formation was analyzed on day 8.

Sheep red blood cells (SRBCs) were washed in PBS and resuspended at 10^9 cells/ml, followed by intraperitoneal injection of 100 µL into adult *Rosa26*$^{Tir1(F74G)}$ *Tada3*$^{AID/AID}$ mice, and GC B cells were analyzed on day 10.

4.9 ELISPOT analysis

The mice were intraperitoneally injected with NP-KLH (100 µg) in alum. The frequencies of NP-specific IgG1 or IgM antibody-secreting cells (ASCs) in the spleen were determined by ELISPOT assay (Smith et al. 1997). ELISPOT was also performed for non-immunized aged animals, and IgM and IgG1 ASCs were detected in the bone marrow. Goat anti-mouse IgM or IgG1 antibody-coated plates were used for capturing IgM or IgG1 antibodies secreted by individual cells, respectively. Spots were visualized with goat anti-mouse IgM or IgG1 antibodies conjugated to alkaline phosphatase (Southern Biotech), and the color was developed by the addition of BCIP/NBT Plus solution (Southern Biotech). After extensive washing, spots were counted using an AID ELIspot reader system (Autoimmun Diagnostika).

4.10 RT-qPCR analysis of mRNA

CD43$^-$ FO B cells from lymph nodes enriched by immunomagnetic depletion with CD43-MicroBeads (Miltenyi Biotec) were resuspended in complete RPMI-1640 medium (Gibco). The cells were stimulated with 10 µg/mL anti-mouse IgM antibody (goat anti-mouse IgM

F(ab')$_2$ fragment, Jackson ImmunoResearch) for 1 hour at 37 °C. Total RNA was prepared from cells using TRIzol Reagent (Thermo Fisher Scientific), and reverse transcription was performed using the SuperScript III First-Strand Synthesis System (Invitrogen). RT-PCR was performed on a CFX96 Real-Time System (Bio-Rad) with Luna Universal qPCR Master Mix (New England Biolabs). *Myc* transcripts were normalized to *Tbp* transcripts. qPCR primers were designed to span an exon-exon junction.

4.11 Immunoblot analysis and antibodies

Whole cell protein extracts were separated on 4-12% SDS PAGE gels prior to blotting onto polyvinylidene fluoride (PVDF) membranes using the iBlot2 system (Invitrogen). Membranes were blocked in 3% non-fat dry milk for 1 hour at room temperature. Membranes were incubated overnight with primary antibodies in 3% non-fat dry milk at 4°C. Secondary goat anti-rabbit or anti-mouse antibodies conjugated to HRP in 3% non-fat dry milk were incubated for 1 hour at room temperature. The membranes were developed using ECL Prime (Amersham) and ChemiDoc Touch Imaging System (Bio-Rad). The following antibodies were used for immunoblot analysis: anti-TADA3 (HPA042250, Merck), anti-GCN5L2 (C26A10, Cell Signaling Technology), anti-SUPT7L (A302-803A, Bethyl Laboratories), anti-H3K9ac (07-352, Sigma Aldrich), anti-H3, HRP-coupled (D1H2; Cell Signaling Technology), anti-GAPDH, HRP-coupled (14C10; Cell Signaling Technology), anti-HSP90, HRP-coupled (C45G5, Cell Signaling Technology).

4.12 Acute degradation of TADA3^{AID}

5-Ph-IAA (5-phenyl-indole-3-acetic acid; HY-134653; MedChemExpress) was intraperitoneally injected at a concentration of 10 mg/g into *Rosa26*$^{Tir1(F74G)/Tir1(F74G)}$ *Tada3*$^{AID/AID}$ mice. Mice were analyzed by flow cytometry at different time points after 5-Ph-IAA injection, and the 4 hours treatment time point was chosen for GC B cells. TADA3-depleted GC B cells were sorted for total RNA-seq.

Ex vivo cultured FO B cells were treated with 200µM 5-Ph-IAA and protein degradation was assessed by flow cytometry. A 1 hour treatment was chosen to be sufficient for protein degradation.

4.13 *Ex vivo* TADA3^{AID} degradation experiment for total RNA-seq

CD43$^-$ FO and MZ B cells from the lymph nodes and spleen were enriched by immunomagnetic depletion with CD43-MicroBeads (Miltenyi Biotec) and resuspended in RPMI-1640 medium (Gibco). The cultured cells were equilibrated in the medium for 1 hour at 37 °C, followed by 1 hour of 5-Ph-IAA degradation. Next, the cells were stimulated with 10 µg/mL anti-mouse IgM antibody (goat anti-mouse IgM F(ab')$_2$ fragment; Jackson ImmunoResearch) for 1 or 4 hours at 37 °C. Aliquots of cells were analyzed by flow cytometry, and the cells were frozen at -80 °C prior to further analysis.

4.14 Total RNA-seq experiments

Four hours 5-Ph-IAA treated GC B cells were sorted for total RNA-seq. For *ex vivo* depletion experiments from FO and MZ B cells, the following conditions were collected: treated with medium or for 1 hour with 5-Ph-IAA; treated with medium or for 1 hour with 5-Ph-IAA and activated for 1 hour with anti-mouse IgM antibody; treated with medium or for 1 hour with 5-Ph-IAA and activated for 4 hours with anti-mouse IgM antibody. Total RNA was isolated using a lysis step based on guanidine thiocyanate and further processed using the KingFisher Flex Magnetic Particle Processor. The semi-automated procedure included a 15-min DNase I digestion at 37 °C and a 5-min elution in H_2O at 60 °C. Total RNA integrity and concentration were assessed by a fragment analyzer. Depletion of rRNA from the total RNA was performed using a mix of antisense oligonucleotides matching mouse rRNA and the Hybridase Thermostable RNase H (Epicenter), which specifically degrades RNA in RNA-DNA hybrids (Bhat et al. 2023). DNA was subsequently digested with TURBO DNase (Invitrogen), and RNA was purified using RNA Clean & Concentrator-5 (Zymo Research), according to the manufacturer's instructions. Libraries were prepared using a NEBNext Ultra Directional RNA Library Prep Kit for Illumina (NEB), according to the manufacturer's protocol. Paired-end 50-bp sequencing was performed using a NextSeq3000 sequencing instrument.

4.15 Sequences

Type	Sequences (5'-3')
Genotyping *Tada3*^{AID}. Fwd (a)	CCATGACATACGGTCCCCATAG
Genotyping *Tada3*^{AID}. Rev-I (b)	AGGTCCAGGCAGGCGCAGTA
Genotyping *Tada3*^{AID}. Rev-II (c)	TTGCGGTAGGACCTTACTGG
Genotyping *Rosa26*$^{Tir1(F74G)}$. Fwd-I	AGCACTGGAAATGTTACCAAGGAACT
Genotyping *Rosa26*$^{Tir1(F74G)}$.Fwd-II	GAGAGGAGTGTTTGTGGGAAAC
Genotyping *Rosa26*$^{Tir1(F74G)}$.Rev-I	CCATAGTGGCTCATTAGGGAATGCTT
Genotyping *Rosa26*$^{Tir1(F74G)}$.Rev-II	CAACCTCATTTTCTTGGAGGTC
Genotyping *Bhlha15-Gfp*-Cre. Fwd	GGTTTAAGCAAATTGTCAAGTACGG
Genotyping *Bhlha15-Gfp*-Cre. Rev-I	ATAGTAAGTATGGTGGCGGTCAGCG
Genotyping *Bhlha15-Gfp*-Cre. Rev-II	CTCGATGTTGTGGCGGATCT
sgRNA targeting C-ter *Tada3*	GCAGGTCAGGCTACCCGTCT
RT-qPCR primer *Myc*. F, Spanning exon 2-3	TTCCTTTGGGCGTTGGAAAC
RT-qPCR primer *Myc*. R, Spanning exon 2-3	GCTGTACGGAGTCGTAGTCG
RT-qPCR primer *Tbp*. F	TTCGTGCAAGAAATGCTGAA
RT-qPCR primer *Tbp*. R	CAGTTGTCCGTGGCTCTCTT

4.16 Statistical analysis

Statistical analyses were performed using the GraphPad Prism 8 software. Two-tailed unpaired Student's t-test was used to assess the statistical significance of one observed parameter between the two experimental groups. One-way analysis of variance (ANOVA) was used when more than two experimental groups were compared, and statistical significance was determined using Tukey's or Dunn's multiple comparison test.

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