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Role of beta-catenin in self-renewal and differentiation of mouse embryonic stem cells

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Verfasserin:	Natalia Lyashenko
Matrikel-Nummer:	0606945
Dissertationsgebiet (lt. Studienblatt):	Molekulare Biologie
Betreuerin:	Dr. Christine Hartmann

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Whenever I hear, 'It can't be done,' I know I'm close to success.

Michael Flatley, (Lord of the Dance)

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Abstract

The canonical Wnt signaling pathway is important during early embryogenesis and has been implicated in stem cell self-renewal. The essential intracellular mediator of this pathway is β -catenin, which is stabilized in the presence of a Wnt ligand and thus becoming translocated into the nucleus, where it interacts with TCF/LEF transcription factors to activate target gene expression. In addition, β -catenin, as a component of adherens junctions, is involved in calcium-dependent cell-cell adhesion. This role of β -catenin during development has not been well studied.

Ablation of *β -catenin* in mouse leads to developmental arrest at the primitive streak stage of embryogenesis. Conversely, stabilization of β -catenin in the mouse zygote results in premature epithelial-mesenchymal transition in the epiblast of post-implantation embryo. However, these experiments did not address to what extent the phenotypic changes can be attributed to the signaling or cell-adhesion function of β -catenin. In order to gain insight into this problem, I established a mouse embryonic stem (mES) cell based system. This involved the derivation of ES cells deficient for β -catenin (β -cat ^{Δ/Δ}). These cells showed no defects in self-renewal, but during embryoid body differentiation I noticed cell-adhesion defects and increased apoptosis around day 5. These differences occur because of alterations in plakoglobin levels, a molecule highly related to β -catenin. Moreover, plakoglobin knock-down experiments in β -cat ^{Δ/Δ} ES cells suggest that cell-cell adhesion is most likely essential for proper maintenance of ES cell pluripotency. Secondly, in order to distinguish between adhesive and transcriptional functions of β -catenin, I re-expressed signaling-defective isoforms of β -catenin in β -cat ^{Δ/Δ} ES cells. When these ES cell lines are differentiated *in vitro*, the adhesion defect and increased apoptosis observed during β -cat ^{Δ/Δ} ES cell differentiation are rescued. Furthermore, marker analysis of differentiated EBs revealed that β -catenin cell-adhesion function is required for endoderm lineage formation. In addition, I found that β -catenin cell-adhesion function is essential for cellular survival, but not for formation and terminal differentiation of the neuroectoderm lineage.

My studies also revealed that the canonical Wnt pathway is not active in self-renewing mES cells in the presence of leukemia inhibitory factor (LIF), but can sustain to some extent pluripotency in a LIF-independent manner when activated extrinsically.

In conclusion, my findings show for the first time the importance of β -catenin cell-adhesion function in endoderm and ectoderm formation and suggest that cell-cell adhesion is essential for self-renewal of mES cells.

Zusammenfassung

Der kanonische Wnt-Signalübertragungsweg ist wichtig für die frühe embryonale Entwicklung und es gibt Hinweise, dass er auch für die Selbsterneuerungskapazität von Stammzellen benötigt wird. Das essentielle intrazelluläre Molekül dieses Signalweges ist β -catenin, welches in Anwesenheit eines Wnt-Liganden stabilisiert und in den Kern transportiert wird, wo es dann mit TCF/LEF Transkriptionsfaktoren interagiert und Zielgene anschaltet. Zusätzlich spielt β -catenin als Komponente der Adhäsionsverbindungen, in der Kalzium-abhängigen Zell-Zell Adhäsion eine Rolle. Diese Funktion von β -catenin während der Entwicklung ist bisher nicht gut charakterisiert.

Mäuse in denen β -catenin deletiert wurde zeigen einen Entwicklungsarrest im Stadium der Bildung der Primitivrinne. Im umgekehrten Fall kommt es durch die Stabilisierung des β -catenin Proteins, d.h. Aktivierung des Signalweges, in der Mauszygote zu einem vorzeitigen Übergang vom epithelialen in den mesenchymalen Zustand im Epiblasten des eingenisteten Embryos. Diese Experimente geben jedoch keinen Aufschluss darüber, ob die phänotypischen Veränderungen durch die Funktion von β -catenin im Wnt-Signalweg oder in der Zelladhäsion bedingt sind. Um Einblick in dieses Problem zu gewinnen, habe ich ein zelluläres System basierend auf der embryonalen Mausstammzelle (mES) entwickelt. Dafür war es notwendig mES Zellen zu generieren, die kein funktionales β -catenin haben (β -cat $^{\Delta\Delta}$). Diese Zellen wiesen keine Defekte in ihrer Selbsterneuerungskapazität auf, aber während der Differenzierung in sogenannte embryonen-ähnliche Strukturen zeigten sie einen Zelladhäsionsdefekt und gesteigerten Zelltod am Tag 5. Diese Unterschiede sind durch veränderte Proteinkonzentrationen von Plakoglobin bedingt, einem Molekül das dem β -catenin Protein sehr ähnlich ist. Zusätzlich zeigten Plakoglobin knock-down Experimente in β -cat $^{\Delta\Delta}$ ES Zellen dass die Zelladhäsion wahrscheinlich für die Erhaltung der Pluripotenz der ES Zellen wichtig ist. Um zwischen der Funktion von β -catenin im Wnt-Signalweg und der Zelladhäsion zu unterscheiden habe ich im zweiten Schritt β -cat $^{\Delta\Delta}$ ES Zellen generiert in denen β -catenin Isoformen exprimiert werden die für die Signal- nicht aber für die Zelladhäsionsfunktion defekt sind. Diese Zellen zeigen keinen Zelladhäsionsdefekt oder Zelltod während der Differenzierung. Außerdem ergab eine Markeranalyse dieser differenzierten Zellen, dass die Zelladhäsionsfunktion von β -catenin für die Bildung der endodermalen Keimblattlinie benötigt wird. Zudem weisen diese Experimente darauf hin, dass diese Funktion von β -catenin für das

Überleben der Zellen benötigt wird, dass sie aber nicht absolut essentiell für die terminale Differenzierung der neuroektodermalen Linie ist.

Meine Studien haben außerdem gezeigt, dass der kanonische Wnt-Signalübertragungs-weg in ES Zellen unter Selbsterneuerungsbedingungen in Anwesenheit des Leukemia Inhibitorischen Faktors (LIF) nicht active ist, allerdings kann der aktivierte Wnt/ β -catenin-Signalweg LIF-unabhängig die Pluripotenz der ES Zellen in einem gewissen Ausmaß aufrechterhalten.

Zusammenfassend möchte ich festhalten, dass meine Arbeit zum ersten Mal die Notwendigkeit der β -catenin Zelladhäsionsfunktion für die Bildung der endodermalen und z.T der ektodermalen Keimblätter zeigt und zudem darauf hinweist, dass Zell-Zell Adhäsionsverbindungen für die Selbsterneuerungskapazität der Maus ES Zellen essentiell sind.

Abbreviations

AC	Adenylyl cyclase
AKT1	Protein Kinase B
APC	Adenomatous Polyposis Coli
aPKC	Atypical protein kinase C
Axin	Axis Inhibition protein
BCL9-2	B-cell lymphoma 9-2
BIO	GSK3 inhibitor IX
BMP	Bone Morphogenic Protein
bp	base pairs
BSA	Bovine Serum Albumin
CaCl ₂	Calcium Chloride
CAM	Cell Adhesion Molecule
CamKII	Ca ²⁺ /calmodulin-dependent protein kinase 2
cAMP	Cyclic Adenosine Monophosphate
CBP	Creb-binding protein
CHIR	CHIR99021
CKI ϵ	Casein Kinase 1
c-Myc	Myc proto-oncogene protein
CREB	cAMP response element-binding factor
DEPC	Diethylpyrocarbonat
dH ₂ O	Deionized water
Dkk	Dickkopf
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxid
DTA	Diphtheria toxin A
DTT	Dithiothreitol
Dvl	Dishevelled
EB	Embryoid body
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial-Mesenchymal Transition
EpCAM	Epithelial Cell Adhesion Molecule
ERK	Extracellular Signal-Regulated Kinase 1
ES	Embryonic Stem (cells)
EtOH	Ethanol
FCS	Fetal Calf Serum
fl	floxed
Fz	Frizzled
GSK3	Glycogen Synthase Kinase 3
GTPase	Guanosine Triphosphate enzyme

HCl	Hydrochloric acid
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
ICAM-1	Inter-Cellular Adhesion Molecule 1
ICM	Inner Cell Mass
IF	Immunofluorescence
IHC	Immunohistochemistry
iPS	induced Pluripotent Stem (cells)
Jak	Janus kinase signal transducer and activator of transcription
JNK	C-Jun N-terminal kinase
KCL	Potassium chloride
KOH	Potassium hydroxide
LEF	lymphoid enhancer binding factor-1
Lgs	Legless
LiCl	Lithium chloride
LIF	Leukemia Inhibitory Factor
Lrp 5/6	Low-density lipoprotein receptor-related protein 5/6
M	Molar
MAP1B	Microtubule-associated protein 1B
MAPK	Mitogen-Activated Protein Kinase
MED	Mediator for transcriptional initiation
MEF	Mouse Embryonic Fibroblast
MEK	Mitogen-activated protein kinase kinase 1
MgCl ₂	Magnesium chloride
ml	milliliter
mM	millimolar
mTOR	Mammalian Target of Rapamycin
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NaPi	Sodium phosphate
NFAT	Nuclear Factor of Activated T-cells
ng	nanogram
ON	Over Night
Par	Partitioning-defective
pBARLS	Beta-catenin Activated Reporter, L-Firefly luciferase, S-puromycin selectable
PBS	Phosphate-buffered saline
PCP	Planar cell polarity
PD03	PD0325901
PECAM-1	Platelet Endothelial Cell Adhesion Molecule 1
PFA	Paraformaldehyde
pfuBARLS	fuBAR - found unresponsive Beta-catenin Activated Reporter
PGK	Phosphoglycerate kinase

PI3K	Phosphoinositide 3 kinase
PKA	Protein kinase A
PKC	Protein kinase C
Pygo	Pygopus
Rac1	Ras-related C3 botulinum toxin substrate 1
RAP1	small GTPase
Ror2	Receptor tyrosine kinase-like orphan receptor 2
rpm	revolutions per minute
RT	Room Temperature
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
RYK	Receptor related to tyrosine kinase
SDS	Sodium Dodecyl Sulfate
SIPA1L1	Signal-induced proliferation-associated 1 like 1
Src	Avian sarcoma virus transforming gene
SSC	Sodium Chloride-Sodium Citrate buffer
SSEA-1	Stage-Specific Embryonic Antigen 1
TAE	Tris-Acetate-EDTA buffer
TBE	Tris-Borate-EDTA buffer
TCF	T-cell factor
TE	Tris-EDTA buffer
Tris	Tris(hydroxymethyl)aminomethane
TSC2	Tuberous Sclerosis Complex 2
U	Units
UV	Ultraviolet
WB	Western Blot
Wg	Wingless (Drosophila)
WRE	Wnt Responsive Element
WT	Wild-type
β -TrCP	β -Transducin repeat-Containing Protein
μ g	microgram
μ l	microliter

Introduction

One of the most complex processes in biology is the embryonic development of multicellular organisms. It is based on the cross-talk between evolutionary conserved signaling pathways that provide necessary cell-cell communication signals. Secreted molecules, such as Wnts, BMPs and Hedgehogs, released by one cell population can cause responses in neighboring or distantly located cells. The activation of downstream signaling cascades determines the cell fate or other differentiation parameters. Strict control of the extracellular ligand release is a very crucial cellular process. Insufficient or excess activity can have fatal consequences including severe developmental abnormalities or, later, cancer.

1. Wnt signaling pathway

The Wnt signaling pathway is a molecular mechanism that evolutionary conserved from worms to man (Wodarz and Nusse, 1998). The activation of Wnt signaling is achieved by the extracellular Wnt signals that stimulate numerous intracellular transduction cascades including canonical (also known as Wnt/ β -catenin) and non-canonical signaling pathways. Activation of these pathways coordinates wide variety of cellular processes including cell fate decision, proliferation, differentiation, apoptosis and migration (Logan and Nusse, 2004).

1.1. Wnt ligands

The term Wnt is a combination out of the names of the *Drosophila melanogaster* segment polarity gene *Wingless* (*Wg*) and the mouse protooncogene *Int-1*, which was originally discovered as an integration site for mouse mammary tumor virus (Nusse and Varmus, 1982). The vertebrate *Int-1* was found to be a homolog of the *Drosophila Wg* and renamed to *Wnt-1*, connecting development to cancer (Rijsewijk et al., 1987). Since the discovery of *Wnt-1* in early 1980s, 19 *Wnt* genes in mammals were identified and divided into 12 subfamilies according to sequence similarity (Coudreuse and Korswagen, 2007). Additionally, Wnts were classified into the “canonical” (including Wnt1, Wnt3a and Wnt8) and “non-canonical” (including Wnt5a and Wnt11) classes, depending on the activation of β -catenin-dependent and β -catenin-independent signaling cascades respectively. However, recent discoveries demonstrated that this classification is not absolutely correct (van Amerongen et al., 2008). Wnt activities are likely to depend on receptor complexes on the membrane, presence of specific

signaling molecules and cofactors in the cell. As such the “non-canonical”, Wnt5a is shown to activate or inhibit β -catenin in different studies (He et al., 1997; Mikels and Nusse, 2006; Yang et al., 2009). Another example is Wnt11, which originally has been shown to regulate cell movements in *Xenopus* and Zebrafish gastrulation, and neural tube formation via non-canonical Wnt signaling pathway (De Calisto et al., 2005; Tada et al., 2002). However, recent studies demonstrated that Wnt11 activates the Wnt/ β -catenin pathway in dorsal/ventral axis formation (Tao et al., 2005). Moreover, Wnt11 can form a complex with Wnt5a to activate canonical and non-canonical Wnt signaling via a tyrosine sulfation mechanism in *Xenopus* axis formation (Cha et al., 2008; Cha et al., 2009). Taken together, certain Wnts (if not all) can activate multiple mechanisms, most likely by activating different receptor complexes on the membrane.

1.2. Non-canonical Wnt signaling pathways

In contrast to the canonical Wnt pathway, which signals through β -catenin, the so-called “non-canonical” Wnt pathways do not involve β -catenin transcriptional function (Semenov et al., 2007). Non-canonical pathways are very diverse and in most cases are not well characterized. They are classified, as listed in the following, into several groups according to receptor complex on the membrane:

A) In the Wnt/PCP pathway, binding of Wnt5a or Wnt11 to Fz receptor leads to activation of Dvl and small GTPases (Klein and Mlodzik, 2005; Wang and Nathans, 2007). However, there can be some exceptions in PCP signaling in different organs: this pathway can be activated in a ligand-independent way in the *Drosophila* wing and eye imaginal disc epithelia, via graded activity of the Fz receptor, which determines proximo-distal orientation of the wing hairs and mirror-imaged patterning of ommatidia, respectively (Chen et al., 2008a); (Seifert and Mlodzik, 2007).

B) In the Wnt/ Ca^{2+} pathway, binding of Wnt ligands (e.g. Wnt5a) to Fz leads to activation of heterotrimeric G-proteins and PLC resulting in an increased Ca^{2+} concentration and activation of downstream effectors, such as PKC, NFAT or CaMKII (Kohn and Moon, 2005).

C) In the Wnt/Ror2 signaling pathway, Wnt5a interaction with the Ror2 receptor leads to activation of PI3K-JNK pathway (Schambony and Wedlich, 2007).

D) Wnt/RAP1 signaling is activated by Wnt8 binding to Fz, which triggers activation of CKI ϵ , which can phosphorylate SIPA1L1 and enhance Rap1 activation. This pathway

regulates actin cytoskeleton organization and cell-cell adhesion during vertebrate gastrulation (Tsai et al., 2007).

E) In Wnt/GSK3 signaling, Dvl inhibits and activates GSK3 and JNK phosphorylation of MAP1B, respectively, resulting in increased microtubule stability during axonal remodeling (Salinas, 2007).

F) In Wnt/PKA signaling, a Wnt1/Wnt7a complex binds to Fz and activates G protein and AC resulting in increased cAMP levels, which leads to activation of PKA and CREB for instance in tumorigenesis of endocrine tissues (Rosenberg et al., 2002).

G) In Wnt/aPKC signaling, Wnt/Fz interaction via Dvl induces aPKC activation and leads to inhibition of the Par1/MARK2 kinase by phosphorylation, regulating axon formation via microtubules assembly (Zhang et al., 2007).

H) In the Wnt/RYK signaling, membranous RYK binds to Wnt and regulates axons or cell migration in *Drosophila* and mice, involving probably Src kinase as a downstream factor. This pathway does not involve any Wnt/ β -catenin signaling components apart of Wnts (Bovolenta et al., 2006).

I) In Wnt/mTOR signaling, Wnt/Fz/Lrp5/6 interaction results in inhibition of GSK3 phosphorylation of TSC2, thereby activating mTOR-mediated translational regulation in tumorigenesis. (Inoki et al., 2006).

Taken together, the presence of specific receptor-ligand pairs at the cell membrane directs signaling outcome. Moreover, such classification is not absolutely strict, as these pathways can overlap or intersect with one another.

1.3. The canonical Wnt pathway

Since the first discovery of Wnt proteins 27 years ago the canonical Wnt (also known as Wnt/ β -catenin) pathway has been extensively studied and is so far the best understood Wnt pathway. The existing structural and molecular data has provided detailed information about the sequential steps in the pathway. However, some of the data are controversial and there are still many unanswered questions. In the following I will introduce key components and summarize the up-to-date features of the canonical Wnt signaling cascade.

1.3.1. β -catenin and plakoglobin

The canonical Wnt pathway involves a key mediator – β -catenin (or Armadillo (arm) in *Drosophila*), which as a component of adherens junctions also plays a role in calcium-dependent intercellular adhesion (Gavert and Ben-Ze'ev, 2007). β -catenin or Arm protein was first discovered in *Drosophila* as a product of segment polarity gene *armadillo* (Nusslein-Volhard and Wieschaus, 1980). Later it was shown to play a role in the cell-cell adhesion (Kemler, 1993; Ozawa et al., 1989). The core region of β -catenin contains 12 arm repeats (Fig. 1), which bind to Wnt pathway components (APC and TCF/LEF) and components of adherens junctions (E-cadherin and α -catenin). The phosphorylation sites for the serine/threonine kinases GSK and CKI are located in the amino-terminal domain (NTD) of β -catenin, while the transactivator domain is located in the carboxyl terminal domain (CTD). Crystal structure of β -catenin full-length protein has been resolved recently and a new element has been found following 12 arm repeats, a C-helix, being involved in transcriptional activity, but not in cell-cell adhesion (Xing et al, 2008). The function of β -catenin protein in cell-cell adhesion and Wnt signaling will be discussed later.

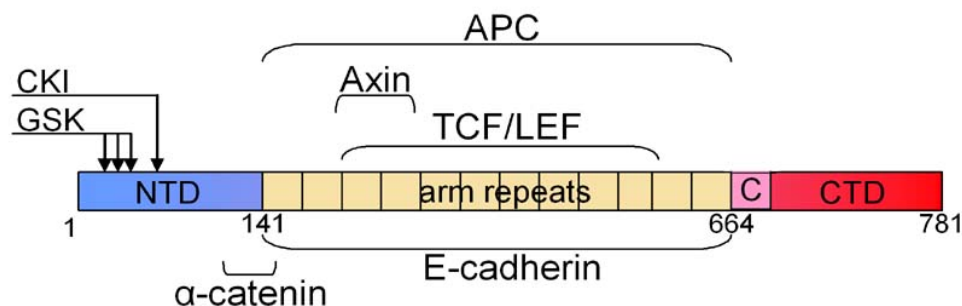


Figure 1 – Structure and interactions of β -catenin protein

Plakoglobin (or γ -catenin) is a close homologue of β -catenin and also contains 12 arm repeats flanked by N-terminal and C-terminal domains. Similar to β -catenin it can be found in adherens junctions and participates in intercellular adhesion (Sacco et al., 1995). Unlike β -catenin, plakoglobin is also a component of another type of intercellular junctions, known as desmosomes, where it interacts with desmoglein and desmocollin (Cowin et al., 1986). Several studies suggest that plakoglobin is capable to participate in the Wnt pathway. However, so far the mechanism by which plakoglobin becomes transcriptionally active is unknown. A signaling role for plakoglobin was first described in *Xenopus* overexpression experiments, where, similar

to Wnt/ β -catenin pathway activation, it caused axis duplication (Karnovsky and Klymkowsky, 1995). Furthermore, plakoglobin transcriptional activity was demonstrated in mammalian F9 cells that lack β -catenin (Maeda et al., 2004). However, deletion studies of *plakoglobin* in mammalian cells revealed that β -catenin and plakoglobin have differential nuclear translocation and transactivation potentials (Simcha et al., 1998; Zhurinsky et al., 2000b). While plakoglobin has been shown to compete with β -catenin for binding to TCF-4 or LEF-1 in different mammalian cell lines (Miravet et al., 2002; Zhurinsky et al., 2000a), the work in F9 cells deficient for *β -catenin* implies that plakoglobin is insufficient for LEF/TCF-dependent transcriptional activation by Wnt3a stimulation (Shimizu et al., 2008). It has to be noted that involvement of plakoglobin in the Wnt signaling machinery was demonstrated only upon plakoglobin overexpression and is not based on *in vivo* data. Thus, up to now it is controversial whether endogenous plakoglobin participates in the canonical Wnt pathway.

1.3.2. The canonical Wnt pathway step by step

The crucial events of the canonical Wnt signaling are: 1) activation by Wnt ligands at the cell surface; 2) tight control of cytosolic β -catenin levels, which is achieved by activation/inactivation of Axin, a component of the destruction complex in the cytoplasm and 3) regulation of β -catenin nuclear function (Fig. 2B).

In the absence of a Wnt ligand (Fig. 2A) β -catenin participates in adherens junctions, while cytosolic β -catenin is targeted for degradation by the 26S proteasome via the Axin complex (Kimelman and Xu, 2006). How β -catenin being recruited to and leaves the Axin complex is currently not well understood. The Axin complex is composed of the two scaffolding proteins AXIN1 and APC, and two kinases CK1 and GSK3 (Fig. 2A). The active Axin complex enables N-terminal phosphorylation of β -catenin first at serine 45 by CKI, followed by threonine 41, serine 37, and serine 33 phosphorylation by GSK3. Probably, concomitantly APC becomes phosphorylated on its 20 aa repeats by CK1 and GSK3 (Liu et al., 2006; Xing et al., 2003). This causes displacement of β -catenin from the Axin complex. Indeed, the phosphorylation of β -catenin at serine 37 and 33 exposes β -catenin protein for ubiquitination by the E3 ubiquitin ligase β -TrCP and 26S proteasome mediated degradation.

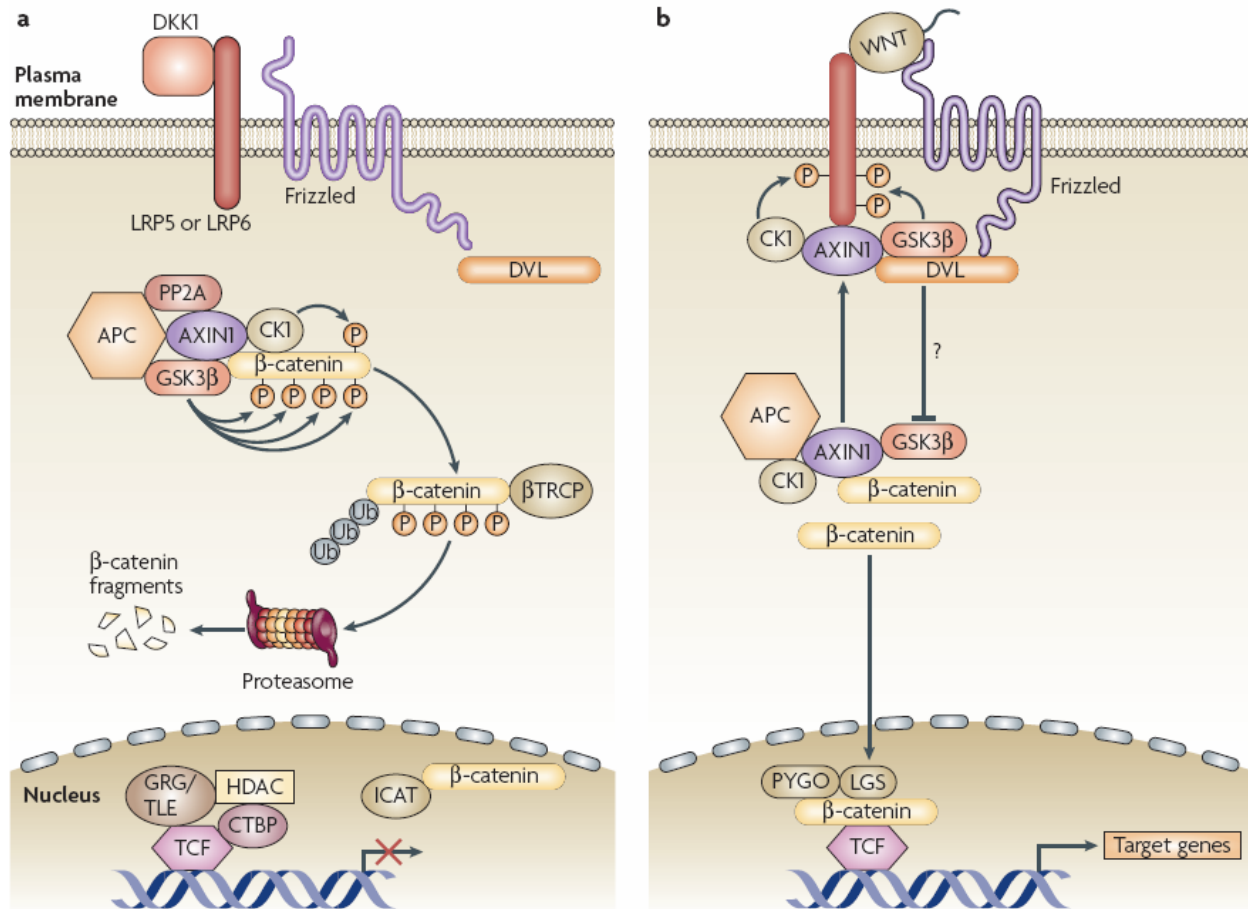


Figure 2 - The “off” and “on” states of the canonical Wnt signaling pathway

(A) In the absence of WNT signaling, (B) Activation of Wnt signaling cascade by Wnt ligand binding to Fz/Lrp5/6 receptors on the membrane (from (Staal et al., 2008)). Explanations can be found in the text.

It is still not entirely clear how Wnt ligands induce the inhibition of the Axin complex. In the current model (Fig. 2B) binding of Wnt proteins to Fz receptor and co-receptors LDL-receptor protein 5 and 6 (LRP5/6) leads to the formation of a Fz/LRP5/6 complex and phosphorylation of LRP5/6 (Tamai et al., 2004). The phosphorylation of LRP5/6 is a key event of Wnt activation and it is carried out by GSK3 and CK1 (Zeng et al., 2005). Subsequently, Dvl is recruited to the C-terminus of Fz and facilitates together with phosphorylated LRP5/6 the translocation of Axin to the cell membrane (Cliffe et al., 2003; Wallingford and Habas, 2005; Wong et al., 2003). However, another model proposes that Fz-Dvl recruitment of the Axin-GSK3 complex initiates LRP6 phosphorylation by GSK3 (Zeng et al., 2008). As a consequence of the Axin complex localization to the cell membrane, Axin cannot be phosphorylated, thus preventing β-catenin phosphorylation leading to its accumulation in the cytoplasm.

The next step in the canonical Wnt signaling cascade is the translocation of stabilized β -catenin from the cytoplasm to the nucleus. The exact mechanism of β -catenin shuffling to the nucleus is still not well understood. An earlier study suggested that β -catenin can enter the nucleus interacting directly with nuclear pore complex (Henderson and Fagotto, 2002). A recent very elegant study, however, demonstrated that β -catenin stabilization induced by Wnt is not sufficient for its nuclear localization (Wu et al., 2008). Here, a new mechanism was proposed involving Rac1-mediated JNK2 activation by Wnt3a triggering β -catenin phosphorylation at serine residues 191 and 605 leading to nuclear translocation (Wu et al., 2008).

When stabilized β -catenin enters the nucleus and interacts with TCF/LEF transcription factors to activate target gene expression. While in *Drosophila* and *C. elegans*, this is mediated by a single TCF gene, four TCF/LEF transcription factors exist in the mammalian genome: TCF1, LEF1, TCF3 and TCF4 (<http://www.stanford.edu/~rnusse/axindshtcf/tcf2.html>). They bind to a specific Wnt responsive element (WRE) on the DNA (Fig. 3). Nevertheless, TCF/LEF molecules can exert different responses on target gene expression: TCF1 and TCF4 can act as repressors or activators; LEF1 is primarily acting as an activator, whereas TCF3 is mostly acting as a repressor, but sometimes also as an activator (Arce et al., 2006; Hoppler and Kavanagh, 2007)

Binding of β -catenin to TCF/LEF transcription factors is accompanied by the interaction of different co-activators with C-terminus domain of β -catenin (Fig. 3). Many co-activators were recently identified including Lgs/BCL9-2 and Pygo (Kramps et al., 2002), MED, p300/CBP, the TIP60 histone acetyltransferases adaptor molecule TRRAP (Takemaru and Moon, 2000), MLL1/2 histone methyltransferases (HMTs), the SWI/SNF family member of ATPases BRG1, involved in chromatin remodeling, and the PAF1 complex, involved in transcription elongation and histone modifications (Mosimann et al., 2009). The interaction with numerous histone modifying enzymes suggests that TCF/ β -catenin binding to WRE results in widespread chromatin remodeling of the target loci (Parker et al., 2008).

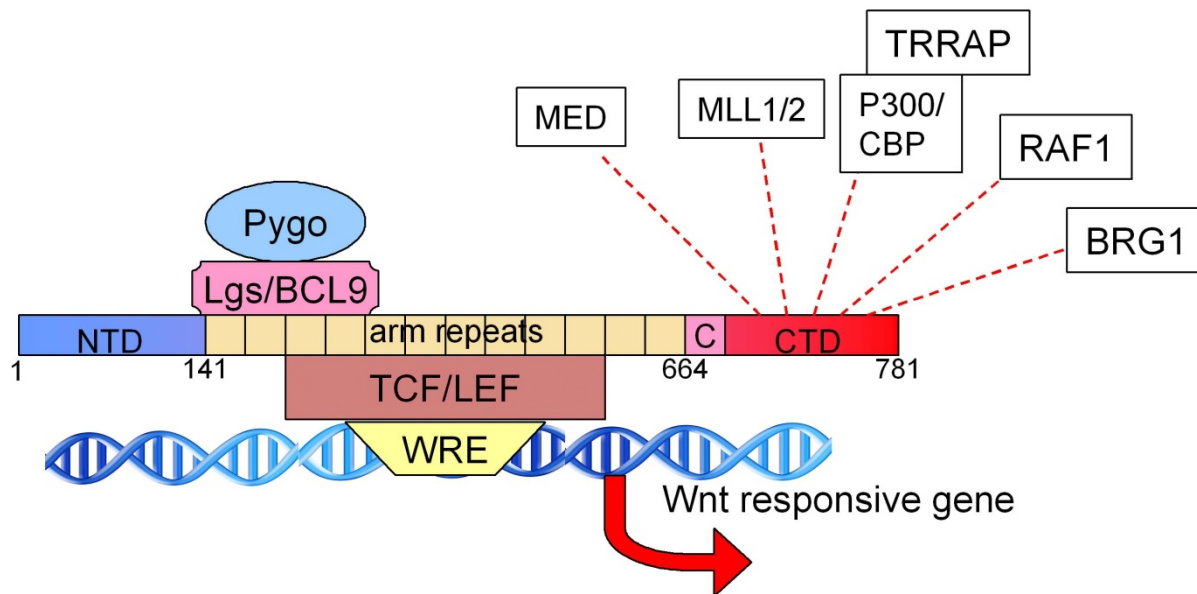


Figure 3 - Nuclear TCF/β-Catenin co-activator complexes

Interestingly, it has recently been shown that the interaction of β-catenin and co-activators with the WRE of the c-Myc gene is dynamic allowing replacement with the transcriptional repressors complex Groucho/TLE1 (Sierra et al., 2006); (Wang and Jones, 2006). Thus, these new data provide an intriguing mechanism of dynamic Wnt-responsive gene activation.

Unlike general co-activators of transcription, the co-factors Lgs and Pygo have been described to have a specific β-catenin-dependent activity in *Drosophila* (Mosimann et al., 2009). Upon Wg stimulation β-catenin binds to Lgs and recruits Pygo, while in the absence of a Wg signal Pygo co-occupies chromatin loci with TCF. In contrast, the mammalian Lgs homologue, BCL9-2 functions as a cell-type specific and Pygo-independent co-activator of Wnt signaling (Sustmann et al., 2008).

Activation of the canonical Wnt pathway coordinates a wide variety of cellular processes including cell fate decision, proliferation, differentiation, apoptosis and migration (Logan and Nusse, 2004). This requires activation of canonical Wnt signaling target genes in a cell-type- and organism-specific manner. So far only Axin2 was described to be a “universal” target gene of the Wnt pathway. As a fine-tuning mechanism Wnt signaling components such as Fz, LRPs, Dkk1, Axin2, TCF/LEF, Naked (a Dvl antagonist) and β-Tcrp are regulated either positively or negatively by the TCF/β-catenin complex (Logan and Nusse, 2004). This kind of regulation creates the feed-back loop mechanisms controlling cell-autonomous coordination of the canonical Wnt pathway.

2. Cell-cell adhesion

Cell-cell adhesion has been extensively implicated in morphogenesis that includes such fundamental biological processes as the regulation of cell shape, cell movement and sorting into complex organization in tissues and organs (Gumbiner, 2005). When cell-cell adhesion is deregulated it modulates tumorigenesis (Logan and Nusse, 2004).

The first cell-cell adhesion components, called CAMs, were discovered almost 30 years ago (Brackenbury et al., 1977; Takeichi, 1977; Thiery et al., 1977). Several types of adhesion molecules can be distinguished including members of the integrin, cadherin, immunoglobulin and proteoglycan families. Integrins and proteoglycans are part of the cell-matrix as well as cell-cell dependent adhesion. In contrast, cadherins specifically control intercellular adhesion. The cadherins are a family of calcium-dependent CAMs. Each member of the cadherin family was found to regulate cell-cell adhesion of particular cell types, thus coordinating the organization of the multicellular organism. This family encompasses more than 30 members, all with a similar molecular weight and common basic structure (Gumbiner, 1996) and includes classical cadherins of type I, closely related cadherins of type II, desmosomal cadherins (desmocollins and desmogleins), protocadherins and a variety of cadherin-related molecules. Several types of cell-cell adhesion complexes can be recognized including adherens junctions, desmosomes, gap junctions and tight junctions (Giepmans and van Ijzendoorn, 2009). In the following I will focus on the calcium-dependent cell-cell adhesion complexes: adherens junctions and desmosomes.

2.1. Adherens junctions

E-cadherin is a member of the type I cadherin family (van Roy and Berx, 2008). It was found to be expressed already in the two-cell stage of embryogenesis and later on its expression increases gradually together with cellular compaction (Shirayoshi et al., 1983). Notably, *E-cadherin*^{-/-} embryos can undergo compaction, because of the presence of maternal E-cadherin. Only later in development, after the blastocyst stage, they exhibit a defect in trophectoderm formation and fail to develop further (Larue et al., 1994).

E-cadherin is part of the adherens junctions (Fig. 4A), where it interacts via its cytoplasmic domain with the central arm domains of β -catenin or plakoglobin, while binding between β -catenin (or plakoglobin) and α -catenin achieved by their N-terminal domains interaction. α -catenin serves as the bridging molecule connecting E-cadherin to the actin cytoskeleton

(Hartsock and Nelson, 2008). Recent data suggest that α -catenin exists in monomeric and dimeric form and it is not continuously bound to β -catenin and actin cytoskeleton (Drees et al., 2005; Yamada et al., 2005). This led to the proposal of a dynamic crosstalk between the α -catenin plasma membrane pool (monomeric, β -catenin bound), the cytoplasmic pool (monomeric) and the cytoskeletal pool (dimeric, actin-bound), where α -catenin switches between the adherens complex and binding to the actin cytoskeleton.

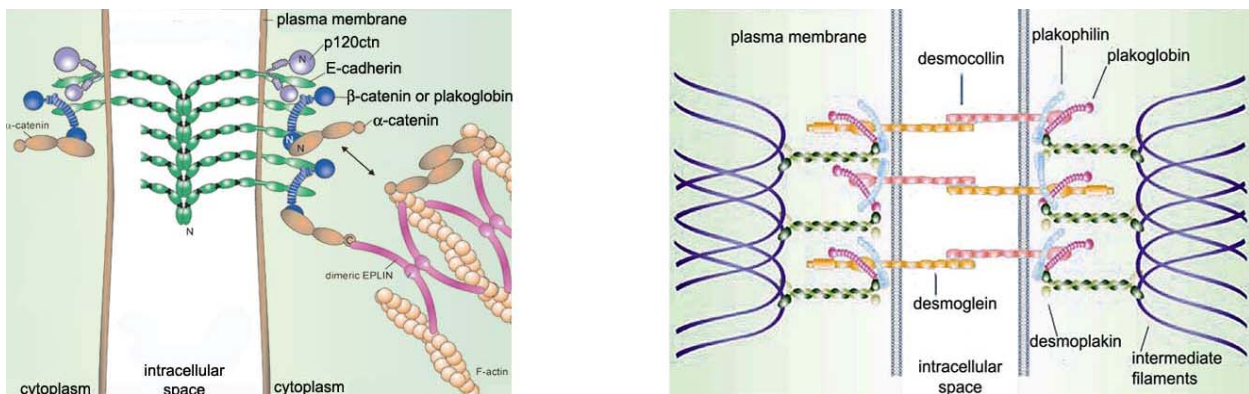


Figure 4 – Adherens junctions and desmosomes

(A) Structure of adherens junctions (Modified from (van Roy and Berx, 2008)) (B) Structure of desmosomes (Modified from Schmidt and Koch, 2007). Explanations can be found in the text.

2.2. Desmosomes

Interestingly, unlike β -catenin, plakoglobin can bind to both classical (E-cadherin) and desmosomal cadherins. Moreover, plakoglobin initially was identified as a cytoplasmic component of desmosomes (Franke et al., 1987). Desmosomes are adhesion complexes specific for cells derived from the ectodermal lineages (Schmidt and Koch, 2007). Based on knock-down experiments of different desmosomal components, desmosomes maintain integrity and stability of the skin and the heart (Bierkamp et al., 1996; Chidgey et al., 2001; Grossmann et al., 2004; Koch et al., 1997; Ruiz et al., 1996). They first appear in the trophectodermal layer of 32-cell-stage embryo during early preimplantation development (Fleming et al., 1991) and are found later also in epithelial layer derivatives.

Structurally, desmosomes comprise of cadherins and cytoplasmic plaque (Fig. 4B). Desmosomal cadherins, desmocollin and desmoplakin are transmembrane proteins connecting two adjacent cells. Their C-terminus end facing the cytoplasmic compartment that connected to intermediate filaments through a cytoplasmic plaque comprised of desmoplakin, plakoglobin and plakophilin proteins (Holthofer et al., 2007).

Interestingly, the analysis of *plakoglobin*^{-/-} mice revealed that β -catenin can partially substitute for plakoglobin in desmosomes of the skin (Bierkamp et al., 1999). Similarly, plakoglobin can substitute for β -catenin in adherens junctions in *β -catenin*^{-/-} mice (Huelsken et al., 2000). These results suggest that β -catenin and plakoglobin can substitute each other in adhesion complexes to support tissue integrity and stability.

3. Balance between cell-cell adhesion and Wnt signaling

As described above, β -catenin and plakoglobin can function in the cell-cell adhesion as well as Wnt signaling. Therefore, nature requires a mechanism to control the balance between the nuclear, cytoplasmic and adhesion pool of β -catenin.

How exactly β -catenin is being released from cell junctions to participate in Wnt signaling?

For instance, in *C. elegans* this problem was solved through the existence of multiple β -catenin forms that participate separately in adhesion and transcription (Schneider et al., 2003). However, in other organisms only one form of β -catenin exists. Findings by Piedra et al. shed some light on the mechanism that regulates disassembly of β -catenin from the adhesion complex (Piedra et al., 2001). This involves phosphorylation of β -catenin at tyrosine 654, which prevents E-cadherin/ β -catenin interaction. Three years later Brembeck et al. presented a detailed mechanism explaining how β -catenin switches from adhesive to transcriptional function (Brembeck et al., 2004). Here it was proposed that the co-factor BCL9-2 binds to and phosphorylates β -catenin at tyrosine 142. It results in a dissociation of β -catenin from α -catenin causing epithelial-mesenchymal transition (EMT), nuclear translocation of the BCL9-2/ β -catenin complex and activation Wnt/ β -catenin target genes. Using zebrafish as a model organism the authors showed that BCL9-2 acts downstream of Wnt8/ β -catenin regulating mesoderm formation (Brembeck et al., 2004). However, in this study the role of BCL9-2-induced switch of β -catenin from adhesion to transcription function in earlier developmental events, such as dorsal organization and posteriorization of anterior neuroectoderm, was not demonstrated.

Interestingly, *in vitro* studies using BCL9, the human homolog of mouse BCL9-2, showed cell-type specific β -catenin activation (Sustmann et al., 2008). Moreover, BCL9/ β -catenin interaction does not involve Pygo like in *Drosophila*. Authors also found that C-terminus of β -catenin is required for this interaction.

In another study, using lysophosphatidic acid to dissociate E-cadherin/ β -catenin complex from the membrane, it was shown that the E-cadherin/ β -catenin complex accumulates in the

perinuclear endocytic recycling compartment and can enter the nucleus upon Wnt stimulation (Kam and Quaranta, 2009). However, in this study the authors did not provide any significant physiological proof for such a mechanism.

4. Self-renewal of mouse ES cells

4.1. Origin of ES cells

During early pre-implantation development after fertilization of the mouse oocyte, the zygote undergoes first three mitotic divisions to form the 8-cell stage embryo (Fig. 5A-C). After the next two rounds of cell division at the developmental stage referred to as morulae (Fig. 5D,E), blastomeres segregates into the smaller inner cells and the larger outer cells (Fleming, 1987). The first segregation of cell, specialized in the development of extraembryonic (derived from the large outer cells of the morulae) versus embryonic tissues, occurs at the blastocyst stage (Fig. 5F) (Gardner, 1983). The population of cells in the blastocyst that gives rise to the whole embryo proper and extraembryonic endoderm is called ICM (Fig. 5F,G). The blastocyst is a spherical structure with polarized ICM and a fluid-filled cavity (blastocoele), surrounded by a cell layer – the trophectoderm. The trophectoderm gives rise to the embryonic part of the placenta, a structure unique to all mammalian species (Niwa et al., 2005).

Remarkably, in 1981 two independent groups of researches isolated and *in vitro* cultured for the first time “pluripotential” cells, later called ES cells, from the ICM of the mouse blastocyst (Evans and Kaufman, 1981; Martin, 1981). After this breakthrough ES cells were isolated from different mammalian species including human (Thomson et al., 1998). One of the defining features of ES cells is **pluripotency**, an ability to contribute to all three germ layers: endoderm, mesoderm and ectoderm. Pluripotency of murine ES cells was demonstrated by injecting them into a host blastocyst, where they can contribute ideally to the whole organism including germ cells (Beddington and Robertson, 1989).

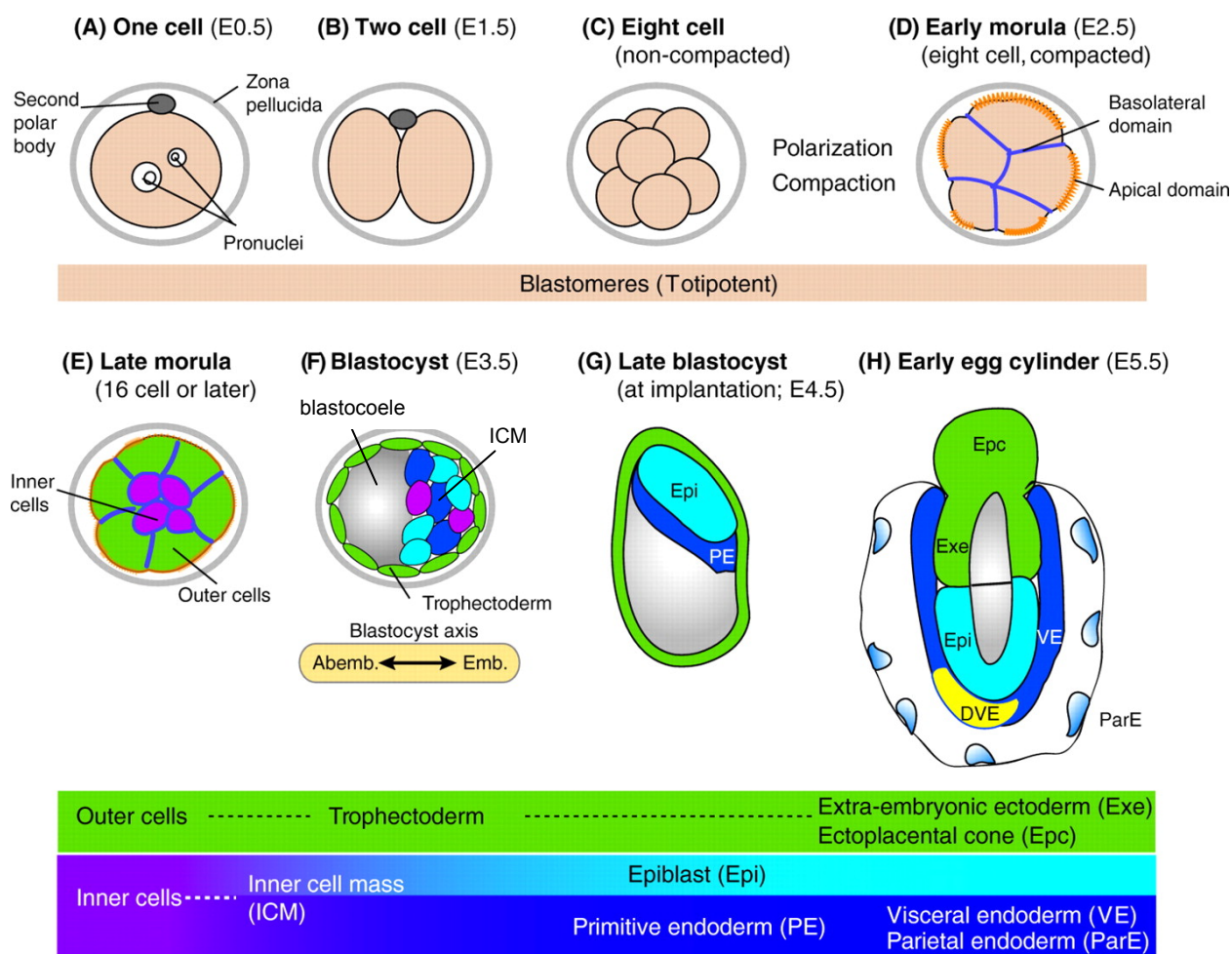


Figure 5 - Cell lineage formation from zygote to egg cylinder stage

(A-G) Schematics of the morphological changes and cell lineage specification that occur in a mouse embryo, from its fertilization at embryonic day 0.5 (E0.5) to the late blastocyst stage (E4.5). The colored bars show the progressive allocation of totipotent blastomeres to outer and inner cells and to the trophectoderm and inner cell mass lineages. The cell types in the embryos are color coded. (H) Egg cylinder stage. DVE, distal visceral endoderm; Abemb (abembryonic) - Emb (embryonic) axis of the blastocyst. (modified from (Rossant and Tam, 2009))

The fact that ES cells are derived from the blastocyst does not mean that they are identical to ICM cells. ES cells do not exist *in vivo*. It is a cell type that is captured at the stage of blastocyst development and can divide *in vitro* under proper culture conditions for indefinite time and produce identical pluripotent progeny. This feature of ES cells was called **self-renewal** (Chambers and Smith, 2004).

4.2. Molecular basis for mouse ES cell self-renewal

4.2.1. Transcription factors preventing differentiation

Recent extensive studies revealed that maintenance of the pluripotent state of ES cells is controlled by transcriptional factor network, including three major regulators: Oct3/4 (POU5F1 or Oct4), Nanog and Sox2 (Boheler, 2009; Yamanaka et al., 2008). The POU family transcription factor Oct3/4 is expressed in blastomeres and later its expression restricted to the ICM. Ablation of Oct3/4 in mice leads to failure in ICM formation (Nichols et al., 1998). Notably, deletion of Oct3/4 in ES cells promotes differentiation towards the trophoblast lineage and overexpression primarily promotes endoderm-like cell differentiation (Niwa et al., 2000). The homeodomain protein Nanog is expressed exclusively in the ICM (Chambers et al., 2003; Mitsui et al., 2003). Overexpression of Nanog in ES cells results in LIF-independent maintenance of pluripotency in serum-containing and serum-free media (Ying et al., 2003).

Moreover, Nanog not only supports pluripotency, but also serves as an inhibitor of ES cell commitment towards neuroectoderm (Ying et al., 2003), primitive endoderm (Mitsui et al., 2003) and mesoderm (Suzuki et al., 2006). The HMG domain-containing factor Sox2 is expressed in the ICM and also in the extraembryonic ectoderm lineage (Avilion et al., 2003). Functional studies revealed that Sox2 cooperates with Oct3/4 to maintain pluripotency and to prevent differentiation into trophectoderm (Li et al., 2007).

Recent groundbreaking work identified that Oct3/4 and Sox2, together with Klf4 and c-Myc (so-called Yamanaka's factors) are essential factors to convert mouse somatic cells to the induced pluripotent stem cells (iPS cells), that have ES cell-like characteristics (Takahashi and Yamanaka, 2006). Only one year later the groups of Yamanaka and Thompson simultaneously succeeded in the establishment of human iPS cells (Takahashi et al., 2007; Yu et al., 2007). Numerous research groups started to work on iPS cell technology since then and most of the efforts are now put to decipher the underlying mechanisms of such reprogramming and to explore the usage of iPS cells in medicine and drug therapy.

Oct3/4, Sox2 and Nanog are probably the most crucial transcription factors governing self-renewal of ES cells (Orkin, 2005). Thus, a lot of effort went into the identification of their downstream targets and self-regulatory mechanisms of this circuit. To gain more inside into the transcriptional regulatory network that maintains the ES cell pluripotent state, several groups combined genomic and proteomic approaches resulting in an expanded circuit of transcriptional

factors and their interactions (Chen et al., 2008b; Kim et al., 2008). Genome-wide mapping data of transcription factor targets by ChIP-Chip and ChIP-sequencing and proteomics data allowed the creation of a complex interaction map (Fig. 6) (Kim et al., 2008; Wang et al., 2006). This map shows the core regulatory circuit of the nine proteins examined in the study by Kim and colleagues: Oct4, Sox2, Nanog, Rex1, Dax1, Zfp281, Myc and Klf4 surrounded by newly identified transcription factors including Sall4 (Zhang et al., 2006), REST (Singh et al., 2008) and Rif1 (Loh et al., 2006).

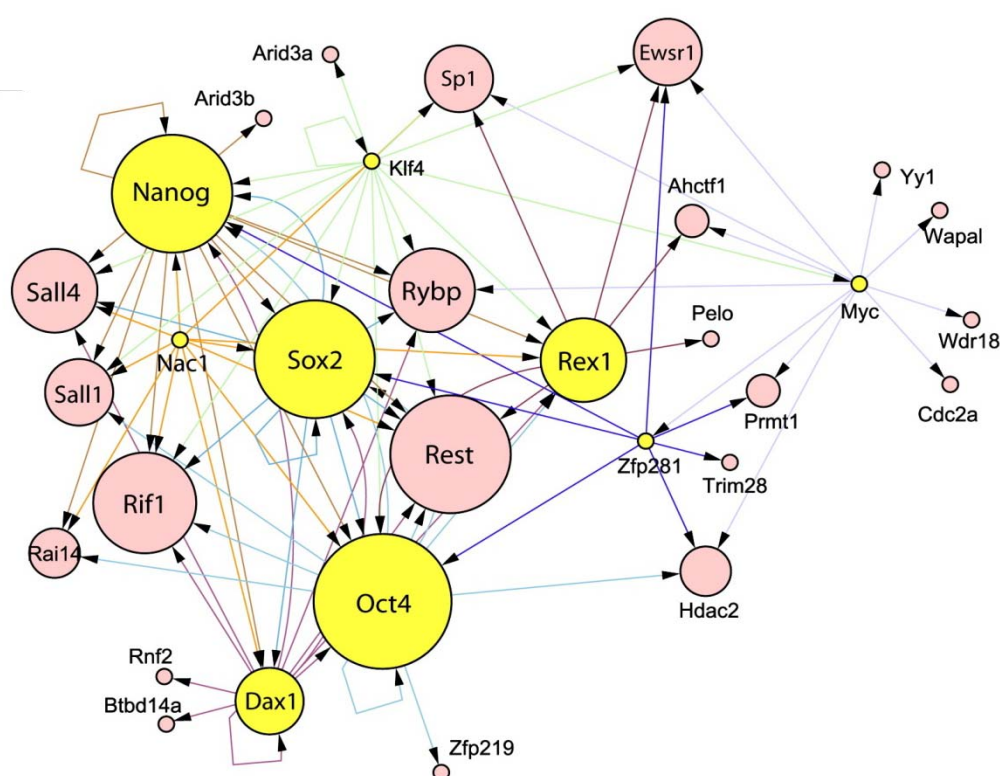


Figure 6 - Expanded regulatory network of transcription factors involved in ES cell pluripotency

This diagram is based on target data and protein-protein interaction data. Yellow circles represent the nine factors examined in the study by Kim et al. The size of each circle reflects the degree of factor co-occupancy of the promoter of the gene encoding each factor. Arrowheads indicate the direction of transcriptional regulation. (From Kim, Chu et al., 2008)

4.2.2. Signaling molecules

Together with transcription factors many intracellular signaling pathways have been implicated to act in the maintenance of ES cell pluripotency.

Originally, ES cells were derived and propagated on a layer of mitotically-inactivated MEFs in the presence of FCS, with the MEFs producing soluble factors that inhibit differentiation of ES

cells (Smith and Hooper, 1983). Later it was possible to substitute the MEF layer through the addition of conditioned media from a Buffalo rat liver cell line (Smith and Hooper, 1987). Fractionation of this conditioned media revealed a presence of secreted protein LIF acting as the main component sustaining mouse ES cells pluripotency (Smith et al., 1988; Williams et al., 1988). In contrast to mouse, LIF is dispensable for the maintenance of human ES cells self-renewal (Humphrey et al., 2004). LIF binds to the LIFR/gp130 receptor complex on the cell membrane and activates the JAK/STAT3 signaling cascade (Niwa et al., 1998). One of the target genes of STAT3 is c-Myc that controls ES cell proliferation (Cartwright et al., 2005). However, culturing of ES cells only in the presence of LIF without FSC is not sufficient to maintain their pluripotency, but the need for FCS can be replaced by BMP4 in combination with LIF (Ying et al., 2003). BMP4 signaling through Smad phosphorylation activates expression of Id proteins, which are negative transcriptional modulators in various cell types (Ruzinova and Benezra, 2003). Additionally, it has also been reported that BMP4-dependent inhibition of the p38 MAPK may also contribute to the maintenance of pluripotency (Qi et al., 2004). Taken together, BMP and LIF are the two cytokines that are sufficient to sustain self-renewal of mouse ES cells.

Recently, LIF-dependent activation of PI3K/AKT signaling pathway was implicated in self-renewal of mouse as well as human ES cells (Kim et al., 2005; Paling et al., 2004). Treatment of mouse ES cells with the PI3K inhibitor LY294002 in the presence of LIF dramatically decreases the proportion of undifferentiated colonies (Paling et al., 2004; Storm et al., 2007). The underlying mechanism was recently uncovered by several independent research groups, which showed that the expression of *Nanog* and *c-Myc* is directly regulated by PI3K through GSK3 β inactivation (Bechard and Dalton, 2009; Storm et al., 2007; Storm et al., 2009). Withdrawal of either LIF or treatment with LY294002 in the presence of LIF, results in the translocation of GSK3 β from the cytoplasm to the nucleus where it phosphorylates c-Myc, targeting it for degradation (Bechard and Dalton, 2009).

Interestingly, the activation of the RAS/RAF/MEK/ERK pathway has been shown to negatively regulate self-renewal of mouse ES cells. Upon withdrawal of LIF, ERK activity increases, but this effect can be suppressed by the addition of inhibitor PD98059 of MEK, a key component of RAS/RAF/MEK/ERK signaling pathway (Burdon et al., 1999). On the basis of these results Ying and colleagues developed defined ES cell culture conditions in serum-free medium making use of the intrinsic mechanisms of ES cells to maintain the pluripotent state (Ying et al., 2008). This does not involve LIF/STAT3 signaling, but propagation of ES cells in serum-free

medium supplemented with MEK inhibitor PD0325901 and very specific GSK3 inhibitor CHIR99021. Under these conditions the inhibition of GSK3 and MEK is sufficient to maintain cellular growth and viability by preventing the intrinsic ES cell differentiation commitment.

4.3. *Wnt signaling and self-renewal*

Recently, activation of the canonical Wnt signaling pathway has been shown to maintain self-renewal of mouse and human ES cells. Sato and colleagues demonstrated that activation of the canonical Wnt pathway through inhibition of GSK3, using the GSK3 inhibitor BIO, was sufficient to maintain self-renewal of both mouse and human ES cells (Sato et al., 2004). Moreover, addition of BIO during early steps of mouse ES cells derivation increased the efficiency to establish ES cell lines (Umehara et al., 2007). Other studies suggest that BIO might have some additional effects in mouse ES cells, that it might not only act through activation of the Wnt/ β -catenin pathway, but also influence the LIF/STAT3 signaling pathway through an unknown mechanism (Ogawa et al., 2006), which as newer studies suggest might be the activation of cMyc (Bechard and Dalton, 2009). However, BIO was not sufficient to maintain self-renewal of human ES cells longer than two passages (Dravid et al., 2005), suggesting that active Wnt signaling is dispensable for self-renewal maintenance in human ES cells.

Another way to stimulate canonical Wnt signaling in ES cells is the addition of Wnt proteins. Wnt3a conditioned medium can support the self-renewal state of mouse ES cells, but only in the presence of low concentrations of LIF (Ogawa et al., 2006). On the other hand, the same study showed that activation of the canonical Wnt pathway by recombinant Wnt3a alone only promotes proliferation and leads to differentiation of mouse and human ES cells (Dravid et al., 2005; Ogawa et al., 2006). Thus, these studies together with other studies suggest that β -catenin might act synergistically with LIF maintaining mouse ES cell self-renewal (Hao et al., 2006; Takao et al., 2007). Takao and colleagues had shown that nuclear β -catenin levels decrease upon LIF withdrawal from ES cell culture. However, in contrast to the study by Ogawa and colleagues, it was shown here that ES cells expressing an activated mutant form of β -catenin can be cultured in the absence of LIF for at least one month. The proposed mechanism was that β -catenin interacts with Oct3/4 to activate Nanog promoter (Takao et al., 2007). Hao and colleagues had shown that the canonical Wnt signaling up-regulates Stat3 gene expression (Hao et al., 2006). In a recent study it was shown that ES cells lacking *β -catenin* have decreased levels of the stem cell markers *Rex-1* and *Nanog* (Anton et al., 2007). In contrast to this analysis

in a very recent study by another group it was shown that β -catenin^{-/-} ES cells can be propagated without LIF in the presence of FCS for 12 days and maintain expression of Oct3/4 (Soncin et al., 2009). However, the source of the β -catenin^{-/-} ES cells in the study by Soncin and colleagues is unclear. Thus it is possible that the ES cells used in the two different studies might vary in their genetic background or behave differently depending on the culture conditions. Altogether, the published data on the role of the Wnt/ β -catenin signaling in ES cell self-renewal are slightly controversial and require further investigations.

4.4. Cell-cell adhesion and self-renewal

Mouse ES cells have specific morphological characteristics that distinguish them phenotypically from differentiated cells. They grow in compact multilayered colonies, consisting of cells with discernible borders and low cytoplasm to nucleus ratio (Evans and Kaufman, 1981). One of the defined features of the ES cell colony morphology undergoing differentiation is a change from dome-shaped multilayered to spread monolayered appearance. Thus ES cell differentiation and the associated changes of the compact colony architecture are probably accompanied by alterations in the expression of cell-cell and cell-matrix adhesion molecules.

Many adhesion-related molecules have been found to be expressed in the ICM of the blastocyst and in mouse ES cells, these include SSEA-1 (Kojima et al., 1994), CD9 (Oka et al., 2002), ICAM-1 (Tian et al., 1997), PECAM-1 (CD31) (Redick and Bautch, 1999), EpCAM, E-cadherin (Larue et al., 1994; Tian et al., 1997), β -catenin and plakoglobin (Ohsugi et al., 1997). Interestingly, the SSEA-1, CD9, PECAM-1, EpCAM and ICAM-1 are also considered ES cell characteristic markers, as their expression is lost upon differentiation (Cui et al., 2004; Furusawa et al., 2004). Only recently the involvement of adhesion-related molecules in ES cell self-renewal was studied and EpCAM (Gonzalez et al., 2009) and PECAM-1 (Furusawa et al., 2004) were implicated in being important for mouse ES cell self-renewal.

The adhesion molecule EpCAM is a transmembrane glycoprotein expressed in normal epithelial and cancer cells (Trzpis et al., 2007). Beside its adhesion function, EpCAM was recently implicated as a target gene of Wnt/ β -catenin signaling and as a signal transducer of the pathway (Maetzel et al., 2009). The EpCAM intracellular domain is being released from the membrane and binds to the LEF/ β -catenin complex in the nucleus to activate target genes transcription. Moreover, EpCAM expression is necessary, but not sufficient for ES cell maintenance (Gonzalez et al., 2009).

PECAM-1 (or CD31) is a glycoprotein that belongs to the immunoglobulin superfamily (Ilan et al., 2000). Originally characterized as an endothelial marker, PECAM-1 isoforms are expressed in ES cells and have been implicated in the activation of STAT3 and as such it may play a role in LIF-mediated maintenance of undifferentiated ES cells (Ilan et al., 2001). Moreover, PECAM-1^{-/-} ES cells are unable to contribute to the epiblast and have only low levels of Oct3/4 expression (Furusawa et al., 2004). Interestingly, PECAM-1 can also associate with tyrosine-phosphorylated β -catenin or plakoglobin and modulate adherens junction assembly (Biswas et al., 2006; Biswas et al., 2005). These interactions link PECAM-1 to cell adhesion and maintenance of self-renewal in mouse ES cells.

5. Early mouse development versus *in vitro* differentiation

5.1. ES cells as a model for developmental studies

A recent potentially therapeutic avenue of ES and, more recently iPS cell research focuses on their directed differentiation into the specialized cells. The generation of pure populations of committed cell types would be beneficial in many ways, to recapitulate development, to model human disease-related issues in culture and might allow eventually replacement cell therapy for treatment of certain degenerative human diseases (Irion et al., 2008).

As described earlier mouse ES cells are derived from the ICM of the blastocyst (see chapter 4.1.). At implantation, the ICM segregates into unpolarized embryonic ectoderm (epiblast) and primitive endoderm (Fig. 5G). Later in development an egg cylinder forms (Fig. 5H), characterized by proximal-distal and anterior-posterior patterning of the embryo and cavitation of epiblast. At this stage the epiblast is polarized in a process referred to as gastrulation, characterized by extensive cell migration and formation of the three primary germ layers. Gastrulation is associated with primitive streak formation, which elongates from the posterior part of the embryo due to organized epiblast cell movements and EMT, giving rise to mesoderm and definitive endoderm. Cells in the anterior part of the epiblast do not migrate and give rise to ectoderm (Tam and Loebel, 2007).

Although the normal process of gastrulation cannot be fully recapitulated during *in vitro* ES cell differentiation, cell types, specific for the different germ layers can form. Hence, *in vitro* ES cell differentiation studies can be used to study early developmental events in more detail and in a quantitative manner, using for instance germ layer-specific reporter ES cell lines. Here in

principle a reporter gene, for example eGFP or LacZ, is inserted into the genomic locus of a germ layer specific marker gene. This enables to monitor the appearance of certain germ layer in culture during differentiation. Germ layer specific loci have been used extensively in developmental studies including *Mixl1* (Davis et al., 2008; Ng et al., 2005), *Brachyury* (Lacaud et al., 2004) and *Axin2* (ten Berge et al., 2008) for primitive streak, *Foxa2* (Gadue et al., 2005) for endoderm and *Flk1* (Fehling et al., 2003) for mesoderm formation.

The culture conditions are very important when studying ES cells differentiation. Upon withdrawal of LIF ES cells start to differentiate. The presence or absence of FCS, growth factors, antagonists of certain signaling pathways and the microenvironment can influence the differentiation of ES cells into specific somatic lineages (Gadue et al., 2005) or even into germ cells (Geijsen and Daley, 2006; Geijsen et al., 2004). One of the commonly used methods to differentiate ES cells into lineage specific derivatives is the embryoid body (EB) aggregates formation (Burkert et al., 1991; Ling and Neben, 1997). The time points of the appearance of specific marker genes during EB differentiation correlate pretty well with those of their endogenous appearance during early developmental stages including gastrulation, cavitation and early organogenesis (Desbaillets et al., 2000; Leahy et al., 1999). Moreover, as shown recently EBs exhibit a high degree of self-organization and axis formation capacity upon Wnt signaling stimuli (ten Berge et al., 2008). However, not only the culture time also the size of EBs is a crucial factor for the induction of the expression of different lineage markers (Karp et al., 2007). Thus, small EBs preferentially differentiate towards endoderm, while big EBs differentiate primarily into mesoderm derivatives due to the differential expression of *Wnt5a* and *Wnt11* (Hwang et al., 2009).

5.2. *Wnt* signaling in early mouse development

Wnt signaling comprises many different components and a variety of intracellular pathways. However, loss-of-function and gain-of-function phenotypes of different components of the Wnt signaling pathways are quite divergent in terms of their stage of developmental arrest (Tam and Loebel, 2007). Moreover, some of the loss-of-function mutants have very mild phenotypes or no phenotypes at all in embryonic development. For example, *Wnt5a* is co-expressed with *Wnt3a* in primitive streak embryos, but *Wnt5a* mutants do not have an early developmental phenotype with regard to the primitive streak (Cha et al., 2004; Yang et al., 2009). Deletion of *β -catenin* in mice leads to an early developmental arrest around E6.5 associated with defects in primitive streak formation, lack of mesoderm formation and increased apoptosis in the

ectoderm (Haegel et al., 1995; Huelsken et al., 2000). Similarly, mice carrying mutations of both *Lrp5* and *Lrp6* fail to establish a primitive streak, but the establishment of anterior visceral endoderm and anterior epiblast occurs normally (Kelly et al., 2004). Similar defects are found in *Wnt3* knock-out mice (Liu et al., 1999). In contrast, *Wnt3a* knockout mice develop through gastrulation stage, but display posterior axis defects and no mesoderm formation (Takada et al., 1994).

On the other hand, constitutive activation of β -catenin in the mouse zygote results in normal blastocyst development, but premature EMT in the epiblast of the post-implantation embryo (Kemler et al., 2004).

Recently an interesting model about the role of Wnt-signaling in development was proposed. This model postulates that Wnt signaling coordinates the transcriptional noise, which defines cell fate decisions rather than influences cell fates directly (Arias and Hayward, 2006). One of the examples supporting this idea is that the expression of the mesodermal gene *Brachyury*, a Wnt/ β -catenin target, still occurs in *Wnt3a* mutant mice, but does not peak at its normal time of development (Galceran et al., 2001).

Overall, the published data would suggest that the canonical Wnt pathway is primarily required for the early post-implantation mouse development, while there is little knowledge about the roles of the non-canonical pathways based on mouse knock-out phenotypes.

Goal of the thesis

The specific aim my thesis was to establish β -catenin-deficient mouse ES cells and β -catenin signaling-defective mouse ES cells in order to distinguish between the adhesion and transcriptional functions of β -catenin in mouse ES cell self-renewal and differentiation.

In my project I have addressed and accomplished the following points:

- 1) Establishment of β -catenin^{fl/fl} and β -catenin-deficient (β -cat ^{Δ/Δ}) mouse ES cells
- 2) Characterization of self-renewal potentials of β -cat ^{Δ/Δ} ES cells
- 3) Utilization of β -cat ^{Δ/Δ} ES cells to examine the activity of different canonical Wnt pathway activators with regard to self-renewal
- 4) Elucidate intercellular junction complexes of ES cells and their influence on self-renewal mechanisms
- 5) Characterization of the differentiation potentials of β -cat ^{Δ/Δ} ES cells
- 6) Rescue experiments by introducing WT and putative signaling-defective forms of β -catenin into β -cat ^{Δ/Δ} ES cells
- 7) Analysis of the differentiation potentials of the signaling-defective ES cell lines
- 8) Generation and preliminary characterization of tamoxifen-inducible β -catenin^{ex3fl/ex3fl} ES cells to study the effects of a gain in Wnt-signaling in ES cell self-renewal and differentiation.

In addition, I was involved in another project that was not included in my dissertation, but of which the manuscript entitled “Mice lacking the orphan receptor Ror1 have distinct skeletal abnormalities and are growth retarded” is included.

Materials and Methods

1. Establishment of ES cells

1.1. In the presence of LIF and serum

1.1.1. MEF isolation

The MEF cells were prepared from MF1 fetuses at 13,5 days post-coitum. Embryos were isolated and cleaned from embryonic membranes and placenta in 37 °C pre-warmed sterile PBS. Using fine sterilized scissors, after removal of liver, embryos were cut into little pieces and incubated in 0,5% Trypsin-EDTA (Gibco) at 37 °C in CO₂ incubator for 15 minutes. Trypsin was inactivated by addition of pre-warmed MEF medium (high glucose DMEM containing 10% FCS, 2mM L-glutamine, 2mM penicillin/streptomycin, all Sigma-Aldrich). Subsequently suspension of cells was seeded onto T75 tissue-culture flasks (1 embryo per flask). After several passages cells were frozen in small aliquots or irradiated using γ -irradiation.

1.1.2. Isolation of β -catenin^{fl/fl} ES cells

Irradiated MEFs were seeded onto 4-well tissue culture plates one day before blastocyst isolation. 3,5 days post-coitum blastocysts were flushed from β -catenin^{fl/fl} females mated naturally with β -catenin^{fl/fl} males (Huelsen et al., 2001) with a syringe in 37 °C pre-warmed sterile PBS and transferred to M2 medium (Sigma-Aldrich) using a sterile glass capillary. Subsequently blastocysts were individually seeded onto prepared 4-well plates in ES cells medium (high glucose DMEM containing 15% FCS, 2mM L-glutamine, 2mM penicillin/streptomycin, 1mM sodium pyruvate, 1x MEM non-essential amino acids, 0,1 mM β -mercaptoethanol, all Sigma-Aldrich; and 1:250 dilution LIF-conditioned medium produced using LIF α -5COS cells, a kind gift of Uta Möle-Steinlein) and incubated at 37 °C, 5% CO₂. After 3-4 days blastocysts hatched naturally from zona pellucida and attached to the MEF layer. After 5-7 days, depending on the growth speed and morphology, a clump of cells (inner cell mass) was transferred to a drop of pre-warmed PBS using a sterile glass capillary and then to a drop of 0,05% trypsin-EDTA (Gibco), incubated for 3 minutes at 37 °C and plated to the fresh well containing MEFs (this step was defined as passage 1). After the first passage,

colonies with ES-like morphology were selected for propagation, storage and further experiments. Two independent ES cell lines were isolated with this method and one (NL β -12) was used for further experiments. Later on this ES cell line was propagated without feeder layer.

1.1.3. Generation of β -catenin $^{\Delta/\Delta}$ ES cell clones

To delete exon2 to exon6 of β -catenin in ES cells, 0.5x10E5 β -catenin $^{fl/fl}$ ES cells were infected with Adeno-Cre virus (University of Iowa) at MOI $\sim$ 150. Next day cells were seeded individually on 96-well plate. Then they were passaged with multichannel pipette (Eppendorf) and one plate was used for genomic DNA isolation (see section “Mini-Southern blot”) and subsequent PCR to screen for deletion. Out of 96 clones 50% were β -catenin $^{\Delta/\Delta}$, 25% β -catenin $^{fl/\Delta}$ and 25% β -catenin $^{fl/fl}$.

1.2. In serum-free LIF-free conditions

1.2.1. Isolation of tamoxifen-inducible β -catenin $^{ex3fl/ex3fl}$ ES cells

4-well plates were covered with 0,1% gelatin for 30 minutes. 3,5 days post-coitum blastocysts were flushed from β -catenin $^{ex3fl/ex3fl};Rosa26^{CreERT2/CreERT2}$ females with a syringe in 37 °C pre-warmed sterile PBS and transferred to M2 medium (Sigma-Aldrich) using a sterile glass capillary. After removal of the zona pellucida by treatment with Tyrode's solution (T1788, Sigma-Aldrich) for 30 seconds, embryos were individually seeded onto gelatinized 4-well plates in serum-free 2i ES cells medium (N2B27 medium (StemCellSciences) supplemented with 1000 U/ml EsgroLIF (Chemicon), Gsk3-inhibitor 3 μ M CHIR and MEK-inhibitor 1 μ M PD03 (both from StemGent)) and placed into a 37 °C CO₂ incubator. After 10 days inner cell mass outgrowths were individually trypsinized and seeded onto fresh gelatinized plates (this step is defined as passage 1). After 5 days ES-like colonies were selected for propagation, storage and further experiments. Six ES cell lines were isolated with this method (E3BR1, 2, 4, 3, 3-2, 5) and two (E3BR3-2 and E3BR5) were used for chimaera generation and further experiments. To induce Cre expression, 1 μ M of Tamoxifen (Sigma) was used.

1.3. Blastocyst injections

Blastocyst injections were performed by IMP/IMBA mouse service facility (Hans-Christian Theussl and Jacek Wojciechowski) using standard techniques. NL β -12 (on feeder, passage 18), E3BR3-2 and E3BR5 (feeder-free, passage 8) ES cell lines were used for injection. Male and female chimeras were crossed to C57Bl/6 mice and their offspring were genotyped for the presence of original mutant alleles.

2. Genotyping of mice and ES cells

2.1. Genomic DNA isolation

Mouse ear or tail samples were digested in 250 μ l lysis buffer (50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris pH8.5, 0.01% gelatin, 0.45% NP40, 0.45% Tween20, 100 μ g/ml proteinase K) at 55 °C ON. Next day samples were heat inactivated at 95 °C for 15 minutes and stored at 4 °C. ES cells pellets were digested in 100 μ l ES lysis buffer (10mM Tris-HCl, pH 7.5; 10mM EDTA; 10mM NaCl; 0,5% sarcosyl; 1mg/ml proteinase K added directly before use) at 55 °C ON. Next day samples were heat inactivated and 95 °C for 15 minutes and stored at +4 °C.

2.2. PCR genotyping

For β -catenin fl/del genotyping a three primer PCR strategy was used. Primers were as follows: forward primer HAR057 AGAATCACGGTGACCTGGGTAAA, reverse primer HAR058 CAGACAGACAGCACCTTCAGCACTC, reverse primer HAR047 CAGCCAAGGAGAGCA GGTGAGG. PCR was performed using 1-3 μ l of genomic DNA and the TAKARA Taq PCR system (Roche) (2.5 μ l 10x MgCl₂-containing PCR buffer, 2 μ l 2.5 mM each dNTPs, 0.5 μ l 10mM primer mix (forward and reverse), 0.2 μ l TAKARA Taq polymerase (5U/ μ l) and dH₂O). Reaction conditions were: 95°C 5', 34x (95°C 10'', 65°C 20'', 72°C 3'), 72°C 5', 4 ° forever. Product sizes were: WT = 310 bp; fl = 350 bp; del = 650 bp.

For β -catenin ex3floxed genotyping a two primer PCR strategy was used. Primers were as follows: forward primer HAR1518 AGAATCACGGTGACCTGGGTAAA, reverse primer HAR1519 CATTCATAAAGGACTTGGGAGGTGT. PCR was performed using 1-3 μ l of genomic DNA and the TAKARA Taq PCR system (see above). Reaction conditions were: 94°C 5', 34x (95°C 30'', 60°C 30'', 72°C 2'), 72°C C5', 4 ° forever. Product sizes were: wt =

350 bp; ex3fl = 650 bp. PCR products were analyzed on 0.8 -1.2% (depending on the product size) agarose gel.

3. Analysis of self-renewal and differentiation of ES cells

3.1. Alkaline phosphatase (AP) staining

For AP staining the alkaline phosphatase kit (Sigma-Aldrich) was used according to manufacturer's instructions. Briefly, TRIS tablet was resuspended in 10 ml H₂O, and then Naphtol tablet was added and mixed thoroughly. Solution was filtered through a paper filter and added to PBS-washed ES cells for 3 hours at 37 °C till WT ES cell colonies appeared intensive red in a control plate. Later staining was stopped by washing with PBS 3 times and plates were stored at 4 °C.

3.2. Immunocytochemistry on round coverslips

ES cells or EBs were seeded onto gelatin-covered 12 mm glass coverslips (Novodirect), fixed in 4% PFA in PBS for 20 minutes. For immunofluorescence fixed ES cells or EBs were permeabilized in 0,5% Triton-X diluted in PBS for 10 minutes, blocked by incubation in 2% BSA in PBS for 1 hour RT and then incubated with primary antibodies for 1 hour RT or overnight at 4°C. The antibodies were diluted in 2% BSA/PBS. Dilutions are listed in Table 1. Samples were then incubated for 1 hour at RT in the dark with goat Alexa 488 anti-mouse or rabbit IgG antibody, donkey Alexa 488 anti-goat IgG antibody or goat Alexa 674 anti-mouse or rabbit IgG antibody (all from Molecular Probes) diluted 1:1000 in 2% BSA in PBS. Afterwards samples were incubated with 10µg/ml DAPI (Sigma) for 5 minutes to stain the nuclei and mounted with ProLong® Gold antifade reagent (Invitrogen/Molecular Probes). Pictures were taken using the LSM 510 Meta/Axiovert200M confocal microscope.

Table 1 - Antibodies used in this study

ANTIBODY	HOST	SUPPLIER	DILUTION	APPLICATION
α-nanog	rabbit	Abcam	1:100	IF
α-nanog	rabbit	Abcam	1:1000	WB

α -Oct3/4	mouse	Santa Cruz	1:100	IF
α - β -catenin C-term	mouse	BD Transductions	1:250	IF
α - β -catenin C-term	mouse	BD Transductions	1:2000	WB
α - β -catenin N-term	mouse	Alexis Biochemicals	1:200	IF
α - β -catenin N-term	mouse	Alexis Biochemicals	1:2000	WB
α - β -catenin N-term	mouse	Alexis Biochemicals	1:200	IHC
α -E-cadherin	mouse	BD Transductions	1:200	IF
α -E-cadherin	mouse	BD Transductions	1:1000	WB
α -plakoglobin	mouse	BD Transductions	1:200	IF
α -plakoglobin	mouse	BD Transductions	1:6000	WB
α -cTnT	mouse	Abcam	1:100	IF
α - α -catenin	mouse	Chemicon	1:100	IF
α -GFAP	rabbit	Dako	1:400	IF
α -GFAP	rabbit	Dako	1:1000	IHC
α - β 3-tubulin	mouse	Santa Cruz	1:200	IF
α - β 3-tubulin	mouse	Santa Cruz	1:100	IHC
α - α -fetoprotein (AFP)	rabbit	Dako	1:200	IF
α - α -fetoprotein (AFP)	rabbit	Dako	1:1000	IHC
α -nestin	mouse	Millipore	1:100	IF

α -myc tag	mouse	Cell signalling	1:200	IF
α -myc tag	mouse	Cell Signalling	1:1000	WB
α -HNF-3 β	goat	Santa Cruz	1:100	IF
α -PECAM-1	rat	BD Biosciences	1:200	IF
α -tubulin	mouse	Sigma	1:6000	WB
α -Stat3	mouse	Cell Signaling	1:1000	WB
α -p-Stat3(705Tyr)	mouse	Cell Signaling	1:1000	WB
α -SMA	rabbit	Thermo Fisher Scientific	1:100	IHC

3.3. Induction of EB formation in vitro

ES cells were dispersed by addition of 0,05% trypsin-EDTA for 5 minutes and counted. $1,0 \times 10^6$ cells per 20 ml of EB medium (ES cell medium without LIF) were plated onto 15 cm² bacterial petri dish. This step is defined as day 0 of differentiation. Medium was changed every second day and EB's were harvested at different time points, fixed for immunofluorescence or pelleted for protein or RNA isolation.

3.4. Teratoma formation in vivo

3.4.1. Injection of ES cells into nude mice

ES cells were dispersed by addition of 0,05% trypsin-EDTA for 5 minutes, washed with PBS and counted. $0,5 \times 10^6$ cells were resuspended in PBS and mixed 1:1 with Matrigel (BD biosciences) on ice. 200 μ l of total suspension were injected subcutaneously in the trunk of MF1 nude mice, control ES cells on the left side and mutant ES cells on the right side of a single mouse. Two weeks after injection, teratomas were recovered and fixed with 4% PFA in PBS ON, dehydrated to 75% EtOH and processed in Tissue-Tek VIP Tissue Processor and embedded in paraffin. Serial sections of teratomas were made at 5 μ m, collected on

SuperFrostPlus slides (Microm), stained with hematoxylin and eosin (both from Sigma-Aldrich) or subjected for immunohistochemistry.

3.4.2. Immunohistochemistry on paraffin sections

Paraffin sections of teratomas were processed using the VENTANA machine (by Vukoslav Komnenovic, Histo lab). Briefly, sections were dewaxed and rehydrated, antigen-retrieval was done using citrate buffer pH6,0. For dilutions of antibodies see Table 1. Slides were counterstained with hematoxylin. Pictures were taken using the Axioplan2/LeicaDFC320 microscope.

3.5. Induction of neural differentiation in monolayer cultures

For low and high density monolayer differentiation $0,9 \times 10^4$ cells/cm² and $4,0 \times 10^4$ cells/cm² respectively of ES cells were plated on laminin-covered coverslips in N2B27 medium (StemCellSciences). Medium was changed every second day. For nestin staining cells were fixed with 4% PFA in PBS at day 4 and for β 3-tubulin staining at day 14 of differentiation. Nestin-positive and β 3-tubulin-positive cells were counted in random fields and standard deviation was calculated in Microsoft Excel program.

3.6. Fluorescence activated cell sorting (FACS)

3.6.1. Detection of apoptotic cells

ES cells and day 5 EBs were dispersed using 0,05 % trypsin-EDTA (GIBCO) for 5 minutes and washed several times with PBS. For apoptosed cell detection cells were stained using Vybrant® Apoptosis Assay Kit #2 (Invitrogen/Molecular Probes) according to manufacturer's instructions. Briefly, PBS-washed cells were stained with 5 μ l of Alexa Fluor® 488 anti-annexinV (AnV) antibodies and 1 μ l of 100 μ g/ml propidium iodide (PI) for 15 min and acquired on FACS Calibur (Becton Dickinson). Analysis of results was done with FlowJo software (Tree Star). The populations of interest were gated as following: live cells - AnV⁻ PI⁻; apoptotic cells - AnV⁺ PI⁻; dead cells - AnV⁻ PI⁺; necrotic cells - AnV⁺ PI⁺

3.6.2. SSEA-1 FACS sorting

ES cells were dispersed using 0,05% trypsin-EDTA (GIBCO) for 5 minutes and washed several times with PBS. After fixation with 4% PFA for 20 minutes cells were permeabilized with 0,5% Triton-X diluted in PBS for 10 minutes, blocked by incubation in 2% BSA in PBS for 1 hour RT and then incubated with mouse anti-SSEA-1 antibodies (Chemicon) (1:500) for 1 hour RT. The antibodies were diluted in 2% BSA/PBS. Cells were then incubated for 1 hour at RT in the dark with goat Alexa 488 anti-mouse IgG antibody (Molecular Probes) diluted 1:1000 in 2% BSA in PBS. Afterwards cells were put on ice and sorted with CellQuest software (BD Biosciences) on CALIBUR machine. Data were analyzed with FlowJo software.

4. RT-PCR and microarray

4.1. RNA isolation

ES cells or EBs were dispersed with 0,05% trypsin-EDTA (GIBCO), washed with PBS several times and pelleted by centrifugation. Cell pellets were lysed in 1 ml TRI Reagent (Sigma) and incubated at RT for 5 min. 300µl of chloroform was added, shook by flicking for 15 seconds, incubated for 5 minutes at RT and centrifuged at 13000 rpm for 15 minutes. The aqueous upper phase was transferred into the fresh tube. The RNA was precipitated by addition of 0.5 ml isopropanol and centrifuged at 13000 rpm for 10 minutes at 4°C. The RNA was subsequently washed in 70% EtOH/H₂O-DEPC, air dried and resuspended in H₂O-DEPC. DNase treatment was performed with DNase A (Roche) according to manufacturer's instruction. RNA concentration was determined by OD measurement using Nanodrop 1000 spectrometer (ThermoScientific). Resuspended RNA was stored at - 80°C.

For the microarray analysis RNA was isolated from ES cells using QIAGEN-mini-RNA kit according to manufacturer's instructions and concentration was measured using the AgilentBioanalyzer.

4.2. 1st strand cDNA synthesis

1-2 µg RNA was mixed with 1µl polydT (500µg/ml, HAR089), 1µg dNTPs (10mM each, Eppendorf) and H₂O at a final volume of 10µl). Then the mix was incubated at 65°C for 5 minutes and cooled on ice for 5 minutes. Afterwards 4µl 5x first strand buffer, 2µl 0.1M DTT

(both Invitrogen), and 1 µl RNase Inhibitor (Roche) were added. The mixture was incubated for 2 minutes at 42°C, before 1 µl of Superscript II Reverse Transcriptase (Invitrogen) was added, and then incubated for 50 minutes at 42°C. The reverse transcriptase was inactivated at 70°C for 15 minutes, and the RNA of RNA:DNA duplexes was digested by incubation for 20 minutes at 37°C with RNaseH (Invitrogen). The cDNA was subsequently diluted 1 in 10 with dH₂O.

4.3. Semiquantitative RT-PCR

RT-PCR was performed using 2-3 µl of the 1 in 10 dilution of 1st strand cDNA and the TAKARA Taq PCR system (see 2.2). Primers were designed to amplify cDNA across exon-intron junctions and are listed in the Table 2.

Table 2 – List of primers used in semi-quantitative RT-PCR

Gene name	# stock	Sequence	Annealing T, °C	Product size, bp
Nanog	HAR1194	CCTCAGCCTCCAGCAGATGC	58	100
	HAR1195	CCGCTTGCACTTCACCCTTTG		
Oct3/4	HAR2113	CACGAGTGGAAAGCAACTCA	60	612
	HAR2114	CTGGGAAAGGTGTCCCTGTA		
Fgf5	HAR2107	GCGACGTTTTCTTCGTCTTC	60	492
	HAR2108	GAAGTGGGTGGAGACGTGTT		
Nestin	HAR2101	GATCGCTCAGATCCTGGAAG	60	491
	HAR2102	GCTCTGGGAGGACACCAGTA		
T	HAR2095	CCGGTGCTGAAGGTAAATGT	60	702
	HAR2096	ATTGTCCGCATAGGTTGGAG		
Mixl1	HAR2188	AGTTGCTGGAGCTCGTCTTC	60	444
	HAR2189	CTCCCAGATCTCCCTGAGTG		
Cer1	HAR2085	GCTTGTTCTCTTGCCTCTGG	60	502
	HAR2086	GTCTTCATGGGCAATGGTCT		

Foxa2	HAR2258double	GTAAAGTATGCTGGGAGCCG	61	219
	HAR2259double	CGCCACATAGGATGACATG		
Hand1	HAR2089	CGCCTGGCTACCAGTTACAT	60	533
	HAR2090	TGGTCTTCTCCAGTCCCATC		
Eomes	HAR2262double	GGCAAAGCGGACAATAACAT	60	300
	HAR2263double	GTTTTGGTAGGCCGTCACAG		
Gata6	HAR2079	GCCAACTGTCACACCACAAC	60	474
	HAR2080	CCTCTTGGTAGCACCAGCTC		

4.4. Real-time PCR

Real-time PCR was performed using 3µl of 1 in 10 diluted 1st strand cDNA in a total reaction volume of 25µl. The reaction was performed using the TAKARA PCR system (see 1.4.2.) with the addition of 0.2µl SybrGreen (Roche, 1:100 dilution in TAKARA PCR 10x Buffer) and dH₂O. Reaction conditions were 94°C 3', 40x (94°C 5'', 60°C 10'', 72°C 15''), plate read at 72°C, 80°C, and 85°C, melting curve: from 60°C-99°C, read every 0.4°C 5''. Products were analyzed by agarose gel electrophoresis and melting curves. Values were calculated using the comparative $\Delta C(t)$ method and normalized to HPRT values. Primers were designed to amplify cDNA across exon-intron junctions and are listed in Table 3.

Table 3 – List of primers used in real-time PCR

Gene name	# stock	Sequence	Annealing T, °C	Product size, bp
Axin2	HAR1430	AAGCCTGGCTCCAGAAGATCACAA	60	134
	HAR1431	TTTGAGCCTTCAGCATCCTCCTGT		
Sox2	HAR1206	GGCAGCTACAGCATGATGCAGGAGC	60	
	HAR1207	CTGGTCATGGAGTTGTACTGC		
Rex1	HAR1196	GGACTAAGAGCTGGGACACG	60	161
	HAR1197	CCTGCTTTTTGGTCAGTGGT		
Oct3/4	HAR1192	GAAGCAGAAGAGGATCACCTTG	58	129

	HAR1193	TTCTTAAGGCTGAGCTGCAAG		
Nanog	HAR1194	CCTCAGCCTCCAGCAGATGC	58	100
	HAR1195	CCGCTTGCACTTCACCCTTTG		
HPRT	HAR1167	AGCTACTGTAATGATCAGTCAACG	60	198
	HAR1168	AGAGGTCCTTTTCACCAGCA		

4.5. Microarray analysis

The in-house 47MM, 51MM and 52MM microarray platforms manufactured by Martin Radolf and Harald Scheuch (Biooptics department, IMP) were used to compare β -catenin^{fl/fl} ES cells versus β -catenin ^{Δ/Δ} ES cells and upon stimulation with recombinant Wnt3a (R&D Biosystems). Microarrays were screened by hybridization with Cy3- and Cy5-labeled probes that were prepared by oligo-dT-primed reverse transcription of 3-5ug of total RNA in the presence of Cy3-dUTP or Cy5-dUTP. RNA was isolated from 2 clones of β -catenin^{fl/fl} ES cells, 2 clones of β -catenin ^{Δ/Δ} ES cells and rWnt3a (R&D Biosystems)-treated ES cells clones. The activation and repression ratios of individual spots were calculated using GenePixPro5 chip software.

5. Luciferase assay

5.1. Generation of stable TOPFLASH and FOPFLASH ES cell lines

One day before infection with virus, ES cells were seeded onto 6-well plates at a concentration of 0,1X10E5 cells/well. The day of infection pBARLS and pfuBARLS lentiviruses (kindly provided by Randall Moon) were thawed on ice, mixed with ES cell medium at a 1:50 ratio and added to the cells. The next day, the medium was replaced with fresh medium containing 2 μ g/ml puromycin (Sigma). After 7 days of selection cells were pooled together, passaged several times, tested for responsiveness to Wnt-signaling activation using BIO and frozen in aliquots. These cells are in the following referred to as TOPFLASH and FOPFLASH reporter ES cell lines.

5.2. Luciferase activity measurement

TOPFLASH and FOPFLASH reporter ES cell lines were seeded onto 12-well plates in concentration $0,5 \times 10^5$ cells/well in triplicates. On the next day the cells were treated with 200ng/ml rWNT3a (R&D Systems), 50% Wnt3a condition medium (CM, kindly provided by R. Latham), 3 μ M CHIR (Stemgent) or 2 μ M BIO (Calbiochem). Cells treated with PBS served as a control. Luciferase assay was performed 24 hours later using Dual-Glo luciferase assay kit (Promega). All subsequent steps were performed in the dark. High glucose DMEM supplemented with 1% FCS (Sigma-Aldrich) was mixed 1:1 with lysis buffer (Dual-Glo luciferase substrate and buffer mix), 100 μ l/well of this suspension was added. The plate was incubated on a shaker for 10 minutes at RT. Cells were resuspended by pipetting several times and 50 μ l of the cell suspension were transferred into the white 96-well reader plate. Lysis buffer without cells was measured as blank control. Luciferase activity was measured in Synergy Fluorescence Plate Reader. For calculations the blank values were subtracted from experimental values and the activity of reporter was normalized to the activity of untreated cells.

6. Western blot analysis

6.1. Protein extraction from ES cells and EBs

Frozen cell pellets were resuspended in appropriate amount of protein extraction buffer (a mix 1:1 Tween20 buffer and NP40 buffer. Tween20-buffer: 50mM HEPES pH7.5, 150mM NaCl, 10mM EDTA, 0,2% Tween20; NP40-buffer: 1% NP40, 50mM Tris pH7.5, 150mM NaCl, 10mM EDTA. Protease/Phosphatase inhibitors (Roche) added immediately before use). The obtained suspension was then incubated on ice for 5 minutes and centrifuged for 10 minutes at 13000 rpm at 4 °C. The supernatant was transferred into a new tube, and protein concentration was measured using following method: 1 μ l supernatant diluted in 1ml 1:5 BIORAD Protein Assay: H₂O incubated for 10 minutes at RT and OD₅₉₅ was measured. Protein concentration was determined using following formula: $23,256 \times OD_{595} + 0,109 = \mu\text{g}/\mu\text{l}$.

6.2. Western Blot

10µg of protein extract was mixed with 2x protein loading buffer (125mM Tris-HCl pH6.8, 4% SDS, 20% Glycerol, β-Mercaptoethanol 1:20, 0.02% Bromophenol blue) and boiled at 95 °C for 5 minutes before it was chilled on ice and loaded onto the polyacrylamid gel. 8-10 % resolving gel and 4 % stacking gel were poured between two glass plates fixed in the plastic holder. The gel was run at 40mA until the dye reached the resolving gel, then at 90mA. After separation the gel was equilibrated in transfer buffer for at least 10 minutes. PVDF membrane was briefly washed in 100% methanol, washed with H₂O and then equilibrated in transfer buffer for 10 minutes. Wet blotting was performed at 400mA for 1 hour. Membrane was washed in TBST and then blocked with 4% non-fat milk in TBST for 1 hour at RT on a shaker before it was incubated with primary antibodies diluted in TBST for 1 hour at RT. Antibodies with dilutions used are listed in the Table 1. After washing 3 times in TBST for 5 minutes each membrane was incubated for 1 hour at RT with secondary antibodies diluted in TBST. After washing 3 times in TBST for 5 minutes, signals were detected with ECL Western Blot detection reagent (Amersham Biosciences) and the membranes were exposed to Hyperfilm ECL.

7. Transmission electron microscopy

Cells were grown for 2 days on gelatinized 12 mm UV-sterilized Aclar coverslips (kindly provided by Marlene Brandstaeter, EM facility). After rinsing in pre-warmed PBS cells were fixed in 2% glutaraldehyde for 1 hour RT, rinsed 3 times with PBS, osmicated with 2% OsO₄ and rinsed again 3x with PBS. Then the coverslips were dehydrated in a graded series of EtOH (40%, 60%, 80%, 95% and 100%) 10 minutes in each step.

For embedding coverslips were incubated in a 1:1 100% EtOH/epoxy resin mixture for 30 minutes at RT. Then they were transferred upside down onto drops of 100% resin in a plastic Petri dish and incubated for 30 minutes. This step was repeated one additional time. After preincubation coverslips were placed onto resin filled lids of Beem capsules and polymerized 48 hours at 60°C. After polymerization Aclar coverslips were pilled off and resin blocks containing the cells were processed for sectioning on Leica Ultracut UCT microtome. Sections were collected on the metal grids, post-stained with Reynolds lead citrate (PbNO₃)₂ and uranyl acetate and images were taken using FEI Morgagni 268 electron microscope.

8. RNAi experiments

8.1. Transient transfection of plakoglobin siRNA

One day before transfection ES cells were seeded onto 6-well plates in concentration $2,5 \times 10^4$ cells/well. The next day 7,5 μ l Lipofectamine2000 (Invitrogen) were mixed with 250 μ l of OptiMEM I (Gibco) and incubated for 5 minutes at RT. Meanwhile, 75 μ l (5 μ M stock) of anti-plakoglobin OnTarget siRNA mix (Dharmacon) were mixed with 250 μ l of OptiMEM I (Gibco), at the same time 75 μ l control anti-LaminA/C siRNA (Dharmacon) were mixed with 250 μ l of OptiMEM I. Then, diluted Lipofectamine2000 was mixed with diluted siRNA and incubated for 20 minutes at RT. Finally, this mix was added to the cells with 2ml of ES cells medium in each well. After 24 hours ES cell medium was replaced.

8.2. Generation of inducible plakoglobin RNAi β -cat ^{Δ/Δ} ES cell line

8.2.1. Generation of β -cat ^{Δ/Δ} R26rtTA ES cell lines

Targeting vector pR26/N-SA-rtTA^SM2-pA was kindly provided by Martin Leeb (Anton Wutz lab, IMP). Schematic map of this vector is shown on the Fig. 7. This targeting vector contains as a backbone pBluescript and is a modified version of the pRosa26-1 vector from P.Soriano lab (Fig. 8), which contains 5' and 3' homology arms of Rosa26 gene and a negative-selection diphtheria toxin (DTA) cassette under control of phosphoglycerate kinase (PGK) promoter downstream of 3' homology arm. rtTA cassette was inserted between the splice acceptor (SA) and polyadenylation (polyA) site. For positive selection a neomycin (Neo) resistance cassette under control of PGK promoter was inserted downstream of polyA site.

8.2.2. Linearization of targeting vector and DNA purification

50 μ g of pR26/N-SA-rtTA^SM2-pA targeting construct were digested in a total volume of 300 μ l 5x with 5 μ l of ClaI restriction enzyme (20 U/ μ l, New England Biolabs) according to the manufacturer's instructions. The digest was performed for 4 h at 37°C. The DNA was purified by the addition of 30 μ l 3M sodium acetate (pH 7,5) and 300 μ l phenol/chloroform/isoamylalcohol (25:24:1). The three reactions (300 μ l each) were merged in 15ml Falcon tube and centrifuged 30 minutes at 13200 rpm at RT. Supernatant was transferred

to a new tube and DNA was precipitated by addition of 1:10 100 % cold ethanol. Precipitated DNA in ethanol was chilled ON at -20°C. After centrifugation at 13200 rpm for 15 minutes at 4°C the supernatant was discarded. DNA pellet was washed with 70% cold ethanol (-20°C) 2 times and air dried for 1 hour. DNA was resuspended in 150 µl TE.

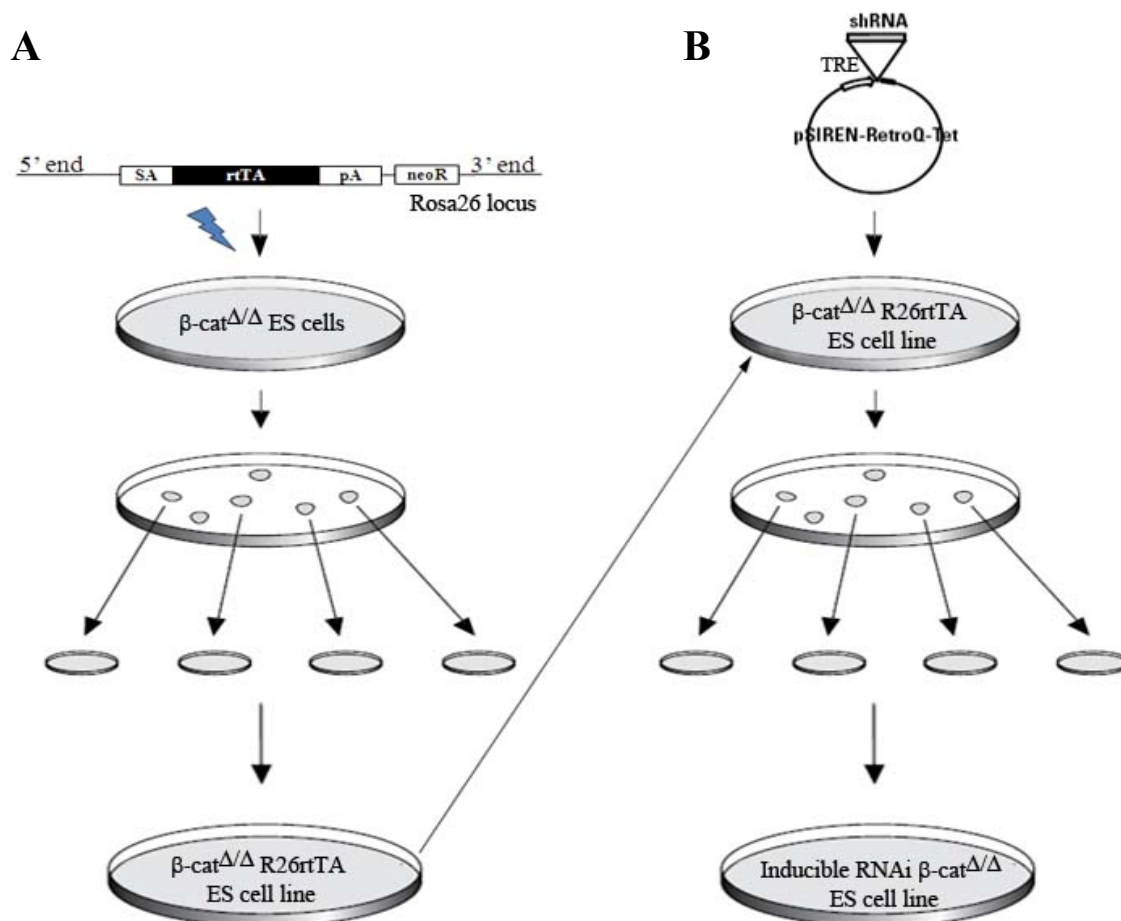


Figure 7 - Generation of inducible RNAi β -cat $^{\Delta/\Delta}$ ES cell line

(A) pR26-SA-rtTA-pA-Neo targeting construct was linearized with ClaI restriction enzyme and electroporated into β -cat $^{\Delta/\Delta}$ ES cells. After homologous recombination in Rosa26 locus correctly targeted clones were screened by Southern blot and several β -cat $^{\Delta/\Delta}$ R26rtTA ES cell lines were expanded (B) pSiren-RetroQ-Tet plasmid was transfected into β -cat $^{\Delta/\Delta}$ R26rtTA ES cell line. Puromycin-resistant colonies were expanded and final lines were named inducible plakoglobin RNAi β -cat $^{\Delta/\Delta}$ ES cells.

8.2.1. Electroporation of β -cat $^{\Delta/\Delta}$ ES cells with targeting constructs

ES cells were expanded in T25 flask until nearly confluent. Cells were trypsinized, spun down at 1000 rpm and resuspended in 10ml of prewarmed 1xPBS. After washing the cells were resuspended in 800µl 1xPBS and transferred into the 0,4mm electroporation cuvette (Biorad). 50 µg of linearized targeting construct were added into the same cuvette. After careful mixing

to avoid bubbles, electroporation was performed at 25 and 400V in two pulses with GenePulserII (Biorad). Afterwards the cells were resuspended in prewarmed ES cell medium and plated on five 15 cm cell culture dishes.

8.2.2. Selection for neomycin-resistant ES cell clones

Electroporated ES cells were cultured ON in ES cell medium. After 24 hours medium was replaced by ES cell medium containing G418 (Gibco) at concentration 750µg/µl. Cells were kept under these selection condition for about 7 days, then single resistant ES cell colonies were picked, trypsinized and plated individually into 96-well plates followed by further expansion, freezing and Southern blot analysis.

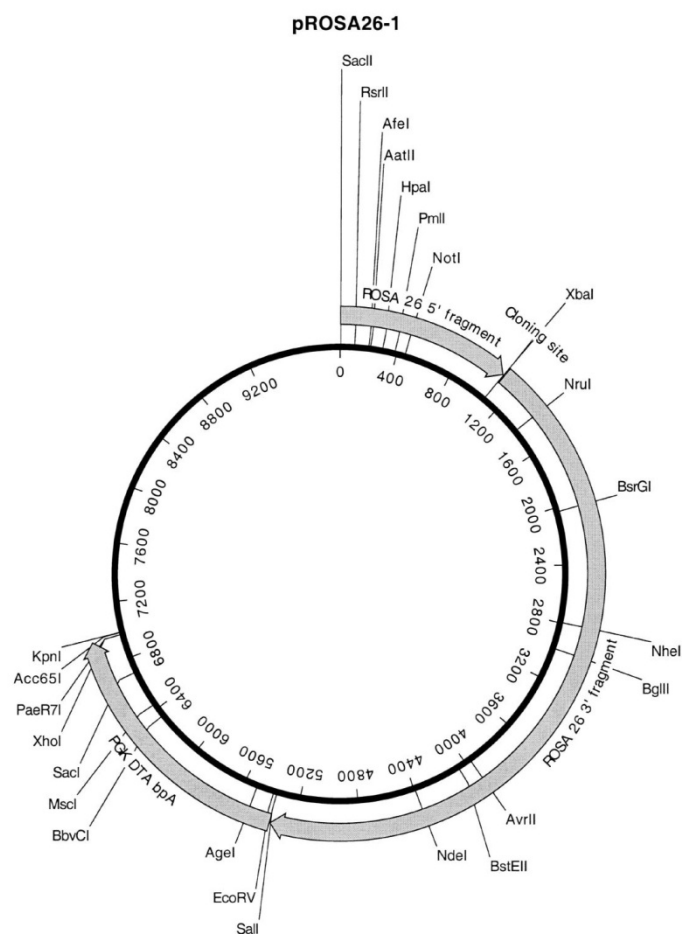


Figure 8 - Map of pRosa26-1 vector (from P. Soriano)

Plasmid contains Rosa26 5' fragment, XbaI – cloning site, Rosa26 3' fragment and PGK-DTA bpA cassette. PGK - phosphoglycerate kinase, DTA - diphtheria toxin A, bpA - polyA sequence

8.2.3. Hairpin design

Design of plakoglobin specific small hairpin RNAs (shRNAs) and further generation of inducible plakoglobin RNAi β -cat ^{Δ/Δ} ES cell line was done using the Knockout Tet RNAi System P (Clontech).

The four hairpins (see below) were designed according to the manufacturer's instructions and contained a sense (green) and antisense (red) sequences for the plakoglobin mRNA, a hairpin loop sequence in the middle, a termination sequence poly(T) and 5' BamHI and 3' EcoRI overhangs (indicated in yellow) for directional cloning into pSiren-RetroQ-Tet vector. ClaI restriction enzyme (grey) was included after poly(T) for the confirmation of cloned insert after ligation and transformation reactions.

Hairpin 1 HAR2055 (Top strand)

5' **GATCCG** **ACACCTACGACTCGGGCAT** TTCAAGAGA **ATGCCCCGAGTCGTAGGTGT** TTTTTT **ATCGATG** 3'

Hairpin 1 HAR2056 (Bottom strand)

3' **G** **CTGTGGATGCTGAGCCCGTA** AAGTTCTCT **TACGGGCTCAGCATCCACA** AAAAAATAGCTAC **TTAA** 5'

Hairpin 2 HAR2057 (Top strand)

5' **GATCCG** **CCAAGCTGCTCAACGATGA** TTCAAGAGA **TCATCGTTGAGCAGCTTGG** TTTTTT **ATCGATG** 3'

Hairpin 2 HAR2058 (Bottom strand)

3' **G** **CGGTTTCGACGAGTTGCTACT** AAGTTCTCT **AGTAGCAACTCGTCGAACC** AAAAAATAGCTAC **TTAA** 5'

Hairpin 3 HAR2059 (Top strand)

5' **GATCCG** **GGAACTACAGCTACGAGAA** TTCAAGAGA **TTCTCGTCGCTGTCGTTCC** TTTTTT **ATCGATG** 3'

Hairpin 3 HAR2060 (Bottom strand)

3' **G** **CCCTTGATGTCGATGCTCTT** AAGTTCTCT **AAGAGCAGCGACAGCAAGG** AAAAAATAGCTAC **TTAA** 5'

Hairpin 4 HAR2061 (Top strand)

5' **GATCCG** **TGAGTGTAGATGACGTCAA** TTCAAGAGA **TGACGTCATCTACACTCA** TTTTTT **ATCGATG** 3'

Hairpin 4 HAR2062 (Bottom strand)

3' **G** **CACTCACATCTACTGCAGTT** AAGTTCTCT **AACTGCAGTAGATGTGAGT** AAAAAATAGCTAC **TTAA** 5'

Annealing of oligonucleotides and ligation the double-stranded oligonucleotides into pSiren-RetroQ-Tet was done according to the manufacturer's instructions. After ligation and purification, pSiren-RetroQ-Tet plasmids (several clones for each hairpin) were sequenced using HAR2187 primer 5'-CTTGAACCTCCTCGTTCGACCCCGCCTC-3' to find the correct insertion of the hairpins.

8.2.4. Transfection of *pSiren-RetroQ-Tet-plako-shRNA* vector

One day before transfection β -cat ^{$\Delta\Delta$} ES cells were seeded onto 6-well plates in concentration 2.5×10^4 cells/well. The next day 9 μ l Lipofectamine2000 (Invitrogen) were mixed with 250 μ l of OptiMEM I (Gibco) and incubated for 5 minutes at RT. Meanwhile, 3 μ g of DNA were mixed with 250 μ l of OptiMEM I (Gibco). Then, diluted Lipofectamine2000 was mixed with diluted DNA and incubated for 20 minutes at RT. Finally, the mix was added to the cells with 2ml of ES cells medium in each well. After 24 hours the medium was replaced with fresh one supplemented with 2 μ g/ml Puromycin (Gibco). After 6 days under puromycin selection resistant colonies were picked and amplified.

9. Generation and analysis of rescued ES cells

9.1. Cloning and transfection of *pCAGGS* constructs

9.1.1. Linearization of *pCaggs-PGKneo* and inserts

The WT β -catenin open reading frame (ORF) was excised from HAR755 plasmid by double digestion with Asp718 and XhoI restriction enzymes in buffer B (Roche) for 2 hours at 37°C. The ORFs of myc-tagged ΔC and M6 human β -catenin were excised from HAR991 and HAR992 plasmids respectively by double digestion with Asp718 and XbaI restriction enzymes in buffer B (Roche) for 2 hours at 37°C. The pCAGGS-PGKneo plasmid (HAR423) was linearized by digestion with XhoI restriction enzyme in buffer H (Roche) for 2 hours at 37°C.

To fill the 5' overhanging ends generated by the restriction enzymes 1 μ l of 10mM each dNTPs (Takara) and 1 μ l Klenow enzyme (1 U/ μ l, Roche) were added to 50 μ l of digested DNA and incubated at 37°C for 15 minutes. Enzyme was inactivated by incubation at 75°C for 10 minutes.

To dephosphorylate the 5' ends after linearization and 5' repair 5 μ l of 10x Antarctic phosphatase buffer and 1 μ l Antarctic phosphatase (both Roche) were added to 50 μ l of linearized vector DNA and incubated for 15 minutes at 37°C.

After blunting and dephosphorylation products were loaded on the 0.8% TAE gel and run at 120V until bands were separated. Correct sized bands were cut out and DNA was extracted with QIAGEN gel purification kit according to manufacturer's instructions.

9.1.2. DNA ligation and transformation of competent *E.coli* cells

For ligation 9,5µl fragment DNA and 0,5µl vector were mixed with 10µl Takara ligation solution I (Takara Inc.) and incubated in a 16 °C water bath ON.

The next day DH5α competent frozen *E.coli* cells (-80°C) were incubated on ice for 20 minutes. 200µl of DH5α cells were then mixed with the ligation reaction and incubated on ice for 30 minutes. The DNA was transformed into DH5α cells by heat-shock for 1 minute 30 seconds at 42°C. After transformation the mixture was left on ice for 5 minutes. Then 700µl LB without antibiotics was added and *E. coli* were incubated for 30 minutes at 37°C and centrifuged for 1 minute at 13500 rpm. The cell pellet was resuspended in 100µl of LB with 50µg/ml ampicillin, plated on LB+Amp plates using glass beads and incubated ON in 37°C incubator.

Single colonies were picked and grown in LB+Amp for subsequently mini-prep analysis (Qiagen mini prep kit). After restriction analysis, positive correctly orientated clones were verified by sequencing and subsequently maxi-preped (Promega Maxi prep kit).

9.1.3. Transfection of β -catenin^{ΔA} ES cells with unlinerized plasmid DNA

One day before transfection ES cells were seeded onto 6-well plates in concentration 2,5X10E4 cells/well. The next day 9µl Lipofectamine2000 (Invitrogen) were mixed with 250 µl of OptiMEM I (Gibco) and incubated for 5 minutes at RT. Meanwhile, 3µg of DNA were mixed with 250µl of OptiMEM I (Gibco). Then, diluted Lipofectamine2000 was mixed with diluted DNA and incubated for 20 minutes at RT. Finally, the mix was added to the cells with 2ml of ES cells medium in each well. After 24 hours the medium was replaced with fresh one supplemented with 600µg/ml G418 (Gibco). After 6 days under G418 selection resistant colonies were picked and amplified.

9.2. Generation of WT, ΔC and M6 targeting vectors

9.2.1. Targeting vector description

Targeting vector pR26-SA-XbaI-pA-loxP-hygro-loxP was kindly provided by Karen Ng (Anton Wutz lab, IMP). This targeting vector contains as a backbone pBluescript and is a modified version of the pRosa26-1 vector from P.Soriano lab (Fig. 8), which contains 5' and 3'

homology arms of Rosa26 gene and the DTA cassette downstream of 3' homology arm. An XbaI cloning site was inserted between the SA and the polyA sites. pRosa26-1 vector was modified by inserting the SA and polyA sites fused to the XbaI site in between the 5' and 3' homology arms. In addition, a hygromycin resistance (Hygro^R) cassette surrounded by loxP sites was inserted downstream of polyA site.

9.2.2. Linearization of targeting vector and inserts

The WT β -catenin ORF was excised from HAR755 plasmid by double digestion with Asp718 and XhoI restriction enzymes in buffer B (Roche) for 2 hours at 37°C. The ORFs of myc-tagged Δ C and M6 human β -catenin were excised from HAR991 and HAR992 plasmids respectively by double digestion with Asp718 and XbaI restriction enzymes in buffer B (Roche) for 2 hours at 37°C. The pR26-SA-XbaI-pA-loxP-hygro-loxP vector was linearized by digestion with XbaI restriction enzyme in buffer H (Roche) for 2 hours at 37°C. Blunting of the insert ends, dephosphorylation of the vector and ligation were performed as described in section 9.1.1.

9.2.3. Linearization of the targeting constructs

50 μ g of WT and Δ C β -catenin targeting constructs were digested in a total volume of 300 μ l with 5 μ l of ClaI restriction enzyme (20 U/ μ l, New England Biolabs) and the M6 targeting construct was digested with AclI restriction enzyme (New England Biolabs) according to the manufacturer's instructions. The rest was done as in the chapter 8.2.2

9.2.4. Electroporation of β -catenin ^{$\Delta\Delta$} ES cells with targeting constructs

The same procedure as in the chapter 8.2.3

9.2.5. Selection for neomycin-resistant ES cell clones

Electroporated ES cells were cultured ON in ES cell medium. After 24 hours medium was replaced by ES cell medium containing G418 (Gibco) at concentration 750 μ g/ μ l. Cells were kept under these selection condition for about 7 days, then single resistant ES cell colonies were

picked, trypsinized and plated individually into 96-well plates followed by further expansion, freezing and Southern blot analysis.

10. Mini-Southern and Southern blot

10.1. Mini-Southern blot

Day1: Cell lysis in 96-well plate

ES cells clones resistant to hygromycin were cultured in a 96-well plate until they were confluent and medium turned yellow twice a day. Before lysis cells were washed 2 times with PBS, 50 µl of ES-cell lysis buffer (Chapter 2.1) were added per well and incubated ON at 60°C in a moist chamber.

Day 2: Isolation of genomic DNA and restriction digest in 96-well plate

150 µl/well of salt/ethanol solution (150 µM NaCl per 10ml 100%EtOH) were added carefully to precipitate DNA, plates were left undisturbed for 2 hours. The solution was removed by flipping the plate upside down and blotting it on paper towels. The precipitated DNA was washed 3 times with 70% EtOH and airdried for 1 hour at RT.

For the DNA restriction digest 30 µl/well of the restriction mix (3 µl of 10 x restriction buffer, 0,3 µl spermidine (100 mM), 0,3 µl RNase A (10 mg/ml), 20 U of BamHI enzyme, H₂O to a total volume of 30 µl) was added. Plates were incubated for 30 minutes at 37°C on a shaker; the digestion was performed ON at 37°C in the incubator.

Day 3: Southern blotting

1 µl/well of 6 x loading buffer were added to the 30 µl of digested genomic DNA and the samples were loaded on 0,8 % agarose gel containing TBE as a buffer. Electrophoresis was performed at 55 V for at least 5 hours. A picture was taken against fluorescent ruler and the DNA was nicked under UV light for 5 minutes. For depurination the gel was incubated for 15 minutes in 0,125M HCl. Then the gel was rinsed with deionized H₂O and incubation for 30 minutes in 0.4 M NaOH to denature the doublestranded DNA. Southern blotting by capillary transfer was set up using a Hybond N+ nitrocellulose membrane (GE Healthcare) for at least 24 hours in 0.4 M NaOH as transfer buffer.

Day 4: Hybridization and exposure

Blot was disassembled and DNA was crosslinked to the membrane using a Stratagene UV Crosslinker.

The 5' external probe was excised from a plasmid HAR730 with EcoRI restriction enzyme (New England Biolabs), separated from the vector by electrophoresis and purified from the gel with Gel purification kit (Qiagen). 25 ng of the probe were labelled with ^{32}P dCTPs using the RediPrime II Random Labelling Kit (GE Healthcare) according to the manufacturer's instructions. The labelled probe was separated from unincorporated radioactive nucleotides with a G-50 ProbeQuant microcolumn (GE Healthcare). The membrane was pre-hybridized in 25 ml Church's buffer (1% BSA, 1mM EDTA, 0.5M NaPi, 7% SDS) for 1 hour at 65°C. The labelled probe was heated to 95°C for 5 minutes, chilled on ice and spun down and then added to the 25 ml Church's in the hybridization tube. The hybridization was carried out at 65°C ON. After discarding the hybridization solution, the membrane was washed three times for 30 minutes each in Southern-Washing buffer (40ml 1M NaPi (pH7.2), 100ml 20% SDS, up to 2L with H₂O)

The membrane was sealed with Saran wrap and exposed in exposure cassette with X-Omat Blue film (Kodak) at -80°C for 4-5 days.

10.2. Southern blot

For Southern Blot analysis 15 µg of genomic DNA were digested with BamHI enzyme. The reactions contained the following components: 15 µg of genomic DNA, 3 µl of 10 x restriction buffer, 2 µl of restriction enzyme (10 U/µl), 0.3 µl of RNase A (10 mg/ml) and H₂O was added to a total volume of 30 µl.

The reaction was incubated at 37°C ON. The next day additional 10U of enzyme were added. After an additional incubation at 37°C for 2 hours, loading buffer was added to the samples and they were loaded on a 0.8% agarose gel containing TBE as buffer. The digested DNA was separated on the gel for at least 5 hours at 55 V. The blot was set up and hybridized as described in the section "Mini-Southern Blot".

Results

1. β -catenin and self-renewal of mouse ES cells

1.1. Generation of new β -catenin-deficient ES cell lines

ES cells were derived from 3.5 dpc old blastocysts of β -catenin fl/fl intercrossed females (Huelsen et al., 2001) and isolated on a feeder layer in the presence of LIF. Initially, two individual β -catenin fl/fl (β -cat^{fl/fl}) ES cell lines were established and tested, but only (NL β -12) was chosen for further experiments described later. The expanded ES cell line, which was injected into blastocysts gave chimaeras and transmitted through the germline (Fig. 9A, B). This experiment confirmed that the chosen ES cell line was fully pluripotent. After the initial establishment of β -cat^{fl/fl} ES cell lines, the lines were propagated without feeder layer in the presence of LIF and serum.

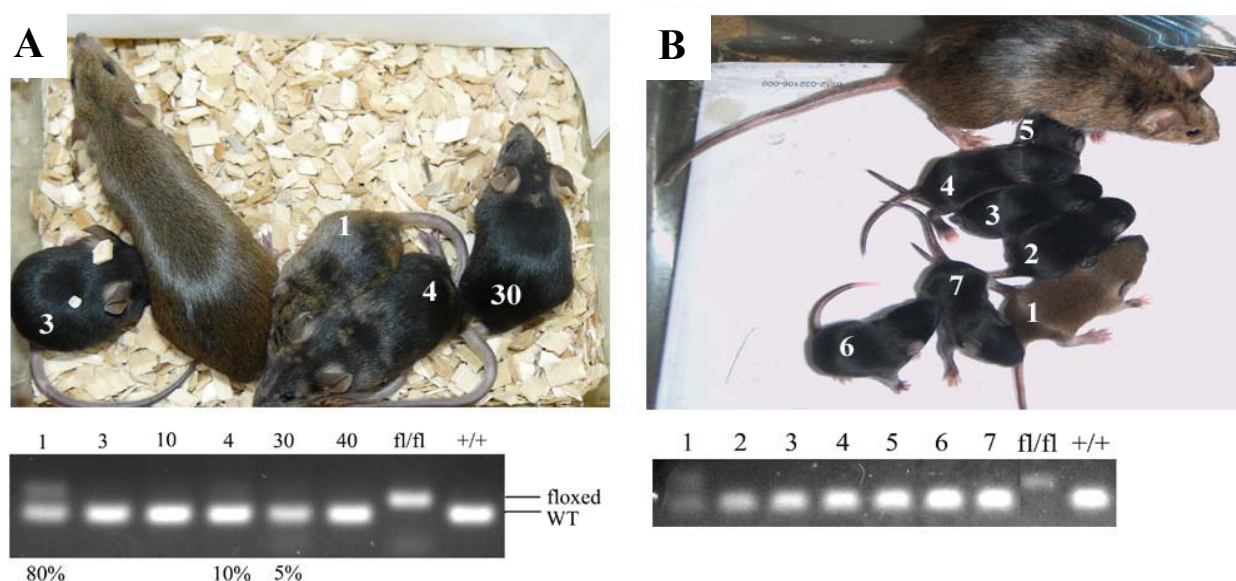


Figure 9- The β -cat^{fl/fl} ES cell line (NL β -12) contributes to chimaeras and is transmitted through the germline

(A) Chimaeric pups showing varying contribution of β -cat^{fl/fl} ES cells. Genotyping of these pups (1, 3, 10, 4, 30, 40) correlates with their coat color, with 1 being highly chimaeric and 4 having low chimaerism. (B) The high (80%) chimaeric female was mated to C57BL/6 (black) male and produced one agouti pup, indicating the transmission of the β -cat^{fl/fl} derived genome. Genotyping of these pups confirmed that the agouti pup (1) is heterozygous for the β -catenin floxed allele.

From the $\beta\text{-cat}^{\text{fl/fl}}$ ES-cells several $\beta\text{-catenin-deficient}$ ES ($\beta\text{-cat}^{\Delta/\Delta}$) cell lines were established through infection with an Adeno-Cre virus. Individual subclones were generated by dilution cloning and genotyped by PCR in order to identify $\beta\text{-cat}^{\Delta/\Delta}$ ES cell clones (Fig. 10A). Two independent $\beta\text{-cat}^{\Delta/\Delta}$ ES subclones (3B and 1B) were used for further characterization (Fig. 10A). The $\beta\text{-cat}^{\Delta/\Delta}$ ES cells showed a similar proliferation behavior as control $\beta\text{-cat}^{\text{fl/fl}}$ ES cells (Fig. 10B). $\beta\text{-cat}^{\Delta/\Delta}$ ES cell colonies resembled those of $\beta\text{-cat}^{\text{fl/fl}}$ ES cells in their appearance, with the exception that an increased number of single cells were noted in the cultures (Fig. 10C).

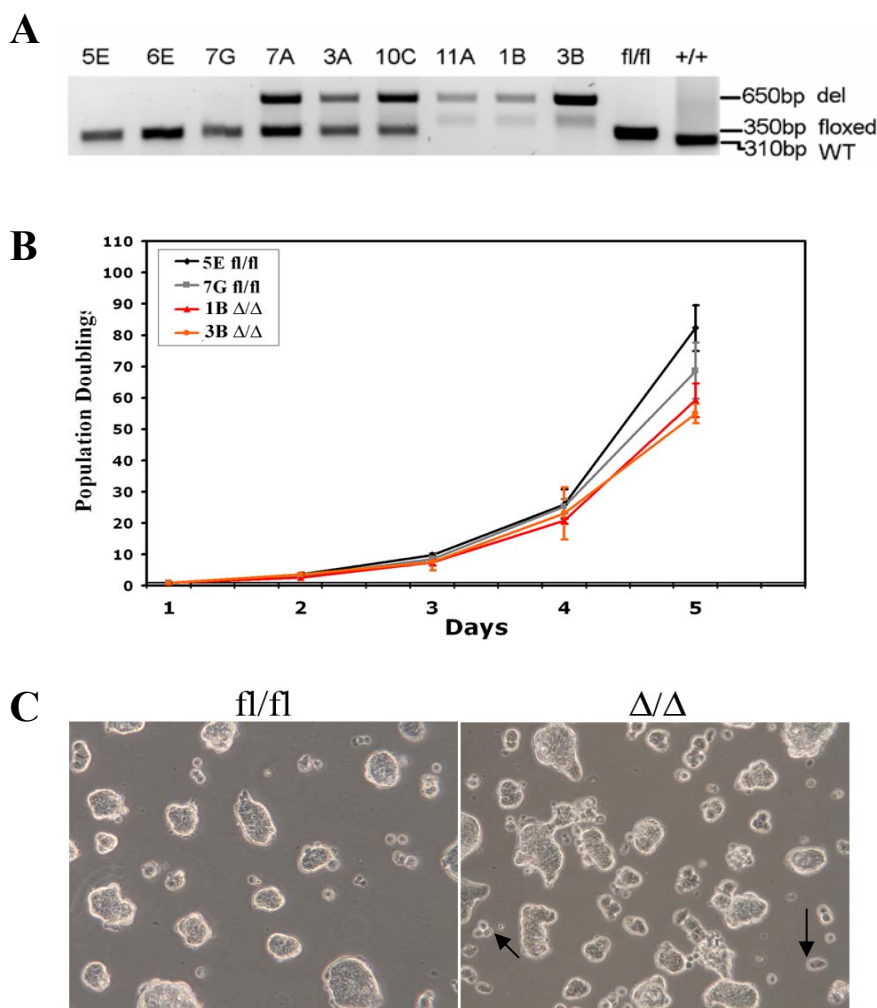


Figure 10 – Genotyping and growth of $\beta\text{-cat}^{\text{fl/fl}}$ and $\beta\text{-cat}^{\Delta/\Delta}$ ES cell lines

(A) PCR genotyping of DNA isolated from subclones after infection with AdenoCre virus and subcloning. (B) Population doublings of cultures of indicated $\beta\text{-cat}^{\text{fl/fl}}$ and $\beta\text{-cat}^{\Delta/\Delta}$ ES cell lines grown for 5 days on gelatinized plates in ES cell media containing LIF without feeder layer. Every day cells were collected and counted. Error bar=SEM

(C) Morphological appearance of $\beta\text{-cat}^{\text{fl/fl}}$ and $\beta\text{-cat}^{\Delta/\Delta}$ ES cell lines. Arrows indicate single cells.

To address whether it is possible to culture $\beta\text{-cat}^{\Delta/\Delta}$ ES cells without serum, I employed the serum-free cell culture system called 2i (Ying et al., 2008). This media consists of N2 and B27 supplements plus 1 μM mitogen-activated protein kinase kinase (MEK) inhibitor PD0325901 and 3 μM Gsk3 inhibitor CHIR99021. Addition of 1000U/ml LIF to this culture media promotes the propagation of WT ES cells (Dr. A. Smith, personal communications). Interestingly, the

morphology of $\beta\text{-cat}^{\text{fl/fl}}$ and $\beta\text{-cat}^{\Delta/\Delta}$ ES cells changed already one day after replacing the normal ES cell medium-containing serum and LIF with the 2i + LIF medium (Fig. 11): for both genotypes the ES cell colonies became more compact. However, after several passages in serum-free medium $\beta\text{-cat}^{\Delta/\Delta}$ ES cells started to detach from the gelatin-coated tissue culture dishes, while control $\beta\text{-cat}^{\text{fl/fl}}$ ES cells were growing normally. This defect could not be rescued by culturing the cells on a more adhesive surface, such as a laminin-coated surface. Thus, this result suggests that $\beta\text{-cat}^{\Delta/\Delta}$ ES cells have a defect in cell-to-surface adhesion in a serum-free cell culture system.

To explain why the slight morphological defect was observed in serum-containing and serum-free cultures, I decided to check the localization of adhesion-related molecules in $\beta\text{-cat}^{\Delta/\Delta}$ ES cells. Colonies of $\beta\text{-cat}^{\Delta/\Delta}$ ES cells stained negative for β -catenin by immunofluorescence (IF) in contrast to control colonies, where β -catenin was expressed in the cell membranes (Fig. 12A). However, the staining for plakoglobin was increased in $\beta\text{-cat}^{\Delta/\Delta}$ ES cells (Fig. 12B), while the E-cadherin staining was unchanged (Fig. 12C). These data were confirmed by Western Blot analysis, which revealed a 2-3 fold increase in plakoglobin levels in $\beta\text{-cat}^{\Delta/\Delta}$ ES cells, while E-cadherin levels were not altered (Fig. 12D). However, no significant change was observed in *plakoglobin* transcript levels by real-time PCR analysis (data not shown). Thus, similar to what has been observed in F9 teratocarcinoma stem cells, plakoglobin probably substitutes for β -catenin in cell-cell adhesion in ES cells (Fukunaga et al., 2005).

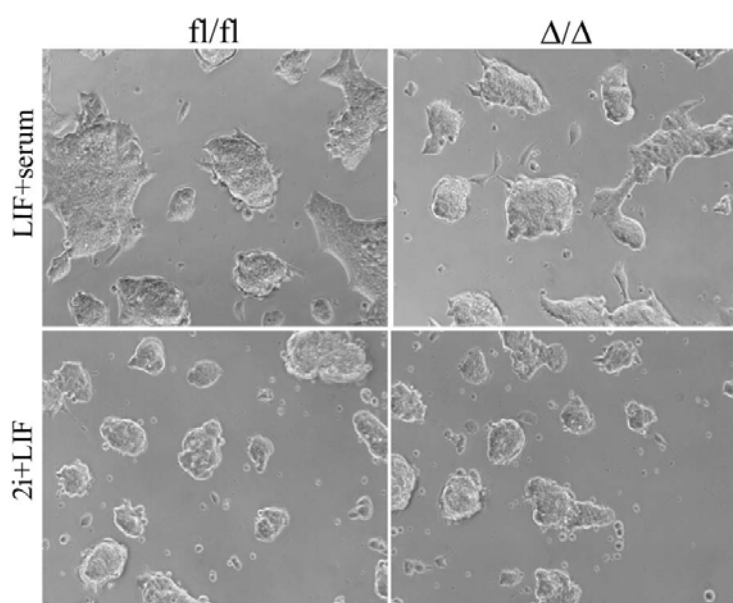


Figure 11 – Culturing $\beta\text{-cat}^{\text{fl/fl}}$ and $\beta\text{-cat}^{\Delta/\Delta}$ ES cells in serum-free medium

Phase-contrast images showing morphology of $\beta\text{-cat}^{\text{fl/fl}}$ and $\beta\text{-cat}^{\Delta/\Delta}$ ES cells in LIF+serum medium or in serum-free 2i +LIF medium after 1 passage. Images were taken with 10x objective.

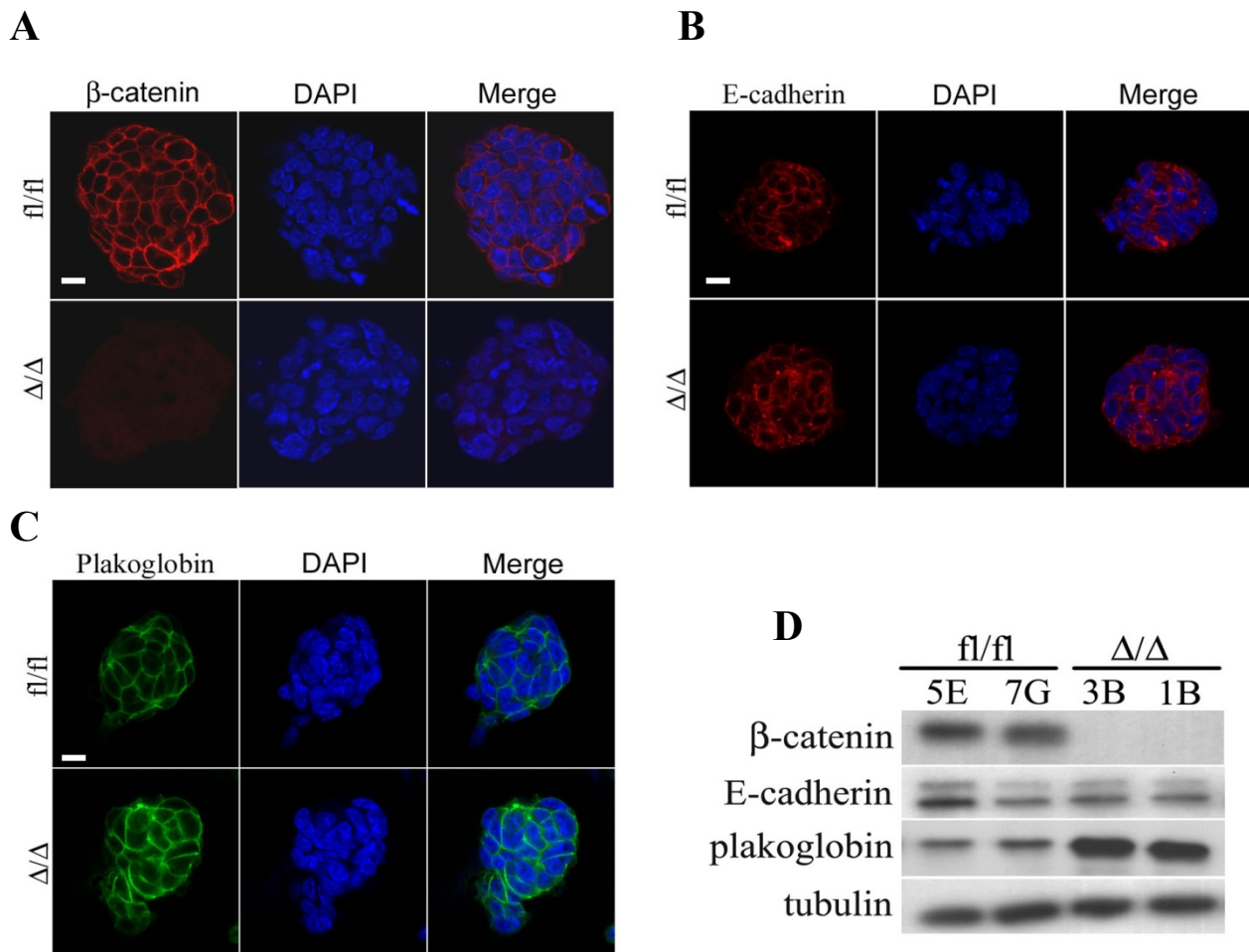


Figure 12 – Adhesion-specific molecules in β -cat^{fl/fl} and β -cat^{Δ/Δ} ES cells

(A) β -catenin localizes to the membrane in β -cat^{Δ/Δ} ES cells, but is absent in β -cat^{fl/fl}. (B) E-cadherin is localized to the membrane in both β -cat^{Δ/Δ} and β -cat^{fl/fl} ES cells. (C) Plakoglobin localizes to the membrane and its levels are much higher in β -cat^{Δ/Δ} ES cells. (D) Western blot analysis showing that β -catenin protein is absent in β -cat^{Δ/Δ} ES cells, E-cadherin levels are not changed and plakoglobin levels are increased in β -cat^{Δ/Δ} ES cells. Scale bars = 10 μ m.

In the literature it has been reported that plakoglobin has a weak signaling activity *in vitro* and that overexpression of *plakoglobin* in a β -catenin deficient (β -cat^{lacZ/lacZ}) ES cell clone can activate a TOPFLASH reporter, suggesting that plakoglobin might also be able to substitute in part for the β -catenin signaling activity (Conacci-Sorrell et al., 2002; Williams et al., 2000). Thus, I analyzed next whether there was a detectable endogenous TCF-mediated gene expression using a new, highly sensitive lentiviral luciferase reporter called BAR (Beta-catenin Activated Reporter), which contains 12 multimerized TCF binding sites and a control reporter fuBAR (found-unresponsive Beta-catenin Activated Reporter), containing mutagenized TCF binding sites (Biechele and Moon, 2008). β -cat^{fl/fl} control and β -cat^{Δ/Δ} ES cells were infected with the BAR (TOPFLASH) or the fuBAR (FOPFLASH) lentiviruses, and selected for

puromycin-resistant ES cell clones. After one week of puromycin selection resistant clones of infected β -cat^{fl/fl} or β -cat ^{Δ/Δ} ES cells were pooled together, respectively, and used for luciferase assay measurements. Only minor reporter activity could be detected in control β -cat^{fl/fl} ES cells as well as in β -cat ^{Δ/Δ} ES cells without external stimulation (Fig. 13B).

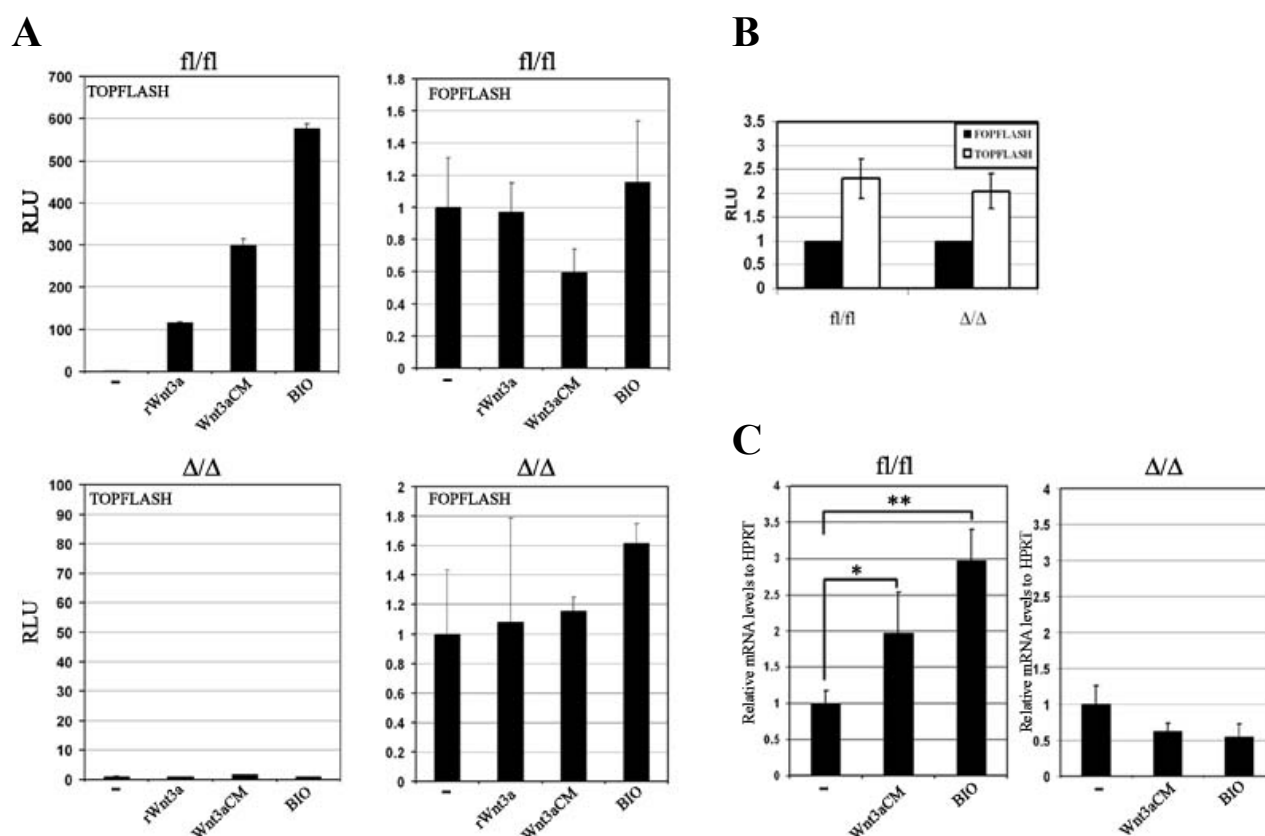


Figure 13 - Activation of Wnt signaling in β -cat^{fl/fl} but not in β -cat ^{Δ/Δ} ES cells

(A) Luciferase assay in puro-resistant, stable β -cat^{fl/fl} and β -cat ^{Δ/Δ} ES cells infected with the lentiviral β -catenin TOPFLASH (BAR) or FOPFLASH (fuBAR) reporter. Results from triplicates after 24h treatment without stimulation (-) or after stimulation with rWnt3a, Wnt3aCM or BIO. At least three independent measurements per sample were performed. Data were normalized to TOPFLASH values of either untreated β -cat^{fl/fl} or β -cat ^{Δ/Δ} ES cells. Control FOPFLASH (fuBAR) luciferase measurements shown on the left. (B) Basic TOPFLASH activity of untreated β -cat^{fl/fl} or β -cat ^{Δ/Δ} ES cells. Data were normalized to FOPFLASH reporter (C) Real-time PCR analysis of *Axin2* transcript levels in β -cat^{fl/fl} and β -cat ^{Δ/Δ} ES cells. Error bars=SEM *p<0.05; **p<0.01 (Anova with Tukey HSD test).

This result is consistent with previously published observations (Anton et al., 2007), where only low or no detectable TOPFLASH activation was detected in unstimulated WT ES cells. However, upon stimulation with either Gsk3 inhibitor BIO (Sato et al., 2004), recombinant To determine the functional consequence of canonical Wnt signaling activation by external stimulation on endogenous target genes, I extended my analysis to a known TCF target gene,

Axin2 (Jho et al., 2002). I measured *Axin2* gene expression levels 24 hours after stimulation with Wnt3aCM and BIO by real-time PCR (Fig. 13B). Control and β -cat^{ΔΔ} ES cells had similar low levels of *Axin2* without stimulation. However, upon stimulation with Wnt3aCM or BIO *Axin2* was two and three-fold increased respectively in control, but not in β -cat^{ΔΔ} ES cells.

Thus, my data suggest that β -cat^{ΔΔ} ES cells are not responsive to Wnt stimulation and that the 2-3 fold increase in total plakoglobin levels cannot substitute for β -catenin/TCF-mediated signaling activity.

1.2. Generation of CAG WT, ΔC and M6 ES cells

In order to address whether reintroduced wild-type (WT) mouse β -catenin would be functional in transcription I stably expressed it in β -cat^{ΔΔ} ES cells. In parallel I established β -cat^{ΔΔ} ES cells expressing two putative signaling-defective versions of human β -catenin, C-terminus deleted (ΔC) Δ696-781 aa and L774A/W776A (M6), which were myc-tagged at their N-termini (Fig. 14A, both gifts from Dr. R. Grosschedl). The C-terminus of β -catenin contains the transactivation domain, which has been reported to interact with multiple transcriptional co-activators. The two point mutations (L774A/W776A) in C-terminus of β -catenin have been shown to impair, in part, the transactivation potential of full-length β -catenin (Sustmann et al., 2008).

For long-term stable expression, I used a neomycin-selectable vector in which the transgene expression is under control of a CMV early enhancer/chicken β actin (CAG) promoter, because in initial experiments driving *β -catenin* expression by a CMV promoter β -catenin was hardly detectable by western blot (data not shown). The CAG promoter has been reported to result in higher levels of transgene expression in mouse ES cells in comparison to the CMV promoter (Alexopoulou et al., 2008). Thus, I cloned WT, ΔC or M6 β -catenin ORFs into pCAGGS-PGKneo plasmid and established several stable ES cells lines for each β -catenin variant. Western blot analysis confirmed that WT, myc-tagged ΔC or M6 β -catenin were expressed at significant levels in the clones that I chose for further experiments (Fig. 14C).

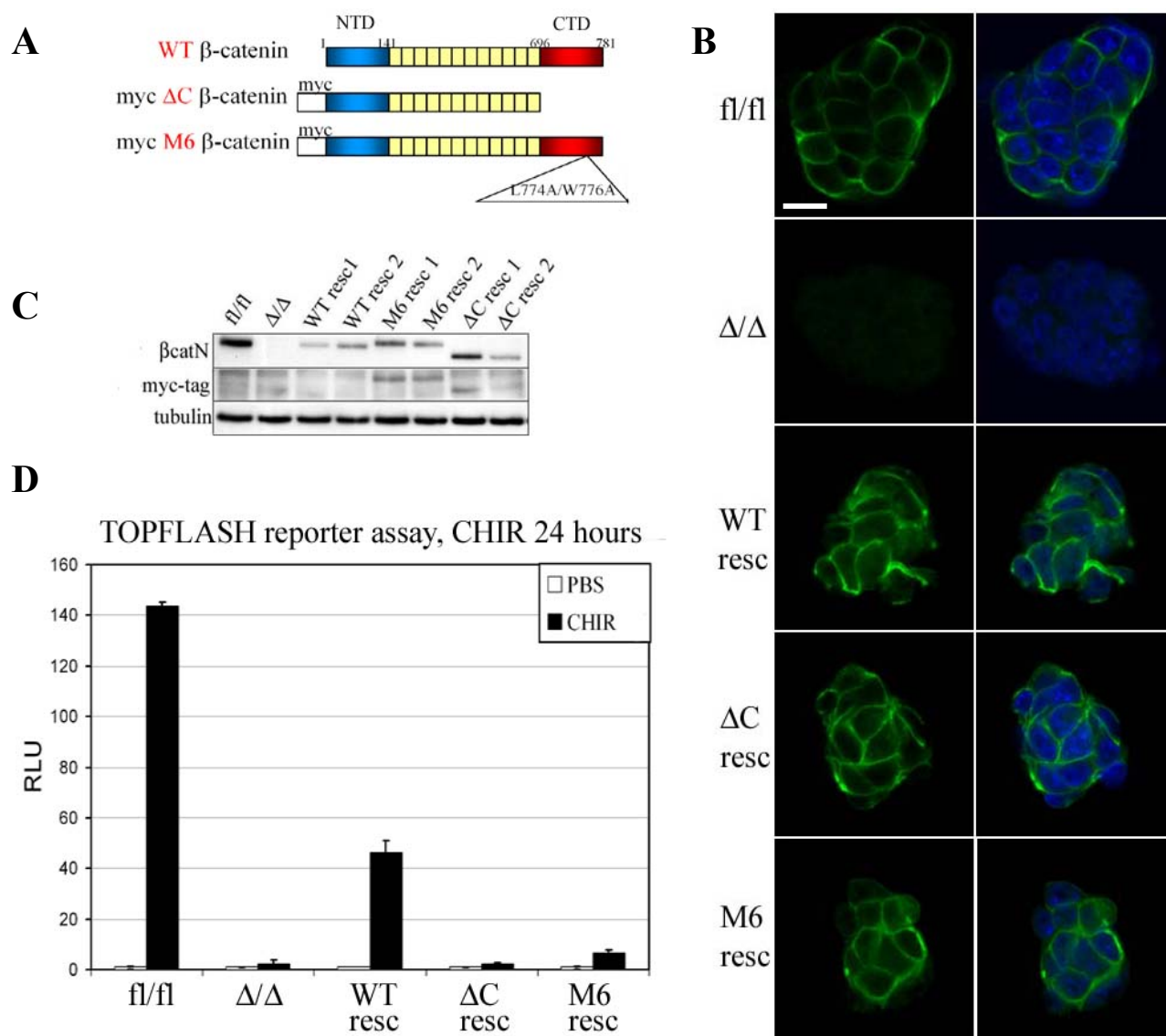


Figure 14 – Generation of pCAGGS WT, ΔC and M6 rescued β-cat^{Δ/Δ} ES cells

(A) Schematic representation of the rescue β-catenin constructs. NTD, N-terminal domain (blue); CTD, C-terminal domain (red); Arm repeats are in yellow. Numbers indicate amino acids. (B) IF images of β-cat^{fl/fl} and β-cat^{Δ/Δ} ES cells as well as β-cat^{Δ/Δ} ES cells rescued with WT, ΔC or M6 β-catenin variants. β-catenin is stained in green, an anti-C-terminal β-catenin antibody was used with the exception of ΔC rescued cells, which were stained with an anti-myc antibody, DAPI nuclear staining is blue. Scale bar = 10 μm. (C) Western blot analysis of β-cat^{Δ/Δ} ES cells rescued with WT, ΔC or M6 β-catenin variants. Membranes were blotted with anti-N-terminal β-catenin antibodies and anti-myc antibodies. Anti-tubulin staining was used as a loading control. (D) TOPFLASH reporter assay in ES cell lines stably expressing TOPFLASH (BAR) reporter. Luciferase activity was measured 24 hours after treatment with CHIR99021 in triplicates. Experiment was repeated at least 3 times. Data were normalized to TOPFLASH values of β-cat^{fl/fl} untreated cells. Error bar=SEM.

Immunocytochemical stainings revealed that WT, ΔC or M6 β -catenin proteins all localized to the cell membrane in β -cat ^{Δ/Δ} ES cells (Fig. 14B), similar to the β -catenin localization in control β -cat^{fl/fl} ES cells.

In order to assess responsiveness to activation of Wnt signaling in the rescued β -cat ^{Δ/Δ} ES cells, I infected WT (clone2), ΔC (clone1) or M6 (clone1) β -catenin rescued ES cells with the TOPFLASH lentiviral reporter. Upon stimulation with CHIR99021, a Gsk3 inhibitor (Bain et al., 2007; Murray et al., 2004), TOPFLASH reporter activity was increased in the β -cat^{fl/fl} control cells and WT β -catenin rescued ES cells, but not detectable in β -cat ^{Δ/Δ} ES-cells or ΔC β -catenin rescued ES cells (Fig. 14D). However, consistent with previous observations slight transcriptional activity was observed in the M6 β -catenin rescued ES cells (Sustmann et al., 2008).

Notably, TOPFLASH reporter activity correlated with β -catenin protein levels in the β -cat^{fl/fl} and WT β -catenin rescued ES cell lines. Transcriptional activity in WT rescued β -catenin cells (WT resc 2 in Fig. 14A), in which β -catenin levels were approx. 3 times lower than in WT (fl/fl in Fig. 14A), was only at 30% of the control β -cat^{fl/fl} cells. My data show that all the products of all three re-expressed *β -catenin* transgenes localize to the cell membrane and I could clearly establish that the ΔC β -catenin variant cannot participate in TCF-mediated signaling.

1.3. Analysis of self-renewal using β -catenin-deficient ES cells

1.3.1. Self-renewal is not affected in the absence of β -catenin

As described earlier, β -cat^{fl/fl} ES cells can contribute to chimaeras and the germline. This knowledge allowed me to study self-renewal and pluripotency potentials of β -cat^{fl/fl} and their derivative β -cat ^{Δ/Δ} ES cells. Therefore, I analyzed transcriptional levels and protein expression of a number of stem cell markers in β -cat^{fl/fl} and β -cat ^{Δ/Δ} ES cell lines. Both ES cell lines are positive for alkaline phosphatase (AP) at early and late passages (Fig. 15A and data not shown). No differences in transcriptional expression levels were found for the stem cell specific transcription factors *Oct-3/4*, *Nanog*, *Rex-1/Zfp42* and *Sox2* using real-time PCR (Fig. 15B). Protein levels of Nanog and Oct-3/4 analyzed by western blot analysis were similar between β -cat^{fl/fl} and β -cat ^{Δ/Δ} ES cells (data not shown). FACS analysis showed no difference in the expression levels of SSEA-1, an antigen expressed specifically in undifferentiated ES cells (Fig.

15C). Immunofluorescent analysis of Nanog and Oct3/4 revealed normal localization in the nucleus (Fig. 15D and data not shown). Furthermore, phospho-Stat3 levels upon LIF stimulation were also not altered (Fig. 15E). These results suggest that there is no defect in self-renewal potentials of β -cat $^{\Delta/\Delta}$ ES cells.

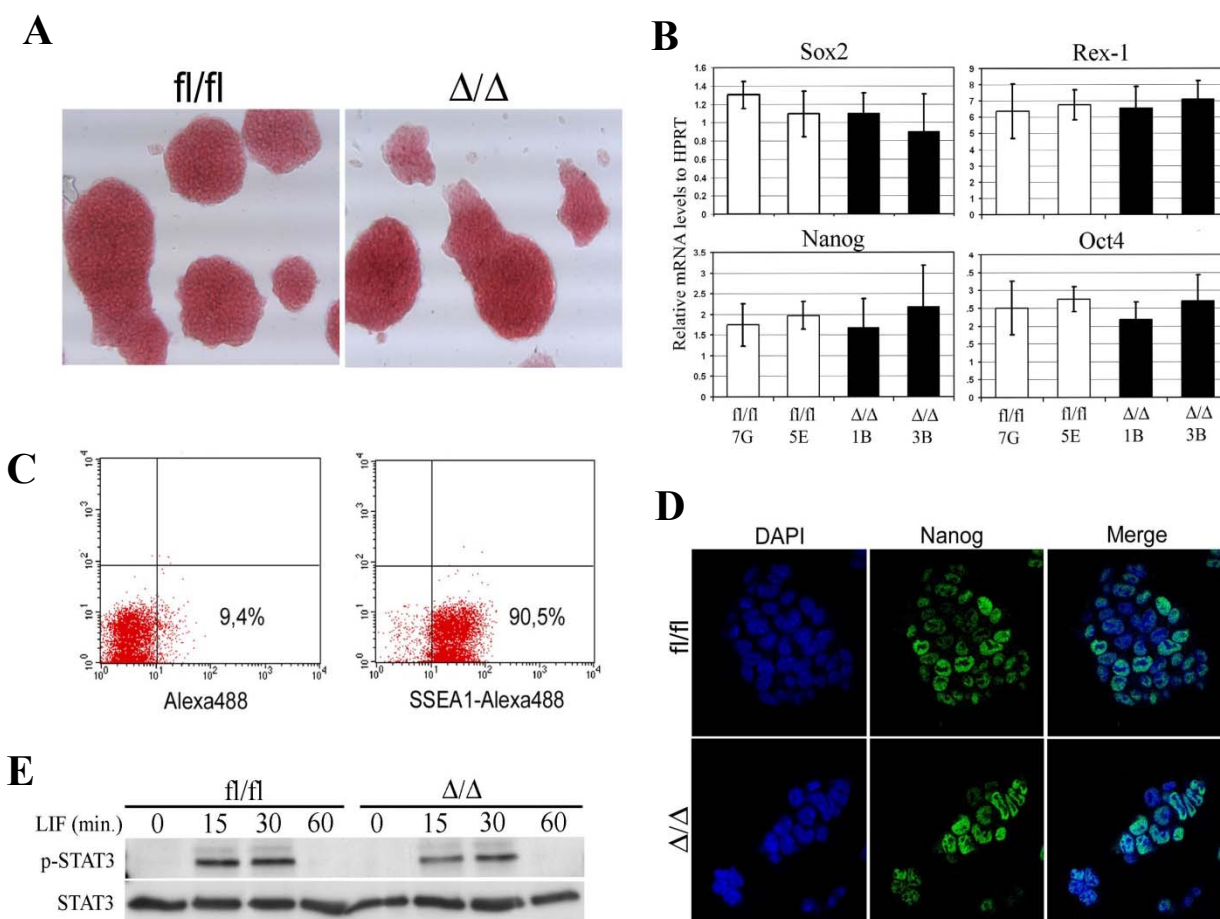


Figure 15 - Self-renewal markers in β -cat $^{fl/fl}$ and β -cat $^{\Delta/\Delta}$ ES cells are not affected

(A) Colony morphology and AP expression at passage 13. (B) Quantitative RT-PCR analysis of transcription factors involved in self-renewal showed no change. mRNA levels of these genes were normalized to HPRT mRNA levels. (C) β -cat $^{\Delta/\Delta}$ ES cells stained with anti-SSEA1 antibodies were analyzed by FACS sorting. As a control (left FACS blot) cells were only stained with the Alexa488-coupled secondary antibodies. (D) IF staining of Nanog showed localization in the nucleus. Nuclei were counterstained with DAPI (blue). (E) Western blot showing normal kinetics of STAT3 phosphorylation (anti-p-STAT3 (705Tyr)) upon treatment with 1000U/ml LIF and the corresponding total STAT3 levels.

1.3.2. Requirement of β -catenin for self-renewal maintenance

Activation of the canonical Wnt/ β -catenin pathway via Wnt3a CM or the GSK3 inhibitor BIO can maintain mouse and human ES identity (Sato et al., 2004). However, none of these factors is absolutely specific for the Wnt/ β -catenin pathway (Bain et al., 2003; Ogawa et al., 2006). Thus, I addressed the question whether the effects mediated by BIO, rWnt3a, Wnt3a CM or CHIR treatment on self-renewal maintenance of murine ES cells require β -catenin activity using our β -catenin-deficient ES cells. For the self-renewal assay, ES cells were plated at low density (20000cells/well) and cultured for 5 days in the presence or absence of LIF and stained for AP. In contrast to 5 day cultures on gelatin in the presence of LIF, withdrawal of LIF resulted in differentiation of β -cat^{fl/fl} and β -cat^{Δ/Δ} ES cells (Fig. 16A). Replacement of LIF with 200 ng/ml rWnt3a maintained self-renewal in control β -cat^{fl/fl}, but not in β -cat^{Δ/Δ} ES-cells (Fig. 16A). Similar results were obtained with 3μM CHIR (Fig. 17, upper panel). In contrast, self-renewal was maintained in β -cat^{fl/fl}, but also in β -cat^{Δ/Δ} ES cells upon treatment with either Wnt3a CM (50%) or 2.5 μM BIO (Fig. 16A). This suggests that Wnt3a CM contains additional components that can activate other self-renewal pathways thereby substituting for the loss of β -catenin and that BIO is probably a less specific inhibitor than CHIR. In addition, I determined the expression levels of the self-renewal marker *nanog* by real-time PCR 24 hours after changing the culture conditions. As expected *nanog* expression levels were reduced upon LIF withdrawal in β -cat^{fl/fl} control and β -cat^{Δ/Δ} ES cells (Fig. 16B). Interestingly, *nanog* expression was maintained in β -cat^{Δ/Δ} ES cells treated with Wnt3a CM or BIO at levels similar to LIF treated ES cells, however, no up-regulation of *nanog* expression as seen in stimulated β -cat^{fl/fl} control ES cells was observed (Fig. 16B). This suggests that β -catenin activity is required for *nanog* transcriptional activation induced by Wnt3a CM or BIO, in agreement with previous reports that *nanog* is regulated by canonical Wnt signaling (Takao et al., 2007). However, after 5 days of treatment with rWnt3a Nanog protein levels were maintained in control ES cells, but not in β -catenin-deficient ES cells (Fig. 16C), suggesting that rWnt3a acts directly through β -catenin to support self-renewal of mouse ES cells.

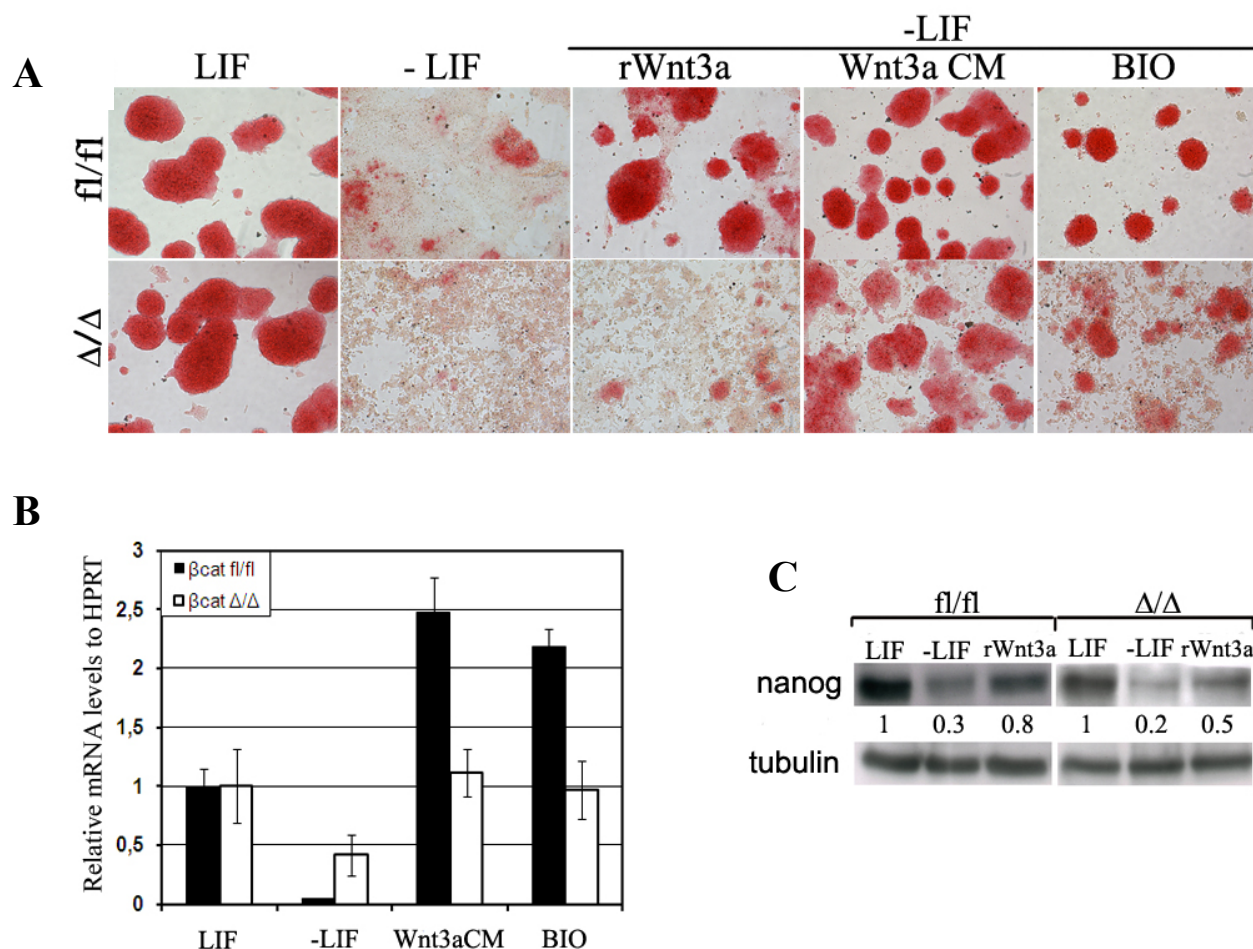


Figure 16 - Self-renewal potentials of β -cat^{fl/fl} and β -cat^{Δ/Δ} ES cells upon activation of Wnt signaling

(A) Morphology of β -cat^{fl/fl} and β -cat^{Δ/Δ} AP-stained colonies. ES cells were plated at low density in FCS-containing medium supplemented with LIF or without LIF, rWnt3a, Wnt3a CM or BIO for 5 days. (B) Real-time PCR analysis of *nanog* expression in β -cat^{fl/fl} and β -cat^{Δ/Δ} ES cells after 24 hours with LIF or without LIF and in the presence of Wnt3a CM or BIO. Error bars = SEM. (C) Western blot analysis of Nanog protein levels in β -cat^{fl/fl} and β -cat^{Δ/Δ} ES cells 5 days after treatment with rWnt3a. Tubulin levels are used as a loading control.

I also tested, whether the β -cat^{Δ/Δ} ES cells would be more sensitive to reductions of LIF concentration by performing LIF titration experiments, but found no differences in the behavior between control and β -cat^{Δ/Δ} ES cells (data not shown). This argues against a previously proposed model whereby LIF and Wnt signaling cooperatively maintain self-renewal (Ogawa et al., 2006).

Next I performed a similar self-renewal assay using the WT, Δ C and M6 β -catenin-rescued ES cells with the GSK3 inhibitor CHIR treatment. Upon LIF withdrawal all 5 ES cell lines were differentiated within 5 days (2nd column in Fig. 17). As expected, most of the WT β -catenin-rescued ES cell colonies were AP-positive after 5 days in culture in the presence of rWnt3a or CHIR (Fig. 17 and data not shown).

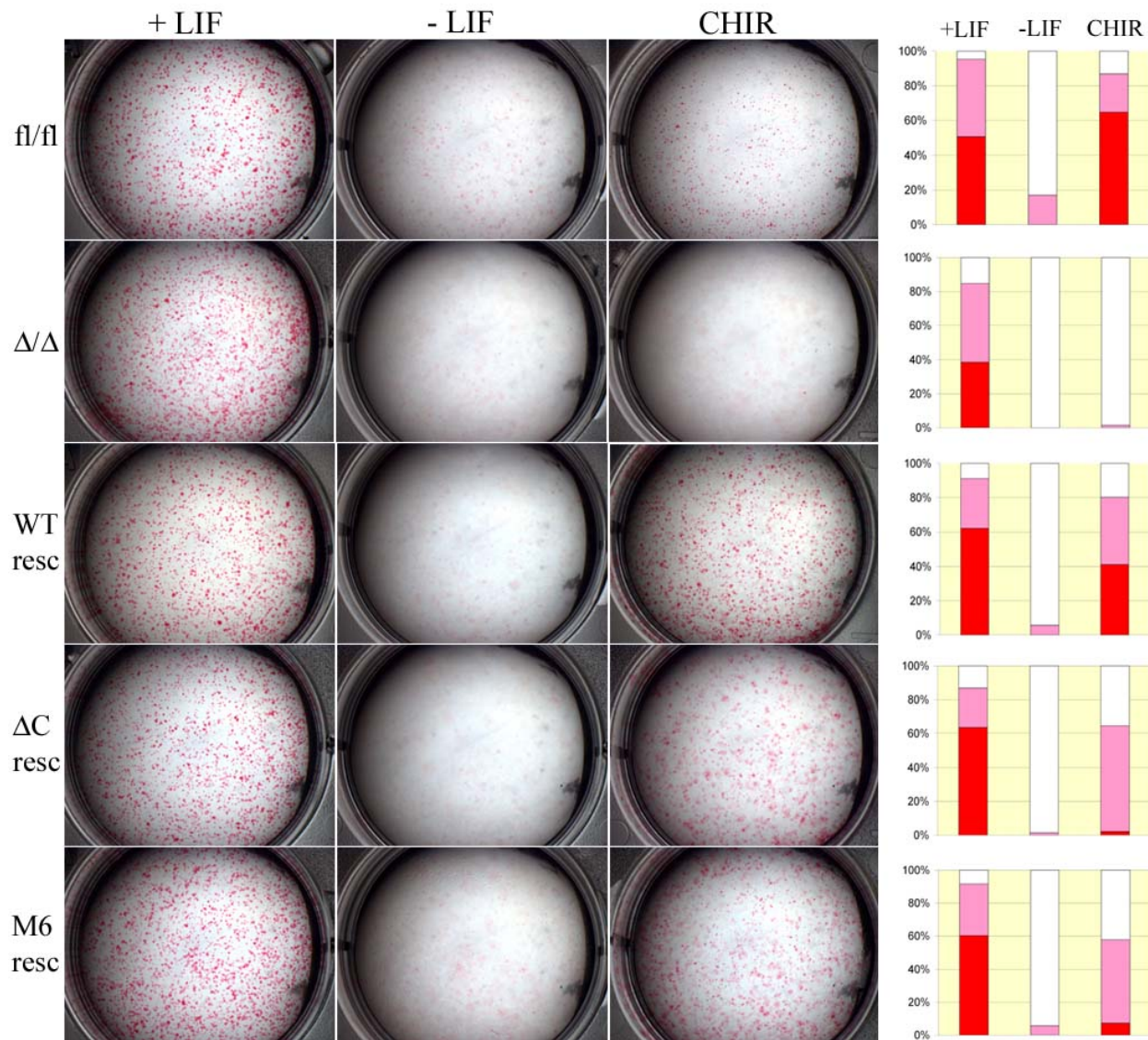


Figure 17 - Self-renewal potentials of pCAGGS rescued β -cat $^{\Delta/\Delta}$ ES cells upon CHIR treatment

AP-stained colonies of β -cat $^{fl/fl}$, β -cat $^{\Delta/\Delta}$, pCAGGS-WT, ΔC and M6 ES cells. ES cells were plated at low density in FCS-containing medium supplemented with LIF (1st column), without LIF (2nd column) or with 3 μ M CHIR (3rd column) in the absence of LIF for 5 days. Percentages of undifferentiated (red), half-differentiated (pink) and differentiated (white) colonies were calculated (right panel) for each of the wells. Total number of colonies in each well was set as 100%.

Not unexpectedly, M6 β -catenin-rescued ES cell behaved similarly (Fig. 17) as they still have some residual Wnt signaling activity according to the TOPFLASH assay (Fig. 14D). However, to our surprise ΔC β -catenin-rescued ES cells also maintained self-renewal under these conditions (Fig. 17). This was quite unexpected since ΔC β -catenin had shown no TCF-mediated transcriptional activity (Fig. 14D). One possible explanation would be that upon stimulation of Wnt signaling ΔC β -catenin might be able to interact with other transcriptional co-factors maintaining self-renewal. This possibility will have to be investigated in the future.

1.3.3. Wnt/ β -catenin target genes in mouse ES cells

My results showed that rWnt3a is able to support self-renewal of mouse ES cells in the presence of serum, but without LIF, to some extent. Therefore, I decided to investigate, which genes are regulated by canonical Wnt signaling in ES cells. In order to do so I performed cDNA microarray analysis comparing the transcriptomes of β -cat^{fl/fl} and β -cat ^{Δ/Δ} ES cells either untreated (PBS) or treated with rWnt3a at the 24 hour time point. RNA from each sample was isolated and hybridized to in-house cDNA microarrays. The experiment was performed in triplicates using two independent β -cat^{fl/fl} and β -cat ^{Δ/Δ} ES cell clones each. Results from independent clones were combined in one table and statistic analysis was performed (by Harald Scheuch). By comparing β -cat^{fl/fl} versus β -cat ^{Δ/Δ} ES cells both treated with rWnt3a, a list of the 300 top up-regulated genes was compiled, which is shown in the Supplementary table 1. Significantly up-regulated genes were filtered using the following thresholds: logarithmic fold change = >0,7, adjusted p-value (adjPvalue) = <0,03. Interestingly, only 5 genes out of 21785 analyzed on the chip were significantly differentially expressed in β -cat ^{Δ/Δ} versus β -cat^{fl/fl} untreated ES cells: 17Rn6, H19, Rhox5 (up-regulated) and Trpv6, Daam1 (down-regulated). This suggests that ablation of β -catenin in undifferentiated ES cells does not influence global gene expression.

In contrast, 2503 genes were up-regulated in β -cat^{fl/fl} ES cells treated with rWnt3a and 2310 down-regulated in comparison to β -cat ^{Δ/Δ} ES cells. The up-regulated genes included established Wnt signaling target genes such as *c-Myc* (He et al., 1998), *Axin2* (Jho et al., 2002; Lustig et al., 2002; Yan et al., 2001) and *Nanog*. With the exception of *Axin2* and *Nanog*, interesting hits would still need to be confirmed by real-time PCR.

1.4. Cell-cell adhesion and self-renewal

1.4.1. Cell-cell adhesion defect in β -catenin-deficient ES cells

As mentioned earlier, in cultures of β -cat ^{Δ/Δ} ES-cells an increased number of single cells could be observed (Fig. 10C). This suggested that there might be a slight cell-cell adhesion defect. To further address this I looked at cell-cell adhesion at the ultrastructural level by transmission electron microscopy (TEM). This revealed that the areas with close attachment between neighboring cells were severely reduced in β -cat ^{Δ/Δ} ES cell colonies, leaving big holes between plasma membranes of two neighboring cells (compare Fig. 18A and B).

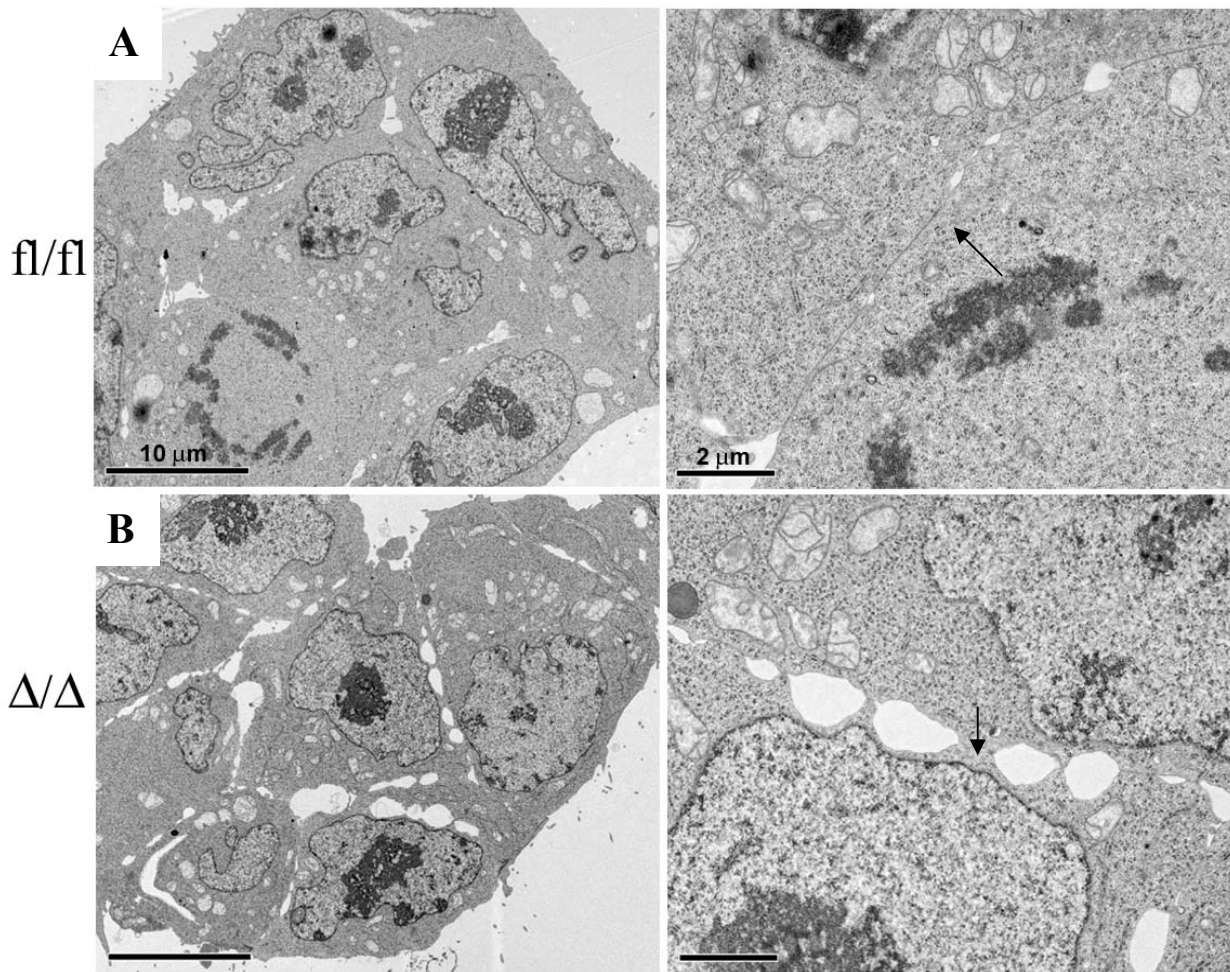


Figure 18 - Cell-cell adhesion defect in β -catenin-deficient ES cells

TEM pictures of ultra-thin sections from $\beta\text{-cat}^{\text{fl/fl}}$ and $\beta\text{-cat}^{\Delta/\Delta}$ ES cells. (A) $\beta\text{-cat}^{\text{fl/fl}}$ ES cell colony consisting of six cells tightly connected via adherens junctions. (B) $\beta\text{-cat}^{\Delta/\Delta}$ ES cell colony consisting of seven cells loosely connected. Images on the right show a higher magnification of plasma membranes (black arrows) of two neighboring ES cells.

Thus, on the ultrastructural level cell-cell adhesion is clearly affected, in contrast to the overall appearance of $\beta\text{-cat}^{\Delta/\Delta}$ ES cell colonies.

However, some cell junctions are still present in $\beta\text{-cat}^{\Delta/\Delta}$ ES cells. This raises the question how this partial cell-cell adhesion is achieved in the absence of β -catenin. As mentioned earlier, plakoglobin levels were upregulated in $\beta\text{-cat}^{\Delta/\Delta}$ ES cells (Fig. 12 C, D). Like β -catenin, plakoglobin can link E-cadherin to actin filament cytoskeletons through α -catenin in adherens junctions (Zhurinsky et al., 2000b). Thus, plakoglobin is the likely candidate to substitute in part for the cell-cell adhesion function of β -catenin in ES cells. To analyze this further, immunoprecipitation (IP) using anti-E-cadherin antibodies was performed to assess complex formation of E-cadherin, plakoglobin, β -catenin and α -catenin (Fig. 19A). In $\beta\text{-cat}^{\text{fl/fl}}$ control

cells, β -catenin, α -catenin and plakoglobin co-precipitated with E-cadherin. In contrast, the amount of plakoglobin associated with E-cadherin was significantly increased in β -cat $^{\Delta/\Delta}$ ES cells, while to our surprise the amount of α -catenin bound to E-cadherin decreased. These data support the notion that plakoglobin substitutes for the loss of β -catenin in adherens junctions.

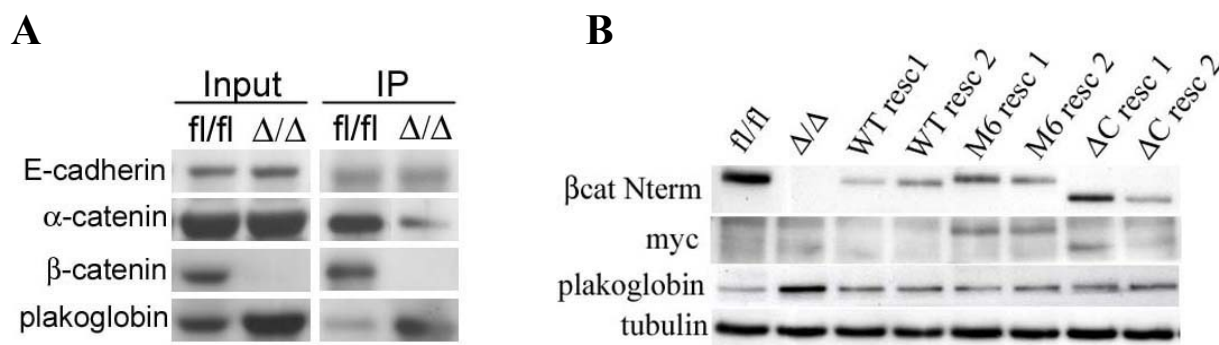


Figure 19 - Plakoglobin substitutes for β -catenin in adherens junctions

(A) IP analysis of adherens junction complex in β -cat $^{fl/fl}$ and β -cat $^{\Delta/\Delta}$ ES cells. Cytosolic fractions from β -cat $^{fl/fl}$ and β -cat $^{\Delta/\Delta}$ ES cells were affinity precipitated with anti-E-cadherin antibody and analyzed by SDS-PAGE and Western blotting with anti- E-cadherin, α -catenin, β -catenin and plakoglobin antibodies. (B) Western blotting analysis of β -catenin, myc-tag and plakoglobin in β -cat $^{fl/fl}$, β -cat $^{\Delta/\Delta}$, WT rescued, Δ C rescued and M6 rescued ES cells. Two independent clones of each rescued ES cell line were analyzed.

To verify that the observed changes in plakoglobin levels in β -cat $^{\Delta/\Delta}$ ES cells were due to an increase of membrane bound plakoglobin levels and dependent on the absence of β -catenin, I examined plakoglobin protein levels in WT, Δ C and M6 β -catenin-rescued ES cells. Reintroduction of WT, Δ C or M6 β -catenin variants into β -cat $^{\Delta/\Delta}$ ES cells resulted in a reduction of plakoglobin levels in all rescued cell lines (Fig. 19B).

In summary, these experiments show that cell-cell adhesion is affected in ES cells lacking β -catenin, however, cells are still connected with each other via adherens junctions, which contain plakoglobin instead of β -catenin.

1.4.2. Loss of adhesion in β -catenin/ plakoglobin-deficient ES cells

What types of cell junctions are present in ES cells? Unfortunately, very little literature exists concerning this question. Based on the observations that β -catenin, plakoglobin and E-cadherin are expressed in cell membranes of ES cells, I can hypothesize that adherens junctions are present in ES cells (Fig. 12A, B, C). Desmosome-like structures were characterized in ES cells by IF and immunoelectron microscopy (Eshkind et al., 2002). However, these structures are not typical desmosomes, because they lack desmoplakin, a component of desmosomal plaque. At

the ultrastructural level I was able to identify a few electron dense structures at cell membranes, similar to what has been described as desmosomes (black arrow heads in Fig. 20). These observations would suggest that ES cells have at least two types of cell-cell junctions: adherens junctions and desmosomes.

Interestingly, unlike β -catenin, plakoglobin plays a role in adherens junctions as well as in desmosomes. In the desmosomal structure, plakoglobin links desmosomal cadherins desmocollin and desmoglein to the intermediate filament cytoskeletons (Franke et al., 1987). However, in mutants lacking plakoglobin, desmosomal localization of β -catenin has been reported (Bierkamp et al., 1999). Thus, I would hypothesize that plakoglobin is present in both adherens junctions and desmosomes in β -cat ^{$\Delta\Delta$} ES cells. If adherens junctions and desmosomes are the only structures mediating cell-cell adhesion in ES cells, ablation of plakoglobin in β -cat ^{$\Delta\Delta$} ES cells should result in a complete loss of cell-cell adhesion.

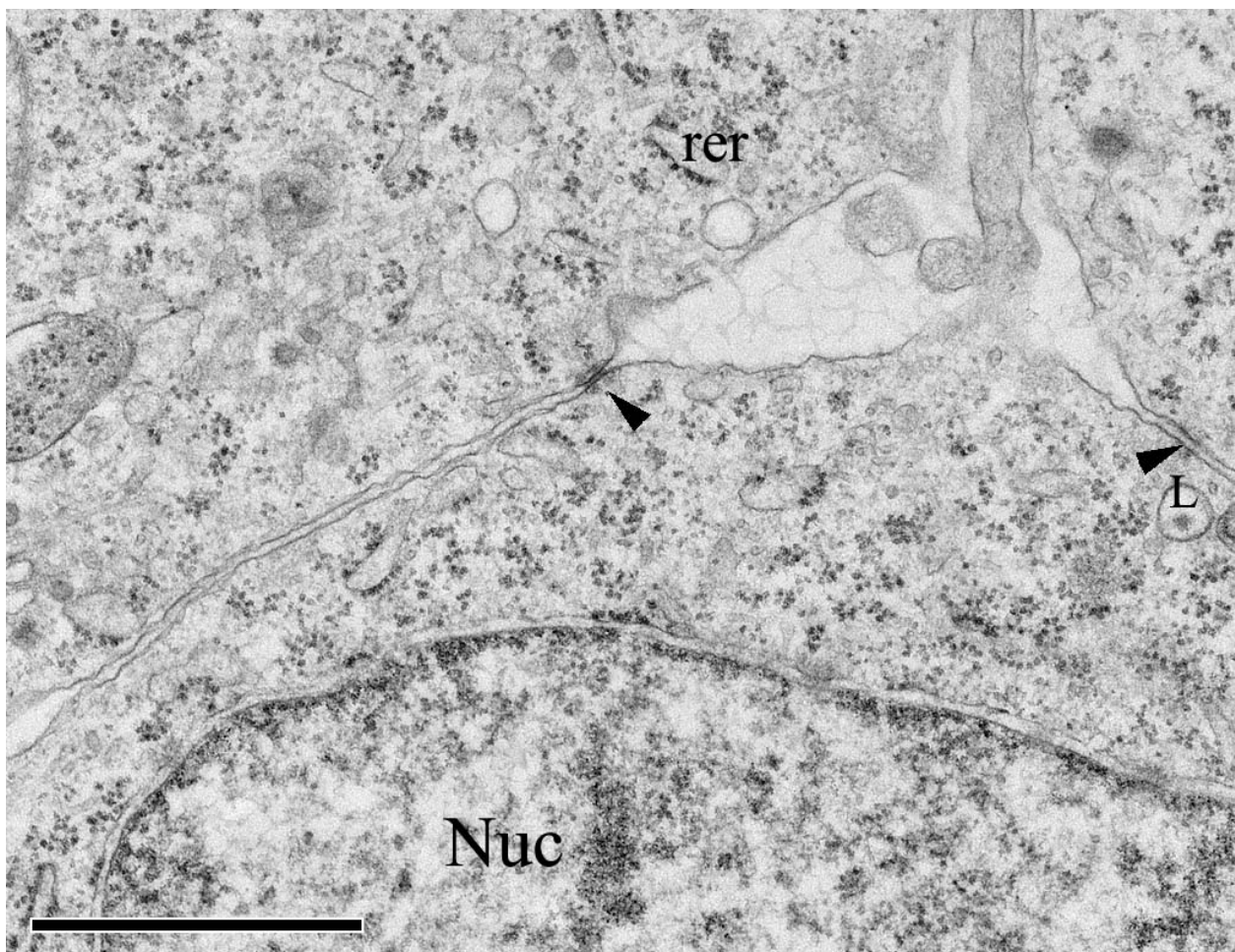


Figure 20 - Cell junctions in undifferentiated WT ES cells

TEM was performed on ultrathin section of WT ES cells. Desmosome-like structures are indicated with black arrow heads. Nuc, nucleus; rer, rough endoplasmatic reticulum; L, lysosome. Scale bar = 100nm.

In order to test this, I performed knock-down experiment using siRNA against *plakoglobin* in β -cat^{fl/fl}, β -cat ^{Δ/Δ} , as well as in WT and Δ C β -catenin rescued ES cells. Knock-down of plakoglobin was confirmed by Western blot analysis (Fig. 21A) and IF staining (Fig. 22).

Already after 48 hours about 80% of the β -cat ^{Δ/Δ} ES cell colonies transfected with *plakoglobin* siRNA displayed a different morphology (Fig. 21B) in comparison to control siRNA treated β -cat ^{Δ/Δ} or β -cat^{fl/fl} ES cells. Instead of the typical dome-shaped compact colonies of ES cells, cells were spindle-shaped and flattened. The percentage of affected colonies correlates with the siRNA-transfection efficiency (data not shown). In contrast, in *plakoglobin* siRNA treated β -cat^{fl/fl} ES cells only 5-10% of the colonies had a different appearance (Fig. 21B). Similarly, knock-down of *plakoglobin* in WT and Δ C β -catenin rescued ES cells did not affect cell morphology, resembling the control situation (Fig. 13B).

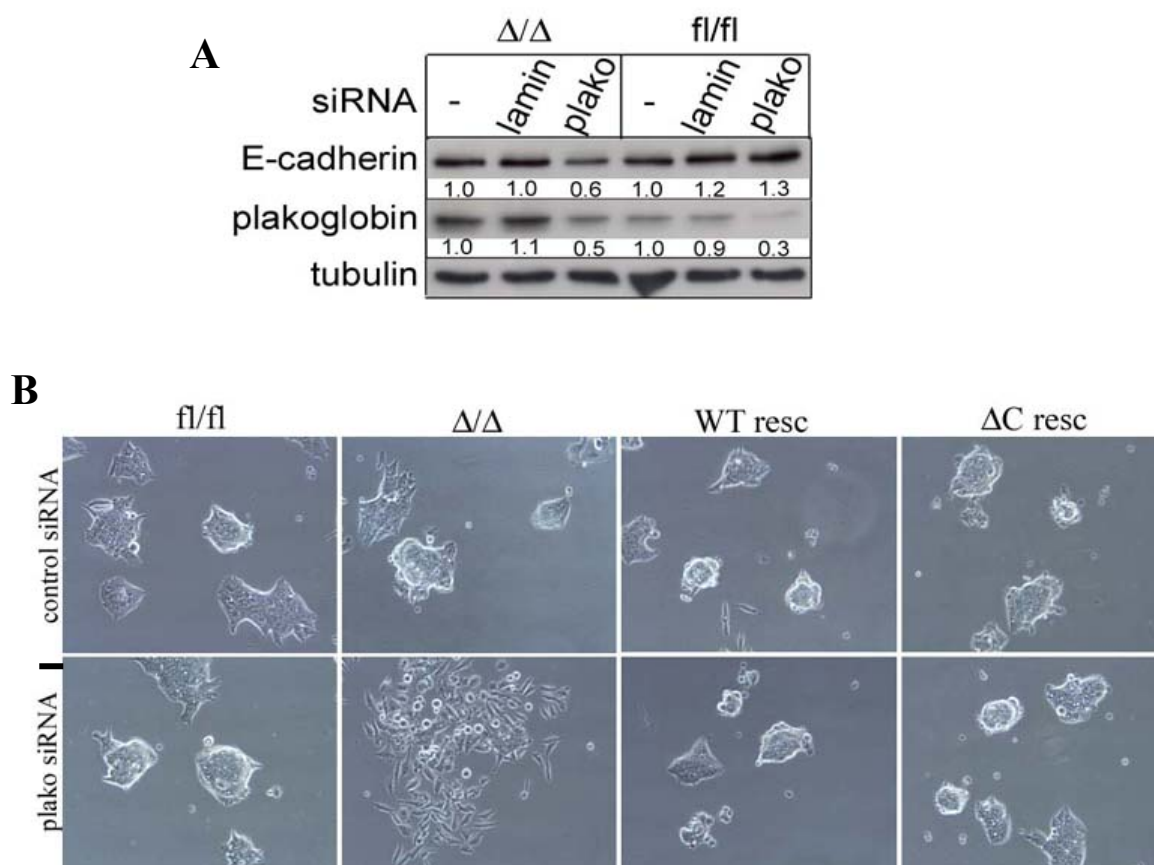


Figure 21 – Knock-down of plakoglobin in β -cat ^{Δ/Δ} ES cells results in loss of cell-cell adhesion

(A) Western blot analysis of E-cadherin and plakoglobin in β -cat ^{Δ/Δ} and control β -cat^{fl/fl} ES cells untransfected or transfected with control siRNA or siRNA against plakoglobin. Cells were collected for analysis after 48 hours post-transfection. (B) Phase contrast live images of β -cat^{fl/fl}, β -cat ^{Δ/Δ} , WT and Δ C rescued ES cells 48 hours after transfection with control or *plakoglobin* siRNA. Scale bar for all images = 50 μ m

E-cadherin has been shown to play an important role during mouse early development, since E-cadherin^{-/-} embryos fail to develop beyond the blastocyst stage (Larue et al, 1994). In addition, loss of E-cadherin has been described as a defining event in epithelial-mesenchymal transition (EMT), which is required for epiblast cell movements during primitive streak formation in early mouse development (Zohn et al., 2006). Notably, E-cadherin protein levels were reduced in *plakoglobin* siRNA treated β -cat ^{$\Delta\Delta$} ES cells compared to the treated control cells (Fig. 21A). By immunofluorescent staining I observed that in *plakoglobin* siRNA treated β -cat ^{$\Delta\Delta$} ES cells, E-cadherin was not located at the cell membrane (data not shown). Thus, this suggests that loss of E-cadherin upon knock-down of plakoglobin in β -catenin-deficient ES cells causes an EMT-like transformation of these cells, which would correspond to my observation that these cells were also motile.

In addition, I analyzed the localization of another cell-cell adhesion molecule, PECAM-1 (also known as CD31), which has been shown to be expressed in ES cells (Li et al., 2005). PECAM-1 can also form a complex with plakoglobin and localizes it to the cell-cell junctions as shown by overexpression experiments in SW480 cells (Ilan et al., 2000). Interestingly, PECAM-1 was absent from the cell membranes in *plakoglobin* siRNA treated β -cat ^{$\Delta\Delta$} ES cells (Fig. 22). However, knock-down of plakoglobin in WT and Δ C β -catenin rescued ES cells did not affect PECAM-1 localization (Fig. 23). These data suggests that β -catenin and plakoglobin are required for PECAM-1 expression and localization to the cell membrane.

Taken together, I was able to show that knock-down of plakoglobin in β -cat ^{$\Delta\Delta$} ES cells results in the complete loss of cell-cell adhesion, that can be rescued by introducing WT as well as the signaling-defective Δ C form of β -catenin. Moreover, loss of both plakoglobin and β -catenin in ES cells interferes with proper expression and localization of cell-cell adhesion molecules.

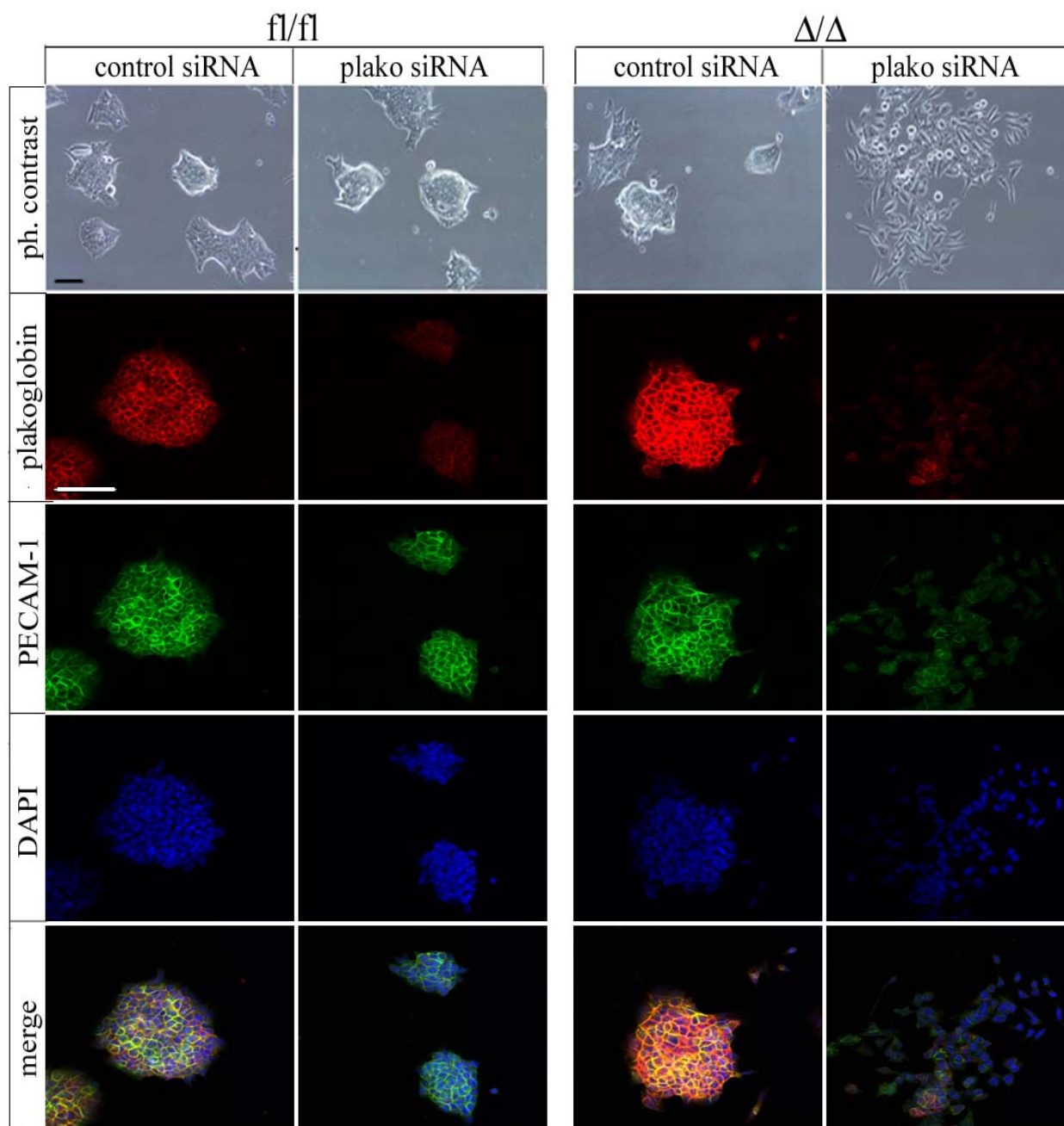


Figure 22 – Knock-down of plakoglobin results in loss of Pecam-1

(A) (Top panel) Phase contrast live images of control β -cat^{f1/f1} and β -cat^{Δ/Δ} ES cells 48 hours after transfection with control or *plakoglobin* siRNA. Scale bar = 50μm. (Lower panels) IF images of β -cat^{f1/f1} and β -cat^{Δ/Δ} ES cells 48 hours after transfection with control or *plakoglobin* siRNA. PFA-fixed colonies were co-stained with anti-plakoglobin (red) and anti-Pecam-1 (green) antibodies. Nuclei were counterstained with DAPI (blue). Scale bar = 50μm.

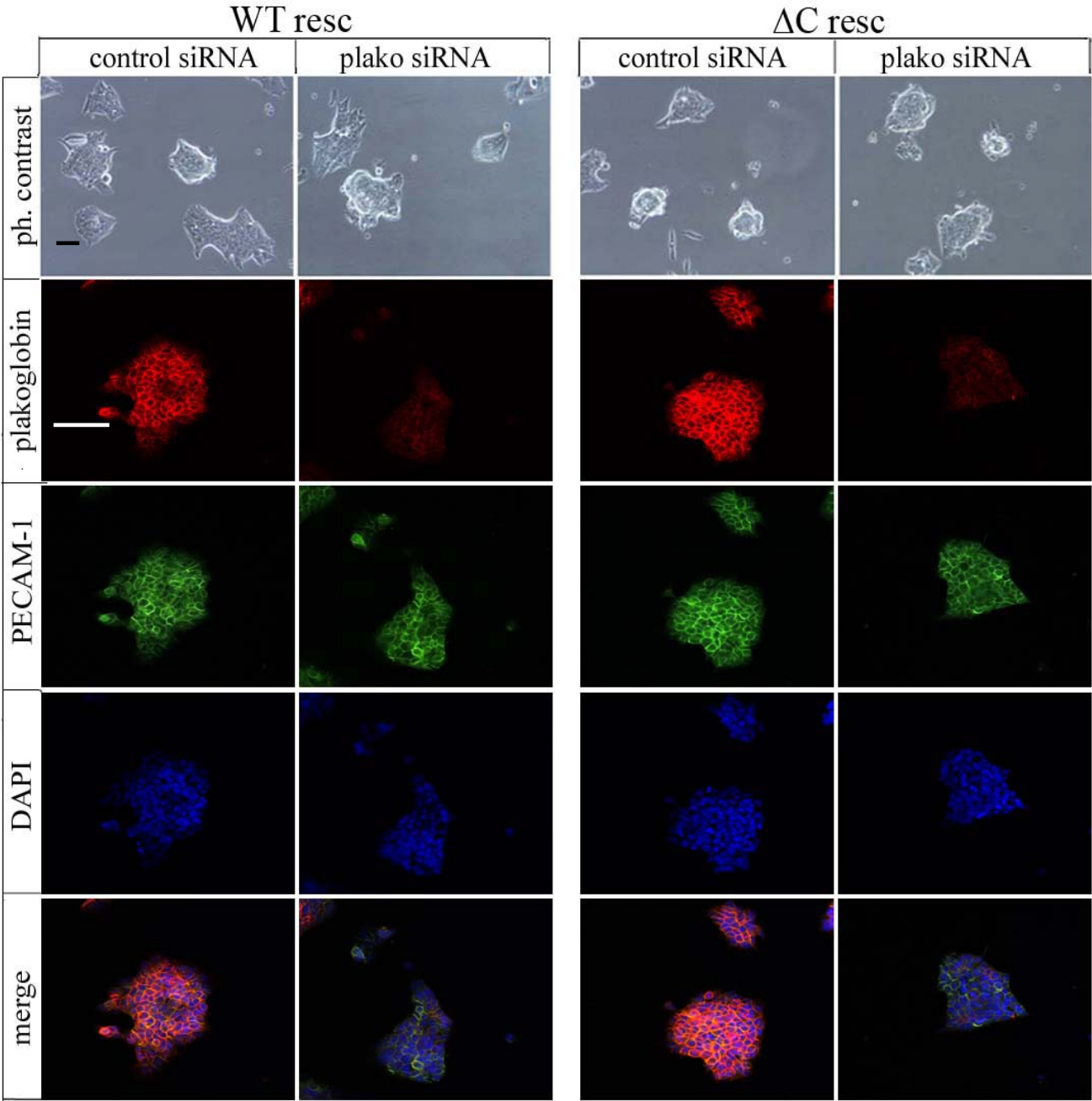


Figure 23 – Knock-down of plakoglobin does not affect Pecam-1 in rescued ES cells

(Top panel) Phase contrast live images of WT and ΔC rescued ES cells 48 hours after transfection with control or *plakoglobin* siRNA. Scale bar = 50μm. (Lower panels) IF images of WT and ΔC rescued ES cells 48 hours after transfection with control or plakoglobin siRNA. PFA-fixed colonies were co-stained with anti-plakoglobin (red) and anti-PECAM-1 (green) antibodies. Nuclei were counterstained with DAPI (blue). Scale bar = 50μm.

1.4.3. Self-renewal is affected in adhesion-defective ES cells

Self-renewal of murine ES cells involves binding of LIF to the cell surface receptor complex LIFR beta/gp130 and activation of STAT3 (Robson et al., 2001). ES cell specific isoforms of PECAM-1 have been implicated in activation of STAT3 and as such may play a role in LIF-mediated maintenance of undifferentiated ES cells (Ilan et al., 2001). Therefore, changes in the expression of PECAM-1 in ES cells may lead to defects in ES cell maintenance (Li et al., 2005).

Since *plakoglobin* siRNA-treated β -cat ^{$\Delta\Delta$} ES cell colonies resembled differentiated ES cells and showed down-regulation of the cell-adhesion molecules, E-cadherin and PECAM-1, I asked next whether down-regulation of plakoglobin in β -cat ^{$\Delta\Delta$} ES cells affects self-renewal marker expression. Real-time PCR analysis revealed a decrease in *nanog* mRNA levels in β -cat ^{$\Delta\Delta$} ES cells 48 hours after transfection with *plakoglobin* siRNA in comparison to cells transfected with control siRNA (Fig. 24A). This result was confirmed by double-fluorescent staining for Nanog and plakoglobin (Fig. 24B). Cells that were negative for plakoglobin expressed only low levels of Nanog in the nucleus. In contrast, knock-down of plakoglobin in WT and Δ C β -catenin rescued ES cells did not affect Nanog expression (Fig. 25). However, I did not see significant changes of protein levels of Nanog and Oct3/4 by Western blot analysis 48 hours after transfection (data not shown). It has to be mentioned, that between 48 and 72 hours ES cell-like colonies started to reappear in β -cat ^{$\Delta\Delta$} ES cells transfected with *plakoglobin* siRNA and no significant knock-down of plakoglobin protein levels could be detected at these time points by western blot analysis (data not shown). This observation can be explained in two ways: 1) siRNA was transfected transiently, therefore it was not working longer than 72 hours; 2) only 80% of the cells were transfected, therefore transfected ES cells were eventually overgrown by non-transfected ones. To avoid these complications I decided to generate β -cat ^{$\Delta\Delta$} ES cell lines that stably expressed *plakoglobin* shRNA. Unfortunately, all attempts to establish stable lines upon infection with lentiviral *plakoglobin* shRNA (from Dharmacon) were not successful. All generated stable lines expressed plakoglobin at endogenous level. My speculation is that downregulation of plakoglobin results in low clonogenicity of β -cat ^{$\Delta\Delta$} ES cells due to loss of cell-cell adhesion. Therefore, I switched to a doxycycline-inducible RNAi system (see Materials & Methods). For this I generated β -cat ^{$\Delta\Delta$} ES cell lines that constitutively expressed the tetracycline reverse transcriptional activator (rtTA) under the control of the Rosa26 promoter.

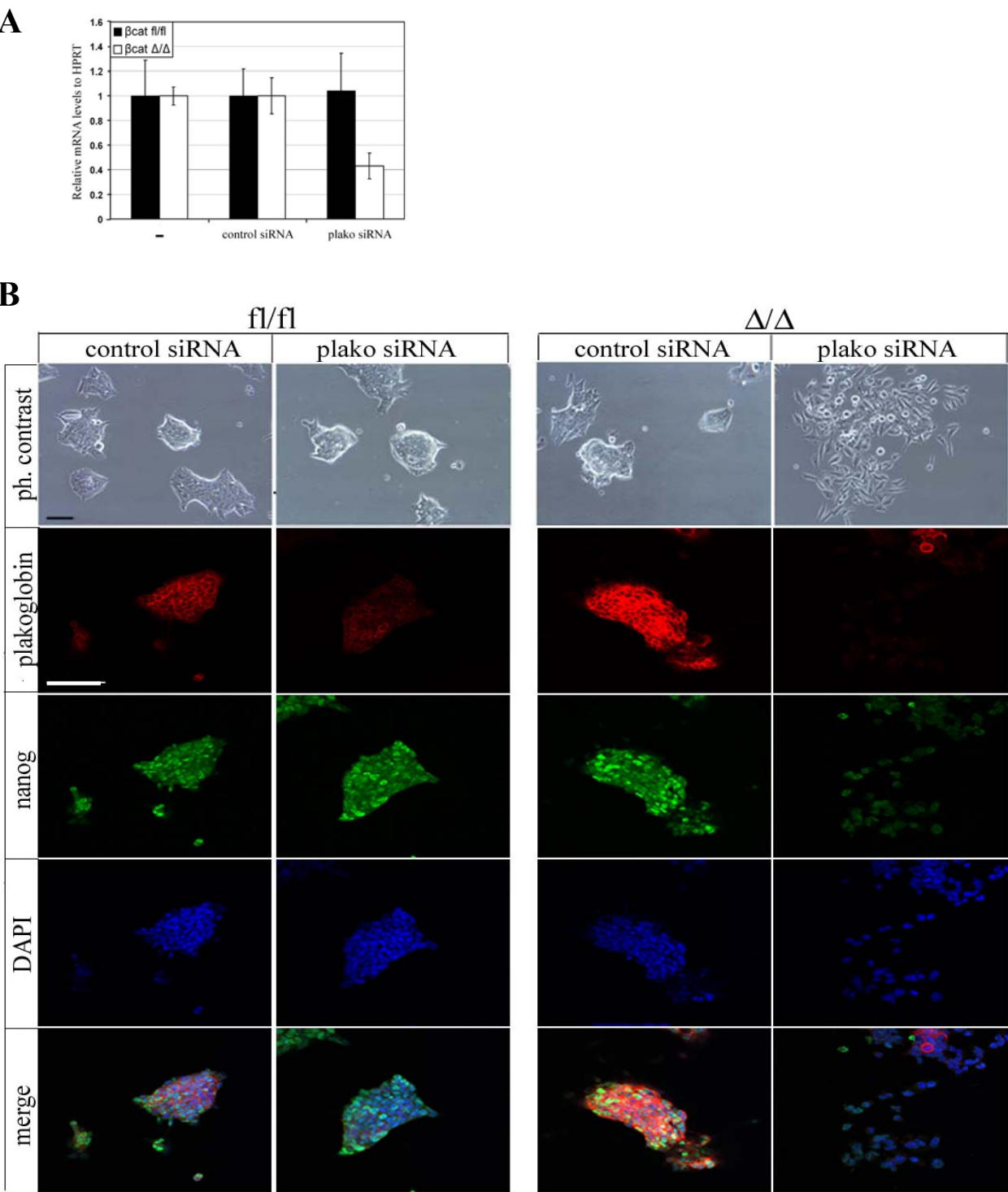


Figure 24 – Knock-down of plakoglobin results in loss of *nanog* expression

(A) Real-time PCR analysis of *nanog* expression in β -cat^{fl/fl} and β -cat^{Δ/Δ} ES cells 48 hours after transfection with control or *plakoglobin* siRNA. Error bars = SEM. (B) (Top panel) Phase contrast live images of β -cat^{fl/fl} and β -cat^{Δ/Δ} ES cells 48 hours after transfection with control or *plakoglobin* siRNA. Scale bar = 50μm. (Lower panels) IF images of β -cat^{fl/fl} and β -cat^{Δ/Δ} ES cells 48 hours after transfection with control or *plakoglobin* siRNA. PFA-fixed colonies were co-stained with anti-plakoglobin (red) and anti-*nanog* (green) antibodies. Nuclei were counterstained with DAPI (blue). Scale bar = 50μm.

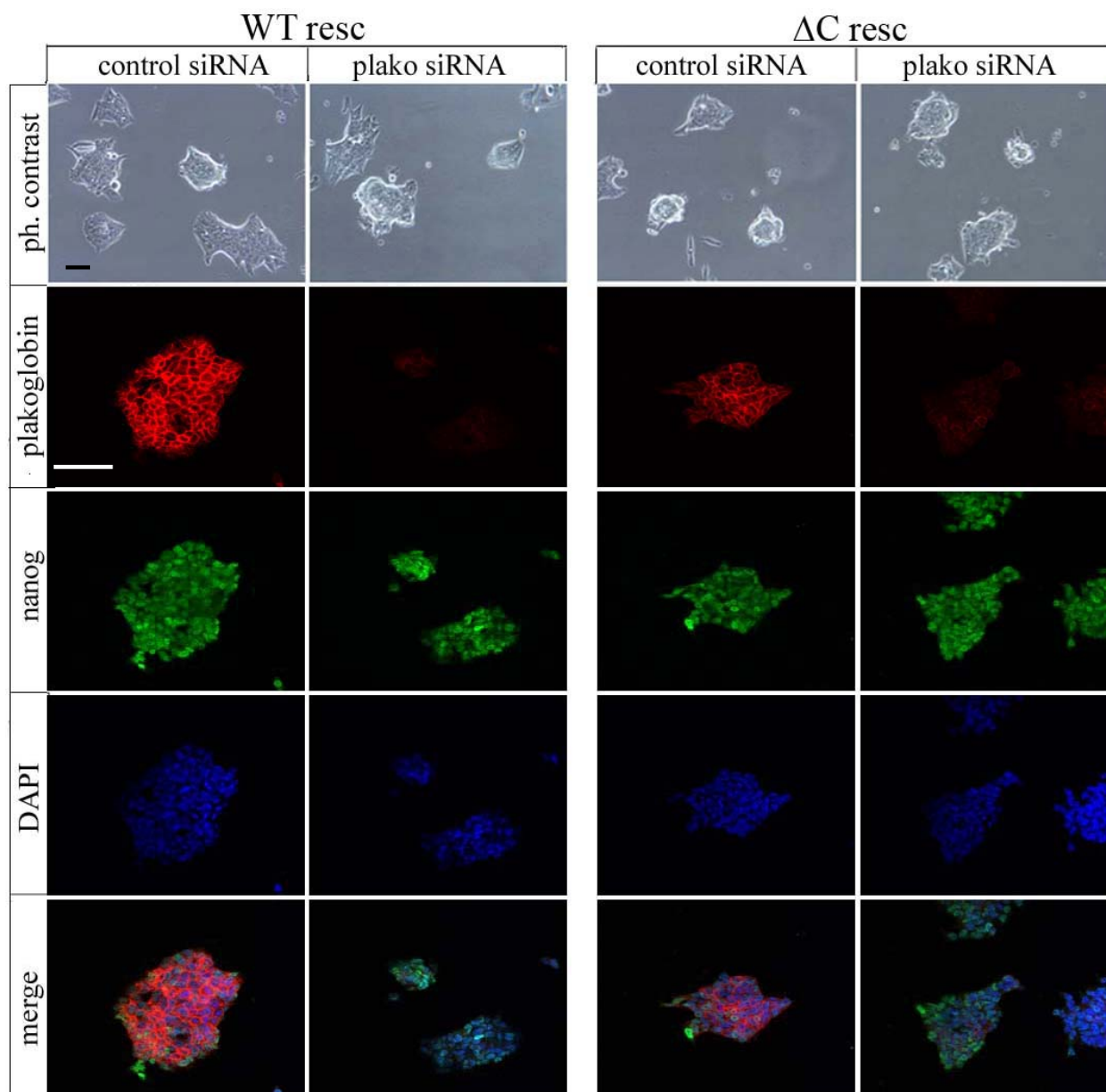


Figure 25 - Knock-down of plakoglobin does not affect Nanog in rescued ES cells

(Top panel) Phase contrast live images of WT and Δ C rescued ES cells 48 hours after transfection with control or *plakoglobin* siRNA. Scale bar = 50 μ m. (Lower panels) IF images of WT and Δ C rescued ES cells 48 hours after transfection with control or *plakoglobin* siRNA. PFA-fixed colonies were co-stained with anti-plakoglobin (red) and anti-nanog (green) antibodies. Nuclei were counterstained with DAPI (Blue). Scale bar = 50 μ m.

In order to do so, I used a construct kindly provided by Martin Leeb (from Anton Wutz lab), which contained the 5' and 3' homology arms of the genomic Rosa26 locus, the rtTA transgene, a neomycin resistance cassette and as a negative selection cassette the diphtheria toxin gene (DTA). This construct was electroporated into β -cat^{Δ/Δ} ES cells and neomycin-resistant ES cell clones were analyzed for correct integration by Southern blot. This yielded in the identification of two positive β -cat^{Δ/Δ}-R26rtTA ES cells clones out of 192.

Next, four shRNAs directed against mouse *plakoglobin* RNA were designed and cloned into pSiren plasmid, containing tetracycline operator elements (TetO) under the control of the U6 promoter (Clontech). Plasmids containing corresponding shRNA were transfected separately into β -cat^{Δ/Δ} rtTA ES cells, selected with puromycin and several ES cell clones for each shRNA were established and named inducible *plakoglobin* RNAi β -cat^{Δ/Δ} ES cells. Upon doxycyclin treatment, rtTA should bind to the TetO element in pSiren and shRNA should be expressed. Unfortunately, I was not able to get efficient knock-down with any of four *plakoglobin* shRNA. Still my data from the transient knock-down experiments suggest that self-renewal is compromised in ES cells that lack both β -catenin and plakoglobin.

2. β -catenin and pluripotency of mouse ES cells

2.1. Cell-cell adhesion defects during *in vitro* differentiation

Ablation of β -catenin in the mouse embryo results in defects in primitive streak formation and failure to develop and properly position mesoderm and embryonic ectoderm (Haegel et al., 1995; Huelsken et al., 2000). It has also been observed that β -catenin-deficient embryos have increased rate of apoptosis (Haegel et al., 1995). However, the molecular mechanism explaining why β -catenin-deficient mice fail to develop beyond the gastrulation stage is still unknown. Our β -cat ^{Δ/Δ} ES cells opened the possibility to study in details the role of Wnt signaling in early mouse development using as an *in vitro* approach embryoid body (EB) formation assay.

Thus, using the suspension culture differentiation method, I generated EBs from β -cat ^{Δ/Δ} and β -cat ^{fl/fl} ES cells. During a differentiation time course from day 0 to day 7, β -cat ^{fl/fl} ES cells formed cyst-like round-shaped EBs, while β -cat ^{Δ/Δ} ES cells failed to form this kind of cyst-like structures and, moreover, from around day 5 of differentiation onwards defects in cell-cell adhesion were observed (Fig. 26A).

As mentioned earlier, β -catenin-deficient embryos have increased rate of apoptosis (Haegel et al., 1995). Therefore, I measured the apoptosis in EBs at day 5 of differentiation by FACS sorting (Fig. 26B). Cells were co-stained with anti-annexinV (AnV) antibodies, detecting apoptotic cells (AnV⁺ PI⁻) and propidium iodide (PI), detecting post-apoptotic dead cells (AnV⁻ PI⁺), whereas the necrotic cell population co-stains with both (AnV⁺ PI⁺). In control β -cat ^{fl/fl} EBs at day 5 of differentiation 6,79% of apoptotic cells and 4,37% necrotic cells were sorted in comparison to 2,53% and 2,36% respectively in undifferentiated ES cells. This was expected, because apoptosis is a natural biological process occurring in early development during cavitation process (Choi et al., 2005; Coucouvanis and Martin, 1995), thus formation of cyst-like EBs. In contrast, 11,38% of apoptotic and 6,96% necrotic cells were detected in β -cat ^{Δ/Δ} 5 day old EBs (Fig. 26B), showing an increase in cell death in comparison to the control EBs. This result is consistent with observations from β -catenin-deficient embryos.

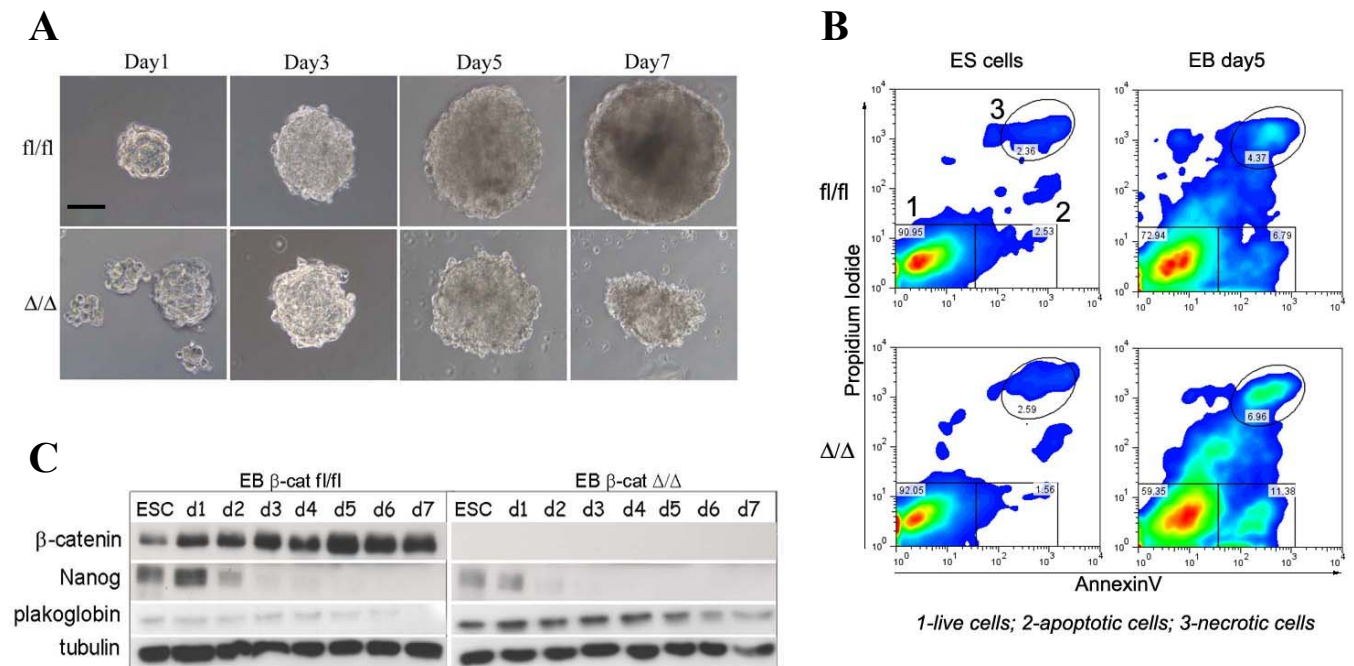


Figure 26 – Cell-cell adhesion defect during EB differentiation of β -cat $^{\Delta/\Delta}$ ES cells

(A) Morphology of EBs formed from β -cat $^{fl/fl}$ and β -cat $^{\Delta/\Delta}$ ES cells during differentiation time-course. Scale bar = 200 μ m. (B) FACS sorting analysis of β -cat $^{fl/fl}$ and β -cat $^{\Delta/\Delta}$ ES cells and EBs at day 5 of differentiation stained for annexinV (X axis) and propidium iodide (Y axis). Numbers indicate the percent cells in a specific gated cell population (C) Western blotting of β -cat $^{fl/fl}$ and β -cat $^{\Delta/\Delta}$ EBs during time course of differentiation from day 1 to 7. Total proteins were separated by SDS-PAGE and membranes stained for β -catenin, Nanog, plakoglobin and tubulin (loading control). ESC=embryonic stem cells.

I then asked whether plakoglobin protein levels were altered during EB differentiation, which could explain the adhesion defect observed in β -cat $^{\Delta/\Delta}$ EBs. Interestingly, throughout the time course of differentiation I noticed in β -cat $^{fl/fl}$ EBs that β -catenin protein levels gradually increased, while plakoglobin levels were constant until day 5 and then gradually decreased in the control cells (Fig. 26C). A similar downregulation of plakoglobin from day 5 of differentiation onwards was seen in β -cat $^{\Delta/\Delta}$ EBs (Fig. 26C).

These data suggest that plakoglobin cannot support cell-cell adhesion in the absence of β -catenin longer than until day 5 of differentiation and as a consequence cells start to dissociate and die.

2.2. Pluripotency of β -catenin-deficient ES cells is impaired

As mentioned earlier, β -cat $^{fl/fl}$ EBs developed normally into cyst-like structures, while β -cat $^{\Delta/\Delta}$ EBs never formed these transparent cysts under identical conditions. When β -cat $^{fl/fl}$ EBs were

plated at day 7 of differentiation onto gelatin-coated dishes, more than 70% of the EBs contained regions of spontaneously contracting cardiomyocytes by day 9 (data not shown). In contrast, β -cat^{ΔΔ} EBs only formed loose clumps of cells without any characteristic morphology, with the exception of the formation of an epithelial-like cell monolayer, which surrounded the cell clump (data not shown). Therefore, next I asked the question whether the ability to differentiate into three germ layers was impaired in ES cells lacking β -catenin.

In order to find out to which germ layers β -cat^{ΔΔ} EBs can differentiate I performed semi-quantitative RT-PCR analysis of β -cat^{fl/fl} and β -cat^{ΔΔ} EBs at different time points during differentiation (Fig. 27). Prior to the onset of differentiation, β -cat^{fl/fl} and β -cat^{ΔΔ} ES cells expressed *nanog* and *oct3/4*, markers of undifferentiated ES cells. Throughout the time-course in both cell lines *nanog* and *oct3/4* were gradually down-regulated indicating ES cell differentiation. In the absence of β -catenin ectoderm specific genes *fgf5* and *nestin* became up-regulated at day 2 of differentiation and their expression persisted up to day 8 of EB differentiation, very similar to control β -cat^{fl/fl} EBs. In contrast, the mesendoderm-specific gene *mixl1* was not expressed in the β -cat^{ΔΔ} EBs in comparison to control EBs, where it peaked on day 4 of differentiation. Expression of *T* (Brachyury) was initiated in both β -cat^{fl/fl} and β -cat^{ΔΔ} EBs, but it did not peak at day 2 of differentiation in the absence of β -catenin. The endoderm (*cer1* and *foxa2*) and mesoderm (*hand1* and *eomes*) specific markers, also did not peak between day 2 and 4 of β -cat^{ΔΔ} EBs differentiation, when their expression normally peaked in control EBs.

This result suggests that mesoderm and endoderm formation in ES cells lacking β -catenin is impaired; however, ectoderm formation is probably not affected. This conclusion is also supported by earlier observation that attached β -cat^{ΔΔ} EBs form epithelial-like cell layer around. These results are consistent with observations from β -catenin mutant mice (Haegel et al., 1995; Huelsken et al., 2000), which possess severe defects in anterior-posterior axis formation and stop to develop at the egg cylinder stage. Subsequently, mesoderm does not develop, visceral endoderm is displaced, but extraembryonic ectoderm and embryonic ectoderm are formed correctly.

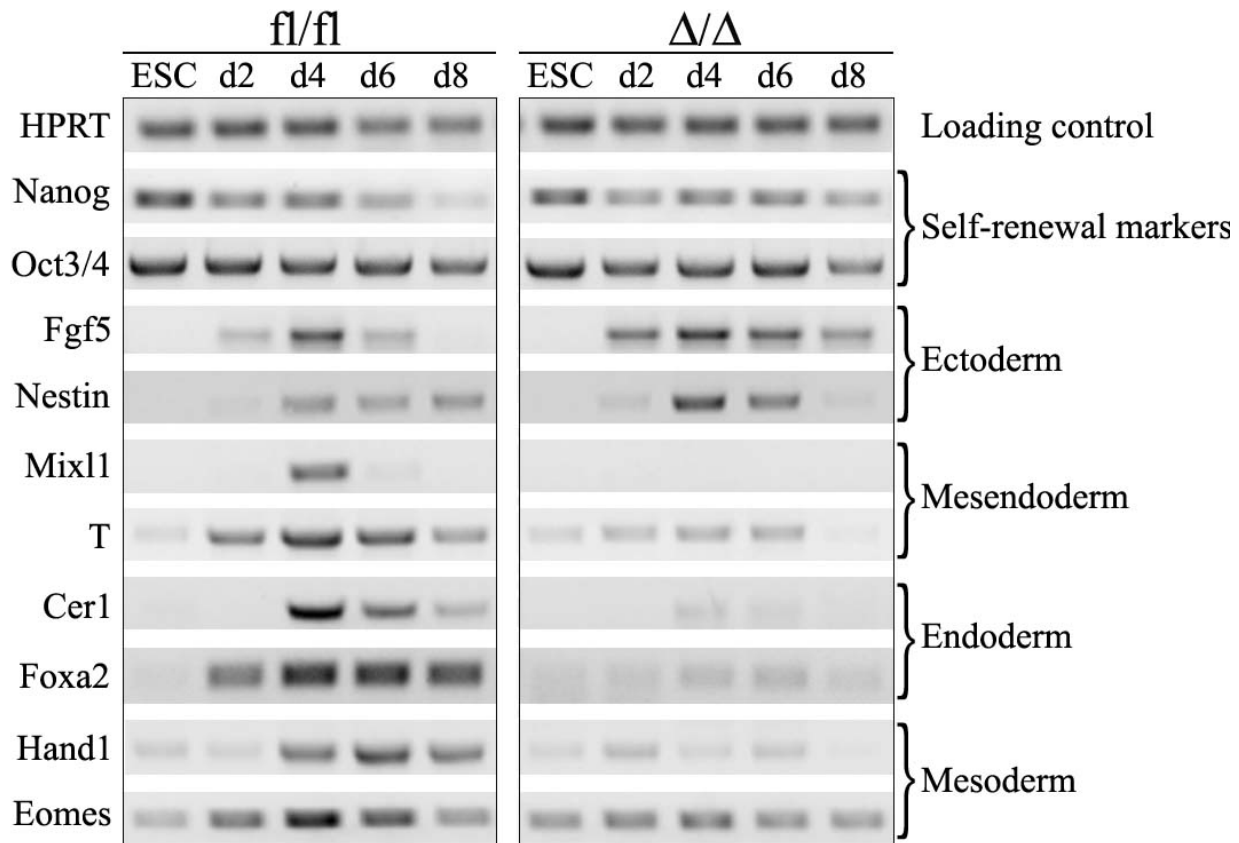


Figure 27 – Mesoderm end endoderm formation is affected during differentiation of β -cat $^{\Delta/\Delta}$ ES cells

β -cat $^{\text{fl/fl}}$ and β -cat $^{\Delta/\Delta}$ ES cells were subjected to EB differentiation time course from day 0 (ESC) to day 8. Mesoderm (*hand1* and *eomes*), endoderm (*cer1* and *foxa2*), mesendoderm (*mixl1* and *T*) and ectoderm (*fgf5* and *nestin*) lineage specific markers and self-renewal (*nanog* and *oct3/4*) markers were analyzed by semi-quantitative RT-PCR. Samples were normalized to HPRT expression.

When injected into immunodeficient mice, ES cells can form tumors, so-called teratomas (Wobus et al., 1984). When these tumors develop from pluripotent ES cells they are show terminally differentiated tissues structures of ecto-, meso-, and endodermal origin.

Thus in order to assess the *in vivo* potential of my ES cells, suspensions of two independent clones of β -cat $^{\Delta/\Delta}$ and two clones of β -cat $^{\text{fl/fl}}$ ES cells each were mixed with Matrigel (as a supportive matrix) and injected subcutaneously into the abdominal flank of MF1 nude mice. Interestingly, three weeks after injection only small tumors with a volume of about 0,5-1,0 cm³ were formed from β -cat $^{\Delta/\Delta}$ ES cells, while very big tumors with a volume of about 2,0-3,0 cm³ developed from β -cat $^{\text{fl/fl}}$ ES cells (Fig. 28A). The teratoma growth curve and the overall appearance of the tumors can be seen in Fig. 28 (B, C). Staining with hematoxylin and eosin revealed several distinct histological features in β -cat $^{\text{fl/fl}}$ teratomas, including glandular epithelial structures indicative of endoderm (Fig. 29A), large regions of neuronal tissues indicative of ectoderm (Fig. 29B), cartilaginous elements and muscles indicatives of mesoderm

(Fig. 29C). In contrast, teratomas derived from β -cat $^{\Delta/\Delta}$ ES cells were much smaller in size and appeared histologically primarily homogeneous with cells embedded in Matrigel (Fig. 29D). The only differentiated structures, which were occasionally observed, were epithelial-like rosettes (Fig. 29E). The other interesting feature of β -cat $^{\Delta/\Delta}$ teratomas was that they were hypervascularized with large areas filled with blood (Figs. 29F).

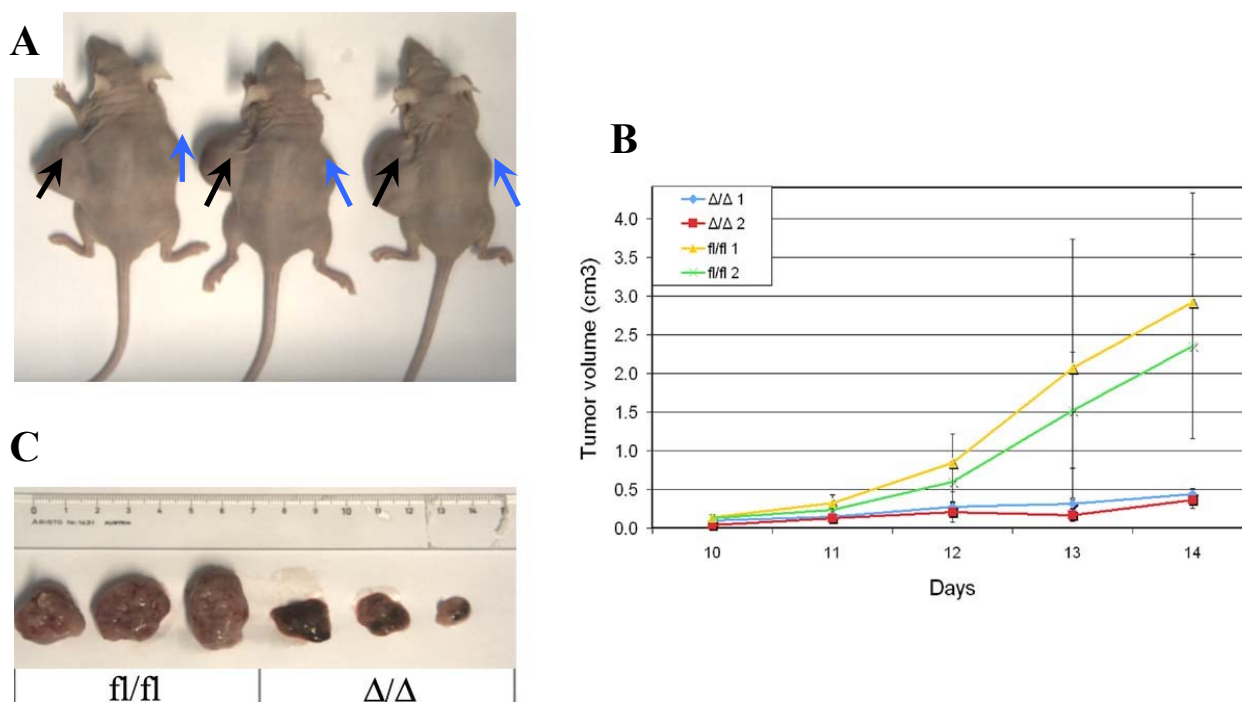


Figure 28 – Tumor growth from ES cells is impaired in the absence of β -catenin

(A) Overall appearance of teratomas in MF1 nude mice three weeks after subcutaneous injection with β -cat $^{fl/fl}$ ES cells (black arrow) and β -cat $^{\Delta/\Delta}$ ES cells (blue arrow). (B) Growth curve of teratomas from day 10 to 14 post-injection of two independent clones of β -cat $^{fl/fl}$ and β -cat $^{\Delta/\Delta}$ ES cells. Teratomas were measured in three dimensions (length, width and height) and total volume (cm³) was calculated. Error bars = SEM. (C) Morphology of teratomas from three independent injections. Note that the β -cat $^{\Delta/\Delta}$ teratomas are highly vascularized (dark red areas).

Whether these hypervascularization originated from the injected ES cells or the host mouse tissues is currently unclear. Immunostaining for β -catenin showed the expected localization in β -cat $^{fl/fl}$ teratomas, predominantly in epithelial structures (Fig. 29G). As expected, no β -catenin staining was observed in β -cat $^{\Delta/\Delta}$ teratomas (Fig. 29H).

To study the tumors in more detail, I performed a marker analysis for the different germ layers. Paraffin sections of teratomas derived from β -cat $^{fl/fl}$ and β -cat $^{\Delta/\Delta}$ ES cells were stained with antibodies specific for the endodermal marker – alpha-fetoprotein (AFP) (Fig. 29J, N), the ectodermal marker – glial fibrillary acidic protein (GFAP) (Fig. 29H, L) and the mesodermal

marker – smooth muscle actin (SMA) (Fig. 29 M). As anticipated given my EB differentiation results, SMA-positive cells (Fig. 29M) were absent in β -cat ^{$\Delta\Delta$} teratomas, while GFAP-positive astrocytes were detected in β -cat ^{$\Delta\Delta$} teratomas. However, in much lower numbers in comparison to the WT teratoma (Fig. 29L). In contrast, to what we expected based on the analysis of early endodermal markers, such as *Foxa2*, *Cer1* (Fig. 27), *Gata4* and *Gata6* (data not shown), which suggest that endoderm formation is not initiated in the absence of β -catenin, we found AFP-positive structures (Fig. 29N), suggesting that visceral endoderm formation can occur even in the absence of β -catenin. An alternative explanation might be that the AFP antibody used in the teratoma analysis is not absolutely specific.

Taken together, these data indicates that β -cat ^{$\Delta\Delta$} ES cells can efficiently recapitulate the developmental defects observed in β -catenin-deficient embryos and can be used to study the role of β -catenin in early mouse development. Thus, I have shown that β -cat ^{$\Delta\Delta$} ES cells fail to differentiate into endoderm (according to the EB results) and mesoderm, but not into ectoderm. Moreover, β -cat ^{$\Delta\Delta$} ES cells fail to form teratomas *in vivo*.

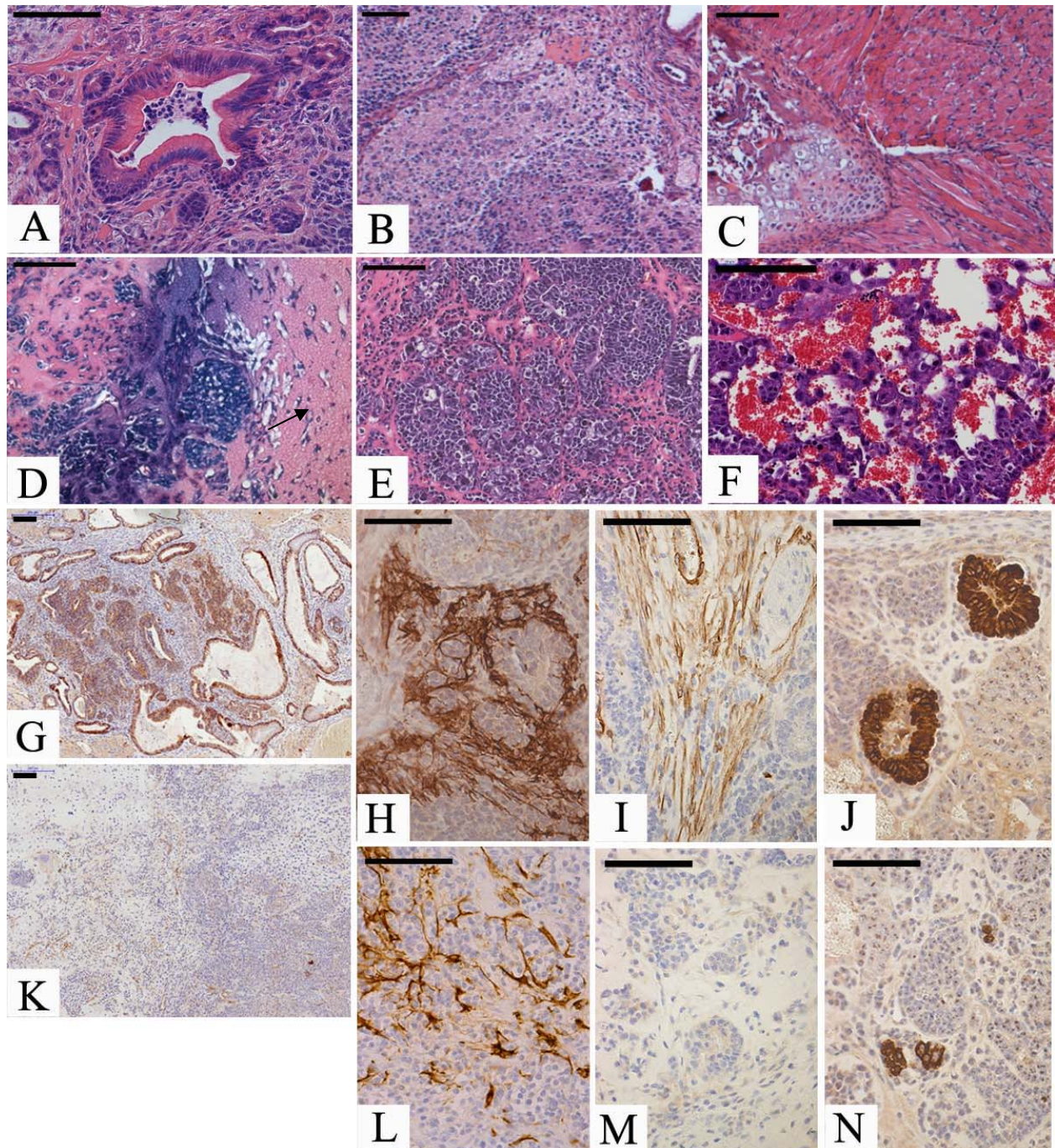


Figure 29 – Analysis of teratomas generated from $\beta\text{-cat}^{\text{fl/fl}}$ and $\beta\text{-cat}^{\Delta/\Delta}$ ES cells

Teratomas generated from $\beta\text{-cat}^{\text{fl/fl}}$ ES cells are heterogeneous (A-C). Histological sections of teratomas stained with hematoxylin and eosin possess many regions of glandular epithelium (A), neuronal tissue (B), muscle and bone (C). Teratomas, generated from $\beta\text{-cat}^{\Delta/\Delta}$ ES cells are homogeneous (D), with supportive Matrigel still present between cells (arrow). However, some epithelial-like structures can be recognized (E) and large regions filled with blood (F). Immunohistochemical staining of paraffin sections from $\beta\text{-cat}^{\text{fl/fl}}$ (G-J) and $\beta\text{-cat}^{\Delta/\Delta}$ (K-N) ES cells with β -catenin (G, K), GFAP (H, L), SMA (I, M) and AFP (J, N). Scale bar = 100 μm .

2.3. CAG β -catenin transgenes rescue cell-cell adhesion, but not pluripotency

In order to see whether the cell-adhesion defects observed in differentiating β -cat ^{Δ/Δ} ES cells can be rescued by either the WT, signaling-defective Δ C or M6 β -catenin transgenes I analyzed the corresponding stable pCAGGS ES cells clones (shown in Fig. 14). As demonstrated earlier these β -catenin variants localize to the cell membrane, indicating they might be able to restore cell-cell adhesion in β -cat ^{Δ/Δ} ES cells (Fig. 14B). In addition, I had shown earlier that Wnt signaling is not restored by the Δ C form, while it is restored by the WT and to certain extend also by the M6 form of β -catenin (Fig. 16D). These tools enabled me to address a very important question in developmental biology that has not yet been tackled: how to distinguish between cell-cell adhesion and signaling function of β -catenin in early mouse development?

In order to address this question I employed initially the *in vitro* differentiation assay of EB suspension culture. As was described before β -cat ^{Δ/Δ} ES cells fail to form cyst-like EBs and cell-cell adhesion defect becomes obvious after day 5 of differentiation, probably due to the observed down-regulation of plakoglobin. First I analyzed, if the pCAGGS rescued lines maintained cell-cell adhesion. For this EB differentiation was performed using standard conditions in the presence of G418 to maintain selection pressure for the pCAGGS expression vector. My initial morphological analysis revealed that at day 5 EBs from WT, Δ C or M6 β -catenin rescued ES cell lines had a round, smooth shape and were indistinguishable from β -cat ^{fl/fl} EBs (Fig. 30A). However, after approximately day 7 of differentiation the EBs from all three different rescued ES cell lines started to disaggregate and many floating cells were observed in the medium (Fig. 30A, day8).

This observation suggested that the CAG promoter might not be able to sustain expression of WT, Δ C and M6 β -catenin variants during EB differentiation. This suspicion was confirmed when I stained EBs for β -catenin at day 8 of differentiation (Fig. 30A, last column). β -catenin was hardly detected in the WT, Δ C and M6 β -catenin rescued EBs. Moreover, when I analyzed β -catenin protein levels by western blot, in contrast to control β -cat ^{fl/fl} EBs where β -catenin levels increased gradually during differentiation, in the three different rescued lines β -catenin levels gradually decreased (Fig. 30B).

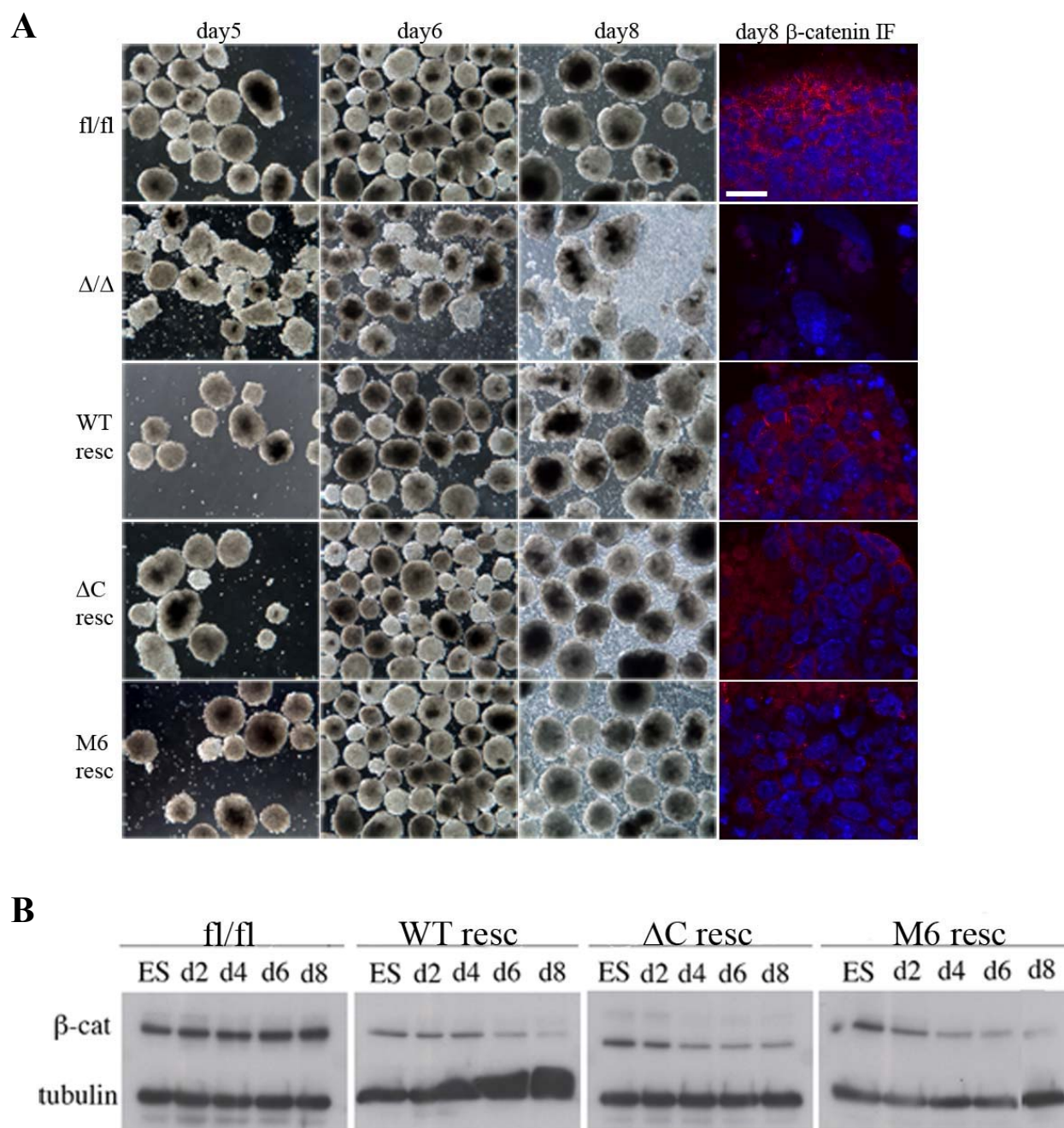


Figure 30 – EB differentiation of β -cat ^{Δ/Δ} ES cells rescued with CAG-driven WT, ΔC and M6 β -catenin variants

(A) Morphology of EBs formed from control and WT, ΔC and M6 β -catenin rescued ES cells during time course of EB differentiation (from day 5 to day 8). Pictures were taken using phase contrast filter with 5x magnification objective. IF staining of EBs at day 8 for β -catenin is shown in the last column. Scale bar = 20 μ m (B) Western blot analysis of total proteins extracted from control β -cat^{fl/fl} ES cells as well as WT, ΔC and M6 β -catenin rescued ES cells subjected to EB differentiation (from day 2 to day 8). Membranes were stained with antibodies recognizing N-terminus of β -catenin. Tubulin is a loading control (Note that tubulin bands in the WT rescued blot expanded due to transfer problem).

So contrary to other published data indicating that CAG promoter is able to maintain long-term expression of transgenes during ES cells differentiation (Alexopoulou et al., 2008), I found that it was not the case with β -catenin.

EBs derived from pluripotent ES cells display clear signs characteristic for self-organization: they are able to establish an anterior-posterior polarity, develop a primitive streak-like domain and undergo EMT followed by mesendoderm formation (ten Berge et al., 2008). Therefore, EBs can recapitulate aspects of normal embryonic development to some extent. Despite the fact that it was not possible to rescue the cell-cell adhesion beyond day 7 of EB differentiation using the WT, ΔC or M6 β -catenin pCAGGS constructs, I analyzed the material to see whether differentiation into mesoderm and endoderm had occurred by performing semi-quantitative RT-PCR for early differentiation markers. Unfortunately, expression of endodermal and mesodermal lineage markers was not rescued during EB differentiation of WT, ΔC or M6 β -catenin ES cells (data not shown). The expression pattern of the analyzed genes was similar to that of β -cat ^{$\Delta\Delta$} EBs. Failure to rescue the pluripotency was probably due to the inability of CAG promoter to maintain β -catenin transgene expression at high levels during differentiation.

2.4. Generation and analysis of R26 WT, ΔC and M6 ES cells

To overcome the problem of unstable transgene expression I cloned WT as well as myc-tagged ΔC or M6 β -catenin variants into Rosa26 targeting constructs (Fig. 31A). The Rosa26 locus has previously been successfully used to drive stable expression of transgenes in ES cells as well as in all tissues of the developing and adult organism (Hohenstein et al., 2008; Nyabi et al., 2009). Targeting constructs were electroporated in β -cat ^{$\Delta\Delta$} ES cells. Homologous recombination was screened by mini-Southern blot and two independent clones from each targeting vector (WT, ΔC or M6 β -catenin) were verified (Fig. 31B) by Southern blot and further referred to as R26WT, R26 ΔC and R26M6 ES cells. Homologously recombined ES cell clones expressed β -catenin at the cell membrane, similar to the control β -cat ^{Δ/Δ} ES cells (Fig. 31C). In later experiments I used only R26 WT and R26 ΔC , but not R26M6 ES cells, because their establishment was postponed due to the fact that the M6 β -catenin variant had shown a minimal response in the TOPFLASH reporter assay, thus being not completely signaling-defective (Fig. 14D).

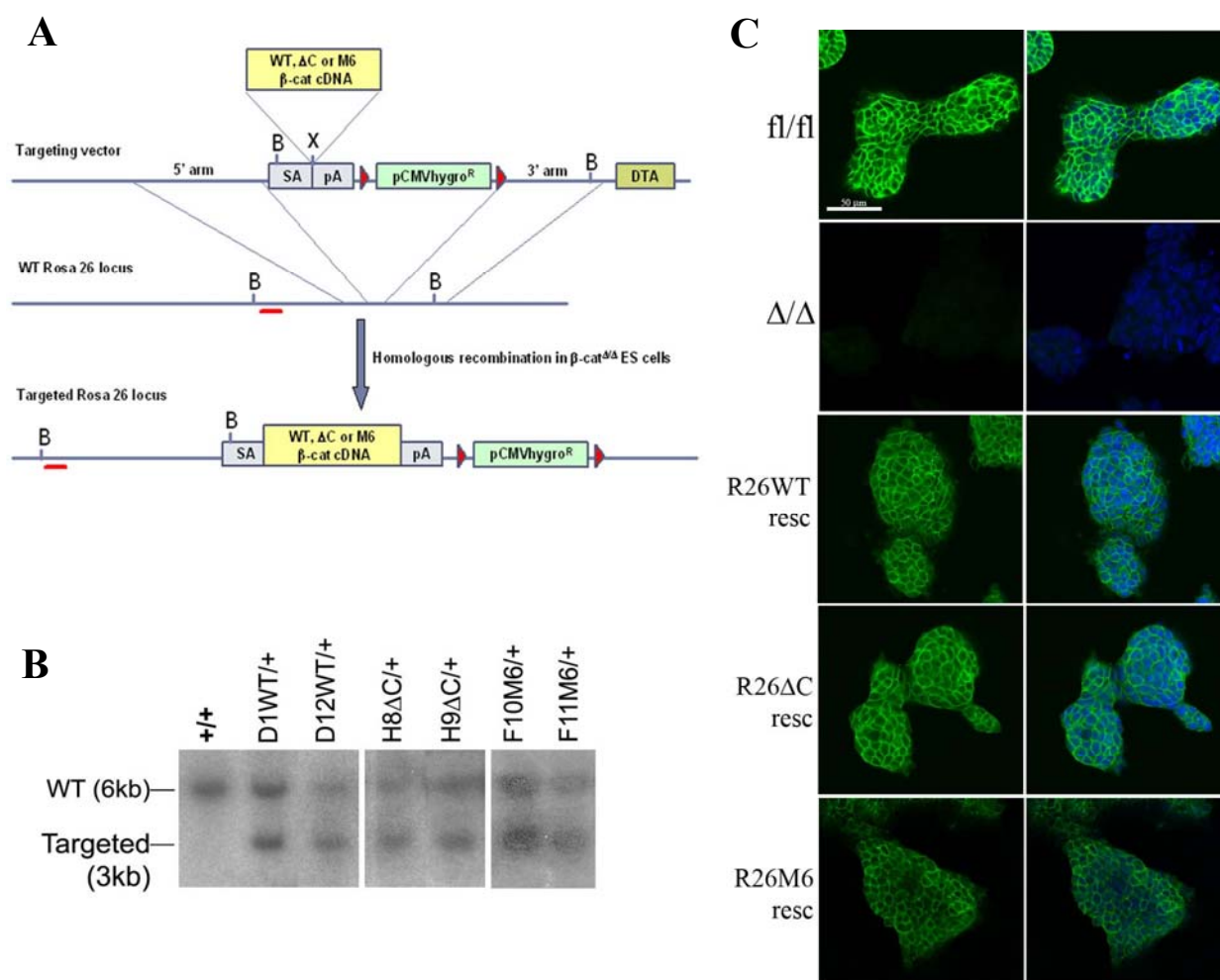


Figure 31 - Generation of R26 WT, ΔC and M6 ES cells

(A) The WT, DC or M6 variants of β-catenin were inserted by blunt-end cloning into the XbaI site of the targeting vector. The targeting constructs were inserted into Rosa26 locus by homologous recombination after electroporation of linearized vectors into β-cat^{Δ/Δ} ES cells. B - BamHI, X - XbaI, DTA - diphtheria toxin A, SA – splice acceptor, pA – polyadenylation site, red bar indicates 5' external probe for Southern blot detection of correct recombination event and red triangles indicate LoxP sites. (B) Correctly targeted ES cell clones (D1WT, D12WT, H8ΔC, H9ΔC, F10M6 and F11M6) were verified by Southern blot. Genomic DNA was digested with BamHI, yielding a 6kb WT band and a 3kb targeted band. (C) IF images of β-cat^{fl/fl} and β-cat^{Δ/Δ} ES cells as well as Rosa26 WT, ΔC or M6 ES cell clones stained with anti-β-catenin antibodies (green), an anti-C-terminal β-catenin antibody was used with the exception of ΔC rescued cells, which were stained with an anti-myc antibody, DAPI nuclear staining is blue. Scale bar = 50μm.

2.4.1. Rescue of cell-cell adhesion and apoptosis

Next I examined the differentiation potentials of R26 WT and R26 Δ C ES cells. As demonstrated earlier β -cat ^{Δ/Δ} ES cells fail to form cyst-like EBs and cell-cell adhesion defect becomes obvious after day 5 of differentiation, probably due to the down-regulation of plakoglobin. First I analyzed, if the expression of transgenes was maintained in R26WT and R26 Δ C ES cell lines during differentiation. EB formation in suspension culture upon withdrawal of LIF was performed as described earlier. In contrast to pCAGGS rescued β -cat ^{Δ/Δ} ES cells, R26WT and R26 Δ C ES cells formed smooth round-shaped EBs throughout the whole differentiation time-course (Fig. 32A). Moreover, β -catenin was expressed at significant level even at day 16 of EB differentiation in contrast to pCAGGS rescued EBs (Compare Fig. 30A to Fig. 32A). This result suggests that cell-cell adhesion defects observed in differentiated β -cat ^{Δ/Δ} ES cells can be rescued by expression of WT as well as signaling-defective Δ C β -catenin transgenes from the Rosa 26 locus.

As shown earlier apoptosis and subsequent death of cells was increased in 5 day old EBs lacking β -catenin. Reintroduction of WT as well as signaling-defective Δ C form of β -catenin was able to rescue the increased rate of apoptosis at day 5 as shown by FACS sorting analysis (Fig. 32B). In contrast to β -cat ^{Δ/Δ} EBs which had 12,14% AnV⁺ PI⁻ apoptotic and 6,78% AnV⁺ PI⁺ dead cells, R26WT EBs had only 7,09% AnV⁺ PI⁻ and 4,29% AnV⁺ PI⁺ cells and R26 Δ C EBs 7,77% AnV⁺ PI⁻ and 4,17% AnV⁺ PI⁺ cells, which were comparable to control β -cat^{fl/fl} EBs (6,79% AnV⁺ PI⁻ and 4,37% AnV⁺ PI⁺) (Fig. 32B).

These findings suggested that the rate of apoptosis and subsequent death of cells was rescued in the β -cat ^{Δ/Δ} EBs by expressing WT and signaling-defective Δ C β -catenin transgenes. Furthermore, my result indicates that the developmental arrest of β -catenin^{-/-} embryos at the gastrulation stage could partially be due to the loss of adhesion function of β -catenin.

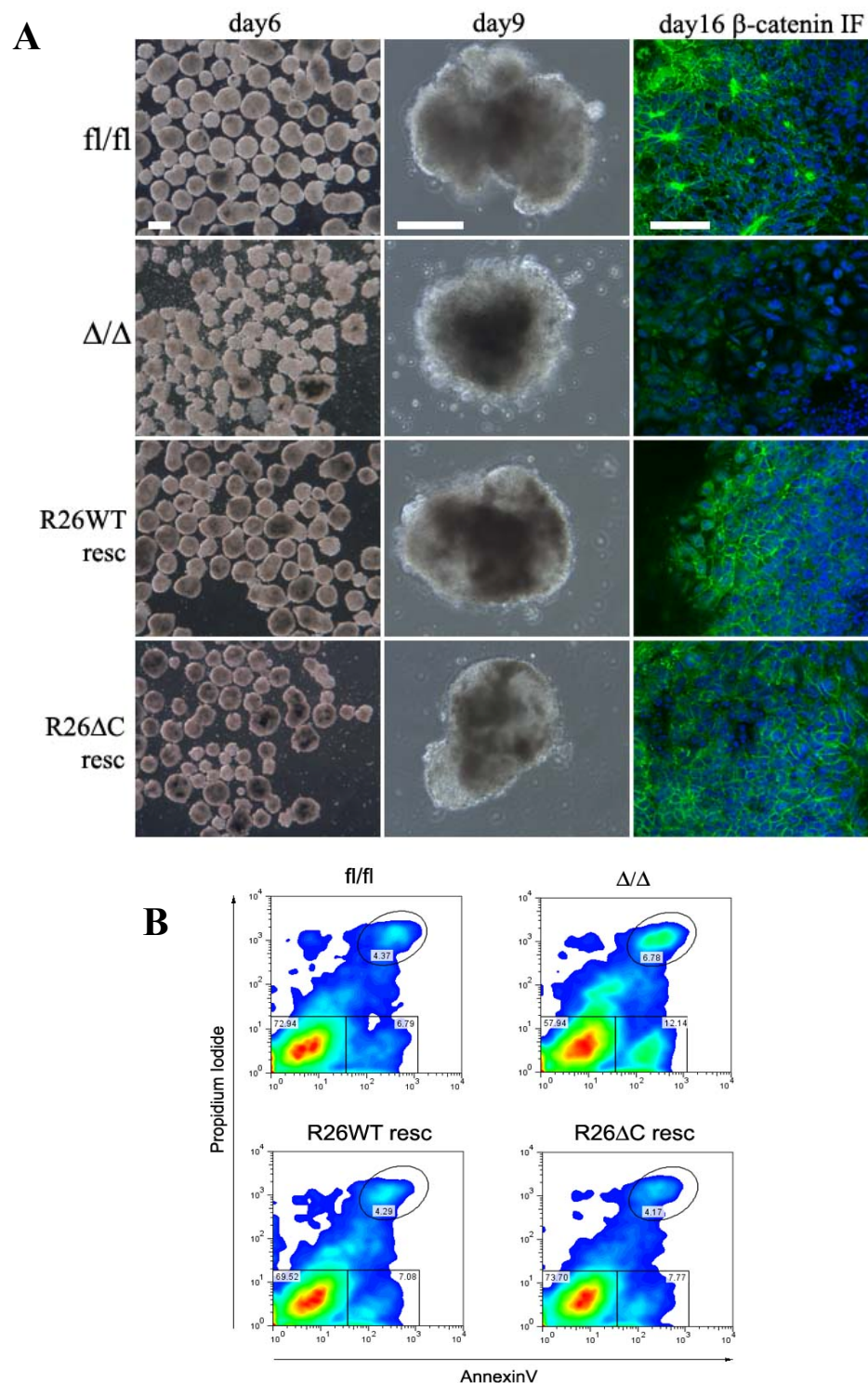


Figure 32 – Rescue of cell-cell adhesion and apoptosis by expression of WT and Δ C β -catenin transgenes from the Rosa26 locus

(A) Morphology of EBs formed from β -cat^{fl/fl}, β -cat ^{Δ/Δ} , R26WT and R26 Δ C β -cat^{fl/fl} ES cells during time course of EB differentiation. Pictures were taken at day 6 and day 9 using phase contrast filter. Scale bar=200 μ m. Immunofluorescent (IF) staining of EBs at day 16 for β -catenin is shown in the last column. DAPI nuclear staining is blue. Scale bar=50 μ m. (B) FACS sorting analysis of β -cat^{fl/fl}, β -cat ^{Δ/Δ} , R26WT and R26 Δ C β -cat^{fl/fl} EBs at day 5 of differentiation stained for annexinV (X axis) and propidium iodide (Y axis).

2.4.2. Rescue of endoderm formation during *in vitro* differentiation

Next I asked the question whether β -cat ^{Δ/Δ} ES cells expressing WT or signaling-defective Δ C forms of β -catenin were able to differentiate into endoderm and mesoderm lineages.

Therefore I performed semi-quantitative RT-PCR analysis using material from β -cat^{fl/fl}, β -cat ^{Δ/Δ} , R26WT and R26 Δ C EBs during differentiation time course (Fig. 33 A). As described earlier in the absence of β -catenin ectodermal markers are expressed, however endodermal and mesodermal markers fail to be expressed during EB differentiation (Fig. 27). Remarkably, not only rescued R26WT, but also R26 Δ C ES cells regained the ability to differentiate towards endoderm and probably mesoderm as judged by the appearance of specific markers for those lineages. However, additional markers would have to be used to verify the results for the mesoderm lineage. Thus, in contrast to β -cat ^{Δ/Δ} EBs, endoderm-specific markers *Gata6*, *Cer1* and *Foxa2* are expressed in R26 Δ C EBs as well as in R26WT EBs (with the exception of *Cer1*) during differentiation.

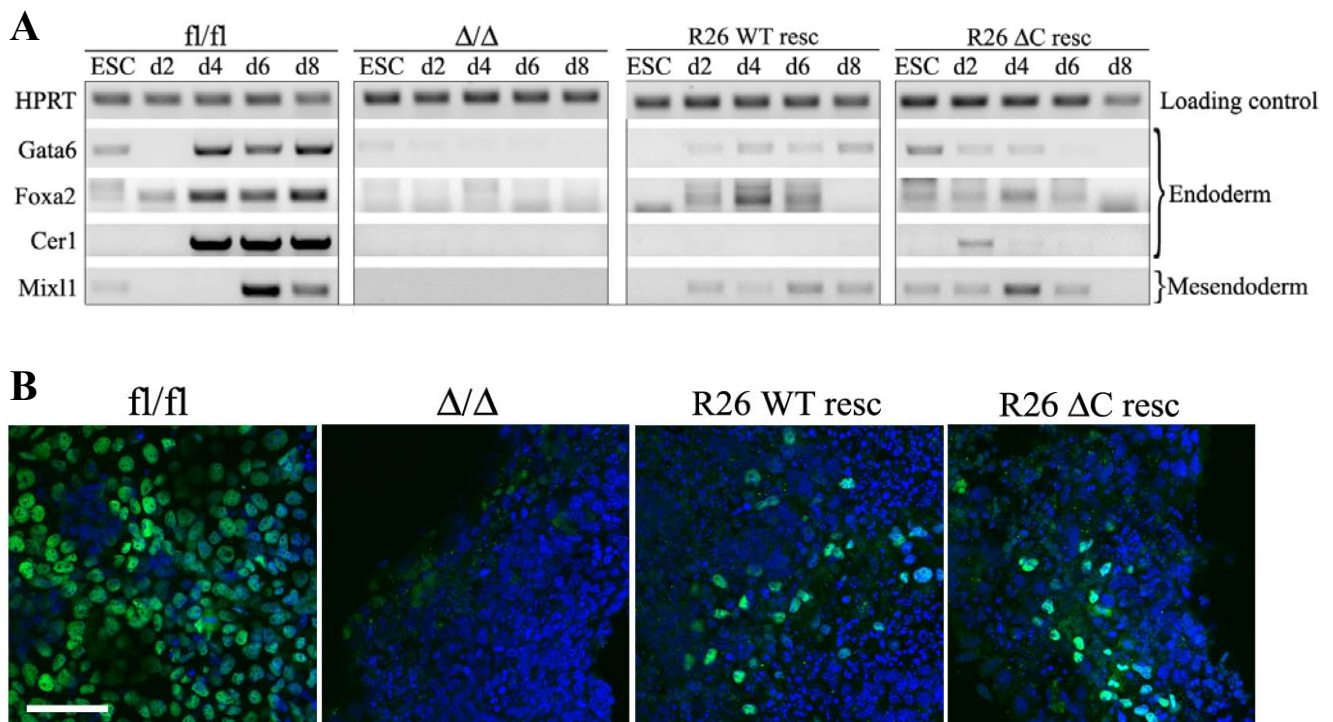


Figure 33 - Endoderm formation is rescued with a WT and Δ C β -catenin transgenes expressed from the Rosa26 locus

(A) β -cat^{fl/fl}, β -cat ^{Δ/Δ} , R26WT, R26 Δ C ES cells were subjected to EB differentiation time course from day 0 (ESC) to day 8. Mesendoderm (*mixl1*) and endoderm (*gata6*, *foxa2* and *cer1*) lineage specific markers were analyzed by semi-quantitative RT-PCR. HPRT expression is a loading control. (B) IF staining of β -cat^{fl/fl}, β -cat ^{Δ/Δ} , Rosa26 WT and Δ C EBs at day 16 with anti-Foxa2 antibodies (green), DAPI nuclear staining is blue. Scale bar=50 μ m.

Similarly, expression of the mesendoderm specific gene *Mixl1* was also rescued. It has to be noted that the intensity of bands was lower and that the onset of expression was premature in comparison to those of β -cat^{fl/fl} EBs. These differences might be due to the constant exogenous expression of the β -catenin transgenes from the Rosa26 locus, which is not absolutely equal to the endogenous levels.

Nevertheless, expression of endoderm-specific genes can be rescued by re-expression of WT as well as signaling-defective ΔC form of β -catenin. In order to confirm that endoderm development can be rescued at late differentiation stages I checked the expression of endoderm-specific transcription factor *Foxa2* at day 16 of EB differentiation by IF (Fig. 33B). In control β -cat^{fl/fl} EBs *Foxa2* was expressed in the nucleus and co-localized with DAPI staining, while no *Foxa2*-positive cells were observed in β -cat ^{$\Delta\Delta$} EBs. Confirming my RT-PCR results *Foxa2*-positive cells could be detected in R26WT and R26 ΔC rescued EBs suggesting that endoderm formation was indeed restored.

2.4.3. Rescue of ectoderm formation during *in vitro* differentiation

As described earlier, ectodermal markers were expressed normally at the early stages of β -cat ^{$\Delta\Delta$} EB differentiation (Fig. 27). However, I was not able to detect at day 16 any ectoderm-derived β 3-tubulin or GFAP-positive cells, which are markers of neuronal and astrocyte cells respectively (Fig. 26 and data not shown). This result is consistent with observations from *β -catenin* mutant mice, which possess normal initiation of ectodermal germ layer, but fail to develop beyond egg cylinder stage due to increased apoptosis in the ectoderm (Haegel et al., 1995; Huelsken et al., 2000). As I was able to show that apoptosis was indeed rescued in R26 WT and R26 ΔC EBs (Fig. 32B), I can speculate that development of ectoderm lineage to terminally-differentiated cells might also be rescued.

In order to test this, I checked the expression of ectoderm-specific marker β 3-tubulin at day 16 of EB differentiation by IF (Fig. 34). No β 3-tubulin-positive neurons were found in β -cat ^{$\Delta\Delta$} ES cell derived EBs in comparison to control β -cat^{fl/fl} EBs, which contained multiple regions with β 3-tubulin-positive cells. As suspected, neuronal differentiation occurred in R26WT and R26 ΔC rescued EBs suggesting that ectoderm (at least neuroectoderm) formation was restored even in the absence of Wnt signaling.

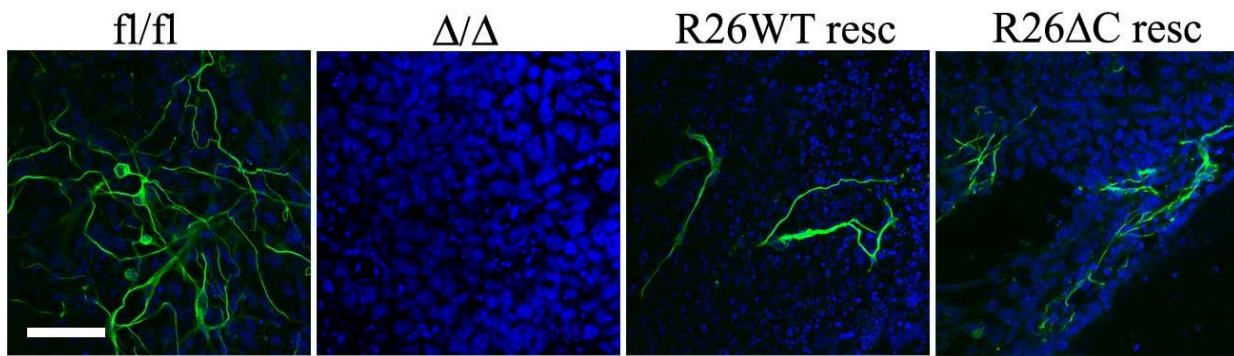


Figure 34 - Ectoderm formation is rescued with a WT and ΔC β -catenin transgenes expressed from the Rosa26 locus

IF staining of β -cat^{fl/fl}, β -cat ^{Δ/Δ} , R26 WT and ΔC EBs at day 16 with anti- β 3-tubulin antibodies (green), DAPI nuclear staining is blue. Scale bar=50 μ m.

2.4.4. *In vivo* differentiation potentials of R26WT and R26 ΔC ES cells

Next I asked the question whether the tumor growth can be rescued in β -cat ^{Δ/Δ} ES cells by expression of WT and signaling-defective ΔC β -catenin transgenes from the Rosa26 locus.

Suspensions of two independent clones of R26WT and two clones of R26 ΔC ES cells each were mixed with Matrigel and injected subcutaneously into the abdominal flank of MF1 nude mice. Tumors were dissected after three weeks (Fig. 35A). As in the experiment performed earlier β -cat ^{Δ/Δ} ES cells failed to form tumors, while β -cat^{fl/fl} ES cell clones formed large tumors (Fig. 28C and Fig. 35A). Interestingly, growth of tumor, originating from R26WT and R26 ΔC ES cells, was restored (Fig. 35A). On the growth curve it is clear to see that, while the control tumors were growing exponentially, β -cat ^{Δ/Δ} tumors failed to increase in size, but that defect could be rescued by expressing WT and ΔC β -catenin transgenes in β -cat ^{Δ/Δ} ES cells (Fig. 35B). Depending on the clones used, the tumor volumes could reach even the volumes of the control β -cat^{fl/fl} ES cell clone (Fig. 35A, B).

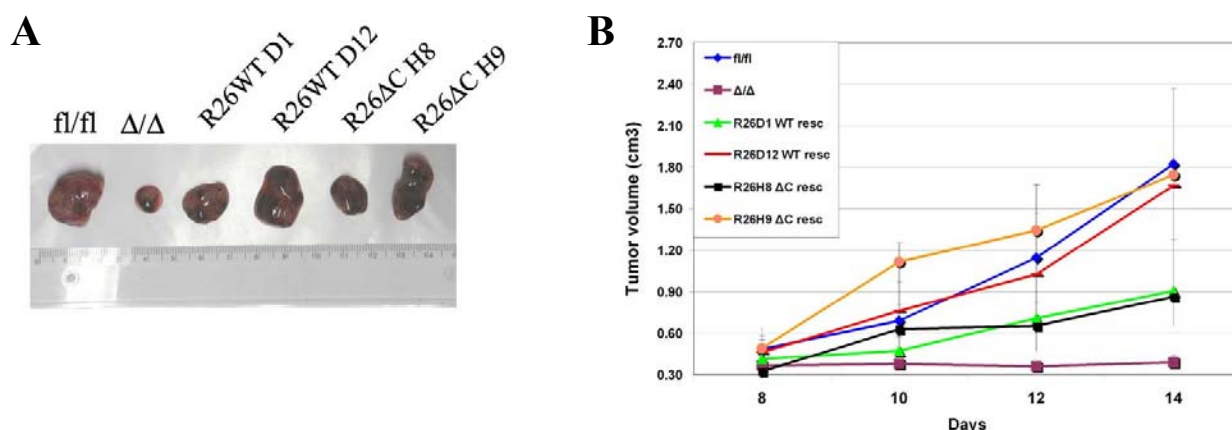


Figure 35 – Tumor growth is restored with WT and ΔC β-catenin transgenes expressed from the Rosa26 locus

(A) Overall appearance of teratomas developed in MF1 nude mice three weeks after subcutaneous injection of β -cat^{fl/fl}, β -cat^{Δ/Δ}, R26 WT (clones D1 and D12) and ΔC (clones H8 and H9) ES cells. (B) Growth curve of teratomas from day 8 to 14 post-injection of β -cat^{fl/fl}, β -cat^{Δ/Δ}, R26 WT (clones D1 and D12) and ΔC (Clones H8 and H9) ES cells. Teratomas were measured in three dimensions (length, width and height) and total volume (cm³) was calculated. Error bars = SEM. n=3.

As already described previously (Fig. 29), staining with hematoxylin and eosin revealed heterogeneous features in β -cat^{fl/fl} teratomas (Fig. 36A, B) and a more homogeneous appearance in β -cat^{Δ/Δ} tumors (Fig. 36D, E). However, R26WT and R26ΔC tumors had a different appearance with primarily loose connective-like tissue (Fig. 36G, J) and some epithelial-like structures (Fig. 36H, K). Hypervascularization observed previously in β -cat^{Δ/Δ} tumors was still present in the ‘rescued’ tumors (Fig. 36D, G). Immunostaining for β-catenin showed the expected localization in β -cat^{fl/fl} teratomas (Fig. 36C), no β-catenin staining above background was observed in β -cat^{Δ/Δ} teratomas (Fig. 36F), while R26WT and R26ΔC tumors stained homogeneously for β-catenin (Fig. 36I and L) as expected.

As described earlier, GFAP-positive cells, which are of ectodermal origin were detected in β -cat^{Δ/Δ} tumors, however in a much lower numbers in comparison to WT teratomas, while mesoderm derived SMA-positive cells were never observed in β -cat^{Δ/Δ} tumors. Thus next I asked the question whether the re-expression of WT and ΔC β-catenin transgenes in β -cat^{Δ/Δ} ES cells rescues the expression of these markers comparable to WT. Although, tumor growth was rescued, the R26WT and R26ΔC tumors were not as heterogeneous as WT teratomas. Instead these tumors were consisting of neural-like tissue with few neuronal rosettes as verified by β3-tubulin and GFAP staining (Fig. 37A, B, D, E). This result indeed confirms the *in vitro* observations that expression of WT and ΔC β-catenin transgenes in β -cat^{Δ/Δ} ES cells restored their potentials to differentiate towards ectodermal lineage.

No mesoderm-derived SMA positive cells could be found in tumors derived from R26WT and R26 Δ C ES cells, with the exception of SMA positive blood vessels that could potentially be of host origin (Fig. 37I, L).

All together, my results from the *in vivo* differentiation assay suggest that (neuro-) ectoderm growth can be rescued in β -cat ^{Δ/Δ} teratomas upon re-expression of WT and most importantly upon re-expression of the signaling-defective Δ C form of β -catenin.

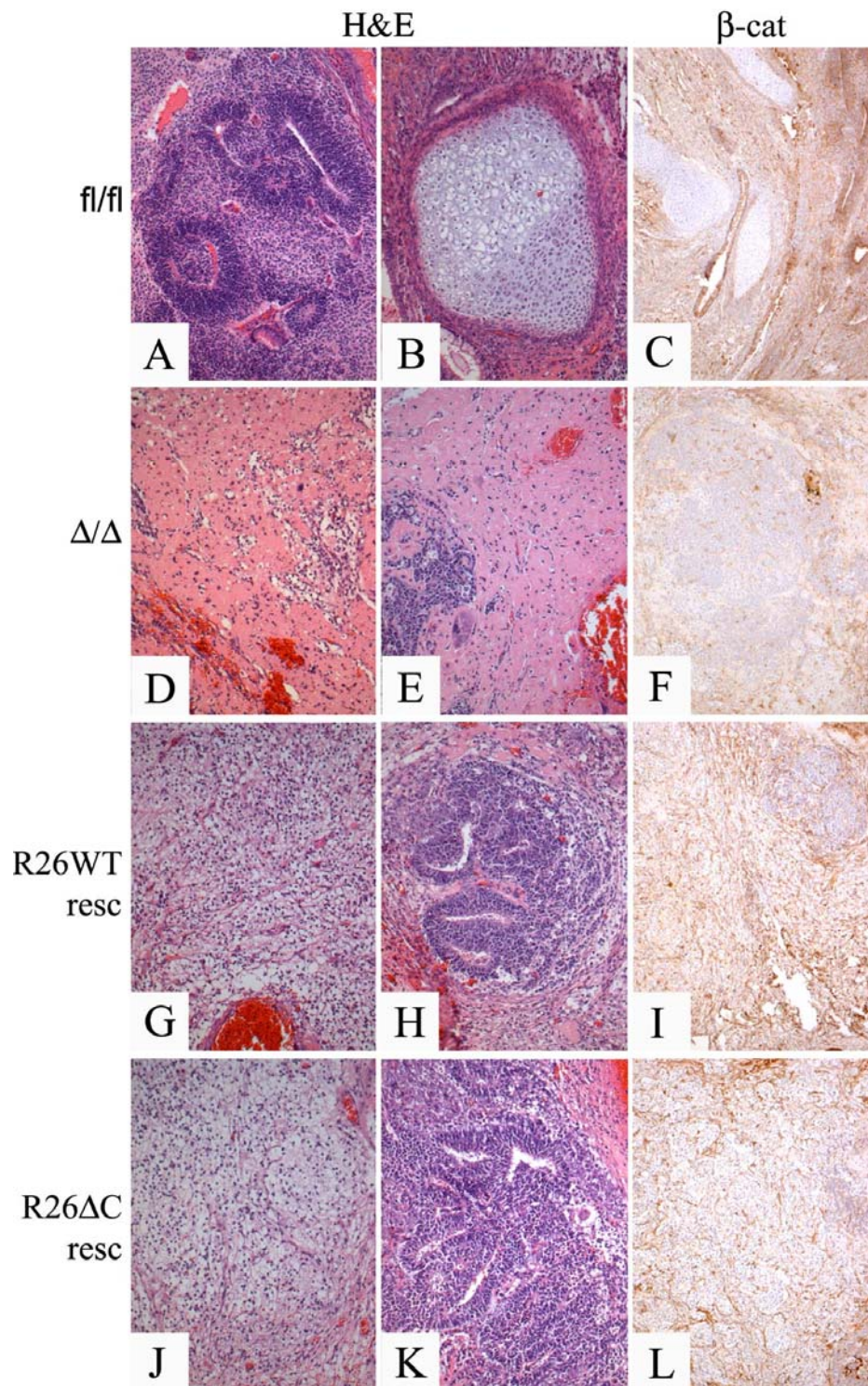


Figure 36 – Morphology of teratomas generated from R26WT and R26ΔC ES cells

(A, B) Histological analysis of sections of teratomas generated from β -cat^{fl/fl} ES cells stained with hematoxylin and eosin. (D, E) Teratomas generated from β -cat^{Δ/Δ} ES cells are homogeneous with supportive Matrigel (pink) still present between cells. Hematoxylin and eosin staining of teratomas generated from R26WT (G, H) and R26ΔC (J, K) ES cells. Immunohistochemical staining of paraffin sections of teratomas generated from β -cat^{fl/fl} (C), β -cat^{Δ/Δ} (F), R26WT (I) and R26ΔC (L) ES cells with anti-N-terminus of β -catenin antibody.

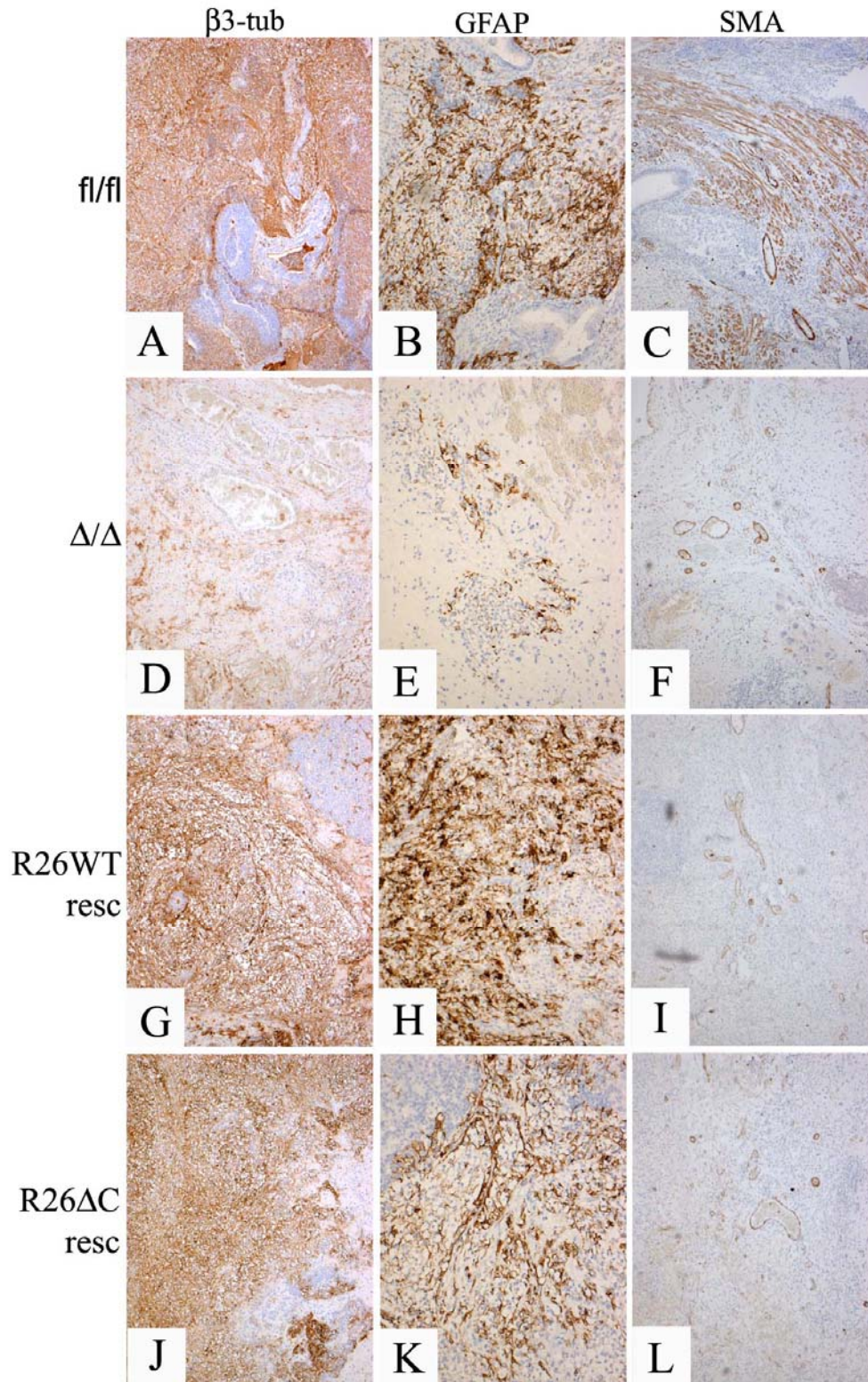


Figure 37 - Analysis of differentiation markers in teratomas generated from R26WT and R26ΔC ES cells

IHC on paraffin sections of teratomas from β -cat^{fl/fl} (A, B, C), β -cat ^{Δ/Δ} (D, E, F), R26WT rescued (G, H, I) and R26ΔC rescued ES cells. Sections were stained with $\beta 3$ -tubulin (A, D, G, J), GFAP (B, E, H, K) and SMA (C, F, I, L) specific antibodies. Nuclei counterstained with hematoxylin.

3. Generation of tamoxifen-inducible β -catenin^{ex3fl/ex3fl} ES cell lines

In order to examine the role of active canonical Wnt signaling in ES cell self-renewal and during differentiation I decided to generate tamoxifen-inducible β -catenin gain-of-function ES cells. For this I used a mouse line homozygous for the β -catenin^{ex3fl} allele (Harada et al., 1999) and the inducible Cre recombinase (Cre-ERT2) allele, targeted to the ubiquitously expressed ROSA26 locus (R26CreER^{T2}; (Seibler et al., 2003)). The estrogen receptor T2 (ERT2) moiety fused to Cre retains the recombinase in the cytosol, until tamoxifen administration releases this inhibition. Thus, addition of tamoxifen allows to induce Cre recombination between the LoxP sites, that surround exon 3 of β -catenin. This results in the generation of a smaller constitutively “active” form of β -catenin, since it cannot be phosphorylated by Gsk3 and targeted for proteasome-mediated degradation.

Initially, I tried to isolate tamoxifen-inducible β -catenin^{x3fl/ex3fl} ES cells from 3.5 dpc old blastocysts, using standard conditions in the presence of serum, LIF and a feeder layer, which I had successfully used for the generation of β -cat^{fl/fl} ES cells. However, all attempts to do so failed due to unknown reason. Therefore, I switched to the recently described MEF-independent serum-free ES culture system, called 2i (Ying et al., 2008). As described earlier, this system is based on N2B27 serum-free medium, supplemented with 1 μ M MEK inhibitor PD03, 3 μ M Gsk3 inhibitor CHIR and 1000U/ml LIF. Using this culture conditions I was able to establish 7 independent ES cell lines out of 5 blastocysts (several ES cell lines can normally be established from one blastocyst after its initial dissociation), referred to as E3BR followed by the number of the line. Morphologically all the established lines resembled normal ES cells, forming dome-shaped compact colonies (Fig. 38A). However, they grow slower than ES cells under serum-containing conditions (data not shown). In order to test their pluripotency potentials, E3BR3-2 and E3BR5 expanded ES cell lines were injected into blastocysts. From the two lines only E3BR3-2 gave chimaeras and transmitted through the germline (Fig. 38B). This experiment indicated that only E3BR3-2 ES cell line was fully pluripotent. One possible explanation for the poor performance of the E3BR5 ES cells might be that they were not fully pluripotent, given that they exhibited lower levels of Nanog (Fig. 38D).

Next I tested whether I could induce recombination in the established ES cell lines using tamoxifen. 24 hours after treatment of E3BR3-2 and E3BR5 ES cells with tamoxifen a shorter β -catenin protein could be detected by western blot analysis (Fig. 38D), suggesting efficient excision of exon3 of β -catenin.

Considering that initial 2i culture system contained Gsk3 inhibitor CHIR and that this results in stabilization of β -catenin as discussed in the introduction, part 1.3, the E3BR ES cells would not be useful for analysis of canonical Wnt signaling effects under self-renewal conditions. Therefore, I tried to switch from culturing them in 2i to normal LIF-dependent serum-containing conditions. This was successful, as judged by the morphology (Fig. 38C). Treatment of E3BR3-2 ES cells with tamoxifen under normal culture conditions resulted in β -catenin localization in cytoplasm and nucleus, as a consequence of β -catenin activation.in comparison to untreated control cells (Fig. 38C).

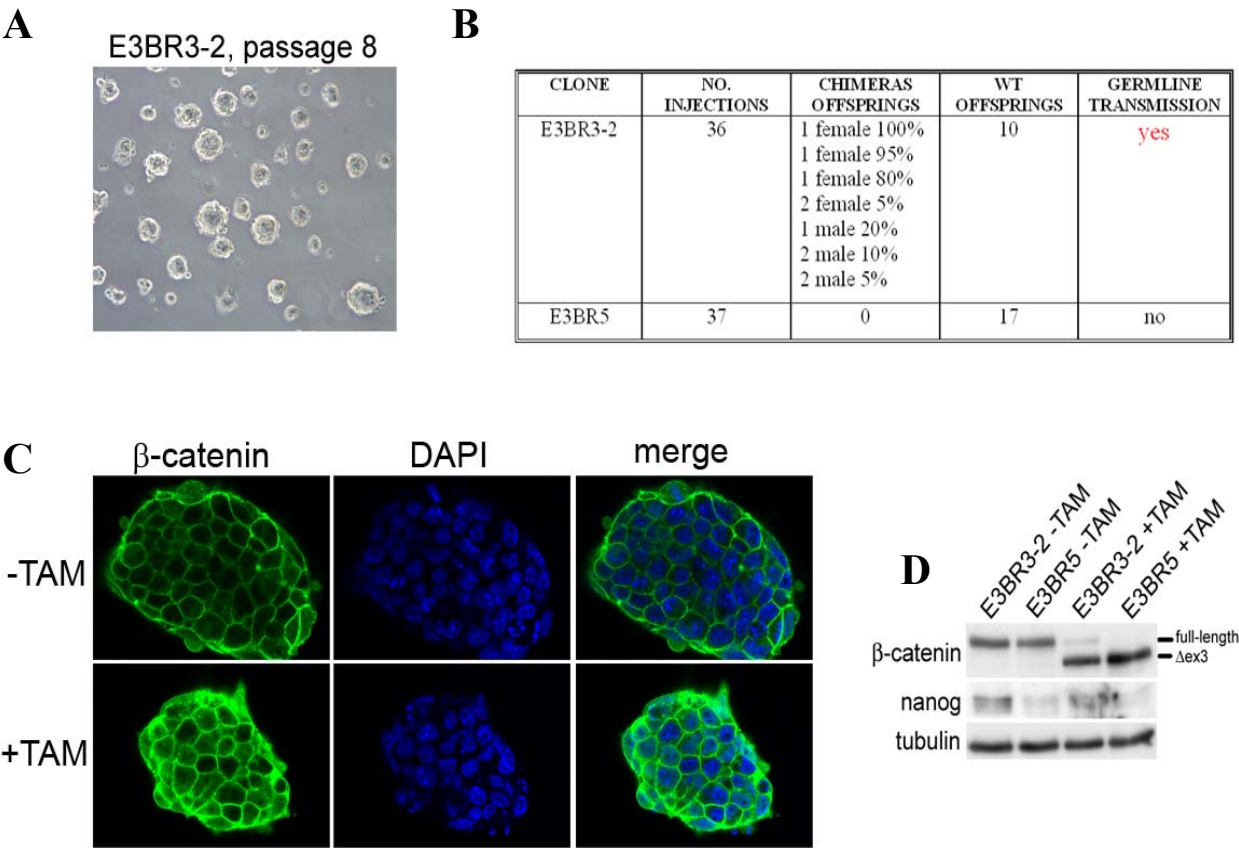


Figure 38 – Characterization of serum-free tamoxifen-inducible β -cat^{ex3fl/ex3fl} ES cells

(A) Morphological appearance of E3BR3-2 ES cells line at passage 8. Image was taken using 10x magnification. (B) E3BR3-2 and E3BR5 ES cells were injected into C57BL/6 blastocyst. Statistics of injection results are shown in the table. (C) IF images of E3BR3-2 ES cells untreated (-TAM) or treated with tamoxifen (+TAM) for 24 hours cultured under normal conditions. β -catenin is stained in green, DAPI nuclear staining is blue. Note the intense green β -catenin staining in the cytoplasm and the nucleus in “+TAM” compared to the primarily membranous green staining in “-TAM”. (D) Western blot analysis of β -catenin and Nanog in E3BR3-2 and E3BR5 ES cells untreated (-TAM) or treated with tamoxifen (+TAM) for 24 hours cultured in 2i medium. Tubulin levels serve as a loading control.

Discussion

Development of multicellular organism is orchestrated by the cross-talk between evolutionary conserved signaling pathways that direct cell-type specific differentiation programs. One of such pathways is the Wnt/ β -catenin signaling pathway. Together with transcriptional regulation of differentiation cell-adhesion plays an important role in regulating cell movements and communication between cells during development. Various studies suggested, that β -catenin, a transcriptional mediator of the canonical Wnt pathway, can also participate in adherens junctions and as such plays a dual role in transcription and cell adhesion. However, little is known how the balance between transcriptional and adhesion functions of β -catenin is achieved. Moreover, knockout studies do not allow to distinguish between the requirement of these two functions during development.

In my efforts to discriminate between the adhesive and transcriptional functions of β -catenin during development, I generated β -catenin-deficient murine ES cell lines (β -cat $^{\Delta/\Delta}$) and re-expressed the full-length (WT) and a signaling-defective C-terminal deleted (Δ C) β -catenin transgene in one of these lines. Using these important tools I was able to show that endogenous β -catenin is dispensable for ES cell self-renewal, but important for differentiation into mesoderm and endoderm lineages. Moreover, during *in vitro* differentiation of β -cat $^{\Delta/\Delta}$ ES cells in EBs cell-cell adhesion defects and apoptosis become apparent, which are probably due to alterations in plakoglobin protein levels, a molecule highly related to β -catenin. By siRNA-mediated silencing of *plakoglobin* in β -cat $^{\Delta/\Delta}$ ES cells I could show that cell-cell adhesion is most likely essential for proper maintenance of ES cell pluripotency. Utilizing the β -cat $^{\Delta/\Delta}$ ES cell lines expressing full-length or signaling-defective Δ C β -catenin transgenes I was able to rescue the cell-cell adhesion phenotype and apoptosis during differentiation. Furthermore, my results from *in vivo* and *in vitro* differentiation studies of the rescued ES cell lines suggest that adhesion function of β -catenin is important for endoderm induction and proper ectoderm differentiation.

1. β -catenin in mouse ES cell self-renewal

1.1. Role of Wnt signaling in mouse ES cell maintenance

ES cells, derived from the inner cell mass (ICM) of the blastocyst, are able to maintain their properties in culture for undefined time. Since the first isolation of mouse ES cells, researchers are focusing on deciphering the mechanisms that control ES cell self-renewal. In addition to already established signaling pathways, like LIF/Stat3, which is sufficient to maintain self-renewal, the canonical Wnt signaling pathway had recently been implicated as well (Dreesen and Brivanlou, 2007; Sato et al., 2004).

1.1.1. Endogenous levels of active β -catenin

When I started this project, one of the open questions in ES cell field was whether the Wnt/ β -catenin pathway is endogenously active in ES cells, cultured under normal LIF-dependent culture conditions.

Active canonical Wnt signaling is normally characterized by a free cytoplasmic pool of β -catenin that can enter the nucleus and act as a co-activator in the transcription of downstream targets. Several experimental tools are usually used to define the active state of β -catenin in cell culture. Among them are the broadly used TOPFLASH reporter constructs, containing varying numbers of multimerized TCF binding sites, inserted upstream of the either the firefly luciferase gene, GFP or LacZ in other cases (Korinek et al., 1997). Active nuclear β -catenin binds to TCF or LEF transcription factors, that occupy corresponding binding sites within the reporter construct resulting in reporter gene expression, such as luciferase. The latter approach allows one to measure levels of β -catenin signaling activity in cells, transfected with a TOPFLASH construct using a conventional luminometer. Sato and colleagues had previously used a yellow fluorescent protein (YFP)-based TOPFLASH reporter line to examine the endogenous transcriptional activity of Wnt/ β -catenin signaling in mouse ES cells. They showed that ES cells expressed YFP under normal LIF-dependent culture conditions, but upon withdrawal of LIF followed by differentiation the cells rapidly lost YFP-TOPFLASH expression (Sato et al., 2004). Consistently, other groups have shown that β -catenin was localized throughout the cytoplasm and in the nucleus of ES cells, and that nuclear β -catenin levels decreased upon LIF withdrawal and furthermore that ES cells express several canonical

Wnt ligands (Anton et al., 2007; Takao et al., 2007). These results indicated that canonical Wnt signaling might be endogenously active in ES cells at low levels.

However, these approaches to measure endogenous levels of the canonical Wnt signaling have some limitations. Concerning TOPFLASH reporter, a possibility of TCF/LEF-independent activation of target genes by β -catenin makes its usage limited (Filali et al., 2002). As shown in the recent study by Takao and colleagues by over-expression of a stabilized form of β -catenin, β -catenin cooperates with Oct-3/4 to activate the *Nanog* promoter (Takao et al., 2007). Based on this data, it was proposed that β -catenin is involved in ES cell self-renewal in a TCF/LEF-independent fashion. Generation of β -catenin fl/fl (β -cat^{fl/fl}) and its derivative β -catenin-deficient (β -cat ^{Δ/Δ}) ES cells, described in my thesis, gave me the possibility to reliably examine endogenous β -catenin activity. Importantly, as shown by blastocyst injection and germline transmission, the newly established β -cat^{fl/fl} ES cell line was fully pluripotent. Comparing the TOPFLASH reporter activity, using the highly sensitive BAR-lentiviral system in β -cat^{fl/fl} and β -cat ^{Δ/Δ} ES cell lines, I observed in both cases only basal levels of the reporter.

Additionally, a functionality of nuclear β -catenin to activate specific target genes has to be proven. Although activation of many Wnt targets is cell type-specific, so far only *Axin2* has been established as a unique target gene in almost all system (Jho et al., 2002). *Axin2* was expressed at the same level in β -cat^{fl/fl} and β -cat ^{Δ/Δ} ES cell lines. These results are in contrary to published data, discussed above, suggesting a possible endogenous transcriptional activity of Wnt/ β -catenin signaling, that, in concert with LIF-dependent stimulation, maintains ES cell pluripotency (Anton et al., 2007; Sato et al., 2004; Takao et al., 2007).

Other studies showed, that expression of *Stat3* (Hao et al., 2006) and *c-Myc* (Cartwright et al., 2005; Hao et al., 2006; He et al., 1998) was activated by β -catenin. Again my data based on the analysis of self-renewal markers, cDNA expression profiling and LIF-induced STAT3-activation experiments with β -cat ^{Δ/Δ} ES cells revealed no differences between the ES cells lacking β -catenin and the parental β -cat^{fl/fl} ES cell lines. Thus, my results are contradictory to the proposed role of active endogenous β -catenin in the maintenance of LIF-dependent self-renewal. However, they further support the notion that Wnt/ β -catenin signaling is not detectable in the pre-implantation embryo, as demonstrated by monitoring the expression of the TCF/Lef-LacZ transgene and non-phosphorylated β -catenin localization (Mohamed et al., 2004).

1.1.2. Exogenous activation of Wnt signaling

Activation of Wnt signaling pathway has been demonstrated by different approaches to maintain mouse and human ES cell in the pluripotent state.

Addition of Wnt3a CM in the absence of LIF was shown to be sufficient to maintain ES cell self-renewal and as confirmed by generation of germline-competent chimaeric mice also pluripotency, (Ogawa et al., 2006; Singla et al., 2006). However, this effect is partially due to the presence of LIF, in an amount similar to 10 Units of recombinant LIF, in Wnt3a CM favoring a potential synergistic effect of Wnt and LIF signaling on self-renewal. Though, these data do not rule out that the Wnt3a CM can contain some additional factors supporting self-renewal. These experiments might in part explain my observation, that Wnt3a CM supports the formation of AP-positive undifferentiated colonies, even in the absence of β -catenin. Given that we have seen in the embryo that canonical Wnt-signaling can regulate the levels of BMPs together with the fact that BMPs can support ES cell self-renewal I had also checked whether the Wnt3a CM contains any BMP4 activity using the C2C12BRA and HepG2BRA reporter cells (Zilberberg et al., 2007); gift from Daniel Rifkin). However, these experiments did not show any increase in BMP4 activity in the CM (data not shown). In contrast, to Wnt3a CM rWnt3a was very specific, as β -cat ^{Δ/Δ} ES cells underwent rapid differentiation in the absence of LIF, while WT ES cells could be maintained undifferentiated at least for 5 days under the same conditions. The reports in the literature on the competence of rWnt3a in supporting self-renewal are mixed. Two studies reported that rWnt3a is not sufficient to keep ES cells in an undifferentiated state (Dravid et al., 2005; Ogawa et al., 2006), while our observations goes along with the finding of another study showing that rWnt3a can maintain compact ES cell colonies up to 3 days (Singla et al., 2006). These discrepancies might be based on genetic variations due to the usage of different ES cell lines and/or serum or might simply be explained by differences in rWnt3a concentrations used (200ng/ml in my experiments and 100ng/ml in other studies). Furthermore, it has to be noted here that I did not test the effects of rWnt3a beyond the 5 day time point.

One of the first studies implicating Wnt/ β -catenin signaling in ES self-renewal was the study by Sato and colleagues, which reported that the Gsk3-specific inhibitor BIO is sufficient to maintain self-renewal of mouse as well as human ES cells through activation of β -catenin (Sato et al., 2004). However, more recent studies, including my own observations, partially disproved this conclusion. As such BIO is only able to support human ES cell propagation short-term and facilitate differentiation long-term (Dravid et al., 2005). Additionally, BIO reduces viability and

proliferation of mouse ES cells (my observations and (Ying et al., 2008). Moreover, using β -cat ^{Δ/Δ} ES cells I demonstrated that treatment with BIO results in AP-positive colonies formation and decreased proliferation of ES cells lacking β -catenin. This result can be explained by additional effects of BIO, which might inhibit also other protein kinases besides Gsk3, thereby eliciting β -catenin independent effects (Bain et al., 2003). This idea is supported by the study of Gsk3 α/β double-knockout ES cells, which possess enhanced retention of ES cell markers under differentiation conditions, but still differentiate eventually (Doble et al., 2007).

Recently the effect of a more specific Gsk3 inhibitor CHIR99021 (CHIR) on ES cell maintenance was demonstrated. Here, addition of CHIR in the absence of LIF could maintain ES cell survival at low density and partially maintain undifferentiated colonies at high density (Ying et al., 2008). Moreover, Ying and colleagues showed that CHIR in combination with MEK inhibitor allows long-term propagation of ES cells in undifferentiated state even in the absence of serum. My data support the notion that CHIR is more specific than BIO in the self-renewal assay, as β -cat ^{Δ/Δ} ES cells rapidly differentiated upon CHIR treatment in the absence of LIF, phenotypically resembling rWnt3a treatment. Intriguingly, when I performed the same experiment using ΔC rescued β -cat ^{Δ/Δ} ES cells, which are defective in TCF/LEF mediated signaling, the ES cells maintained their self-renewal state for 5 days upon CHIR treatment. One possible explanation could be that ΔC β -catenin might still be able to interact with transcriptional co-factors other than TCF/LEF family members in order to maintain ES cell self-renewal. In the literature it has been reported that β -catenin can interact with the specific homeodomain factor, Prop1 (Olson et al., 2006) or Oct3/4 (Takao et al., 2007). This will have to be examined in the future.

Taken together, my data, along with other published studies, suggest that Wnt/ β -catenin signaling supports self-renewal of mouse ES cells, depending on the stimuli used: rWnt3a and CHIR showed clear β -catenin dependency, while BIO and Wnt3a CM medium can mediate self-renewal in part in a β -catenin-independent manner.

1.2. Cell-cell adhesion and the maintenance of ES cell self-renewal

One of the characteristics of undifferentiated ES cells, that distinguishes them phenotypically from differentiated cells, is their ability to grow in compact multilayered colonies, consisting of round cells with low cytoplasm to nucleus ratio (Evans and Kaufman, 1981). Once forced to differentiate, ES cells colonies lose their compact morphology and flatten in the cell culture

dish. This process is normally accompanied by the loss of transcriptional regulators of self-renewal as well as ES cell-specific adhesion-related molecules.

As demonstrated in my thesis ES cells have at least two types of cell junctions: adherens junctions and desmosomes. Components of adherens junction, β -catenin and E-cadherin, are broadly expressed in ES cells as well as plakoglobin, which participates in adherens junctions and desmosomes (Franke et al., 1987). Interestingly, β -cat $^{\Delta/\Delta}$ ES cells had elevated levels of plakoglobin in their cell membranes. Moreover, the amount of plakoglobin associated with E-cadherin was increased. This result is consistent with published data about β -catenin mutant mice and F9 cells lacking β -catenin, where plakoglobin has been shown to substitute for β -catenin in adherens junctions (Fukunaga et al., 2005; Huelsken et al., 2000). However, my ultrastructural analysis revealed that cell membranes were more loosely connected in the absence of β -catenin. This result together with the observation of increased amounts of single cells in β -cat $^{\Delta/\Delta}$ ES cell culture suggests, that plakoglobin might not be sufficient to substitute entirely for the loss of β -catenin in ES cells. Nevertheless, β -cat $^{\Delta/\Delta}$ ES cells appeared to have all self-renewal characteristics that were discussed in chapter 1.1.1. Interestingly, knock-down of *plakoglobin* in β -cat $^{\Delta/\Delta}$ ES cells resulted in a complete loss of cell-cell adhesion. This phenotype did not occur, when pCAGGS WT, M6 or ΔC β -cat $^{\Delta/\Delta}$ ES cells were transfected with *plakoglobin* siRNA. *Plakoglobin* siRNA-treated β -cat $^{\Delta/\Delta}$ ES cells had flattened spindle-shaped morphology and self-renewal markers were affected. Consequently, AP expression was reduced, *nanog* mRNA and protein levels were significantly down-regulated and PECAM-1 expression was lost. Unfortunately, I was not able to generate the inducible *plakoglobin* shRNA β -cat $^{\Delta/\Delta}$ ES cells to study the effects of β -catenin and plakoglobin loss on self-renewal in long-term experiments. Perhaps, a cleaner and more reliable system to study this would be to establish ES cells from β -catenin $^{fl/fl}$; plakoglobin $^{-/-}$ blastocysts. It should be possible to generate β -cat $^{fl/fl}$; plako $^{-/-}$ blastocyst, because plakoglobin $^{-/-}$ mice die only between days 12-16 of embryogenesis due to defects in heart function (Ruiz et al., 1996). Thus, upon Cre recombination double-knockout ES cells, lacking both β -catenin and plakoglobin, could be established.

Despite the fact that more markers should be analyzed, my data suggest that β -cat $^{\Delta/\Delta}$ ES cells, transfected with *plakoglobin* siRNA, have probably lost their self-renewal potentials.

The characterization of E-cadherin-null ES cells has also been reported (Larue et al., 1994). It was shown that loss of E-cadherin in ES cells does not change expression of *oct-3/4* and *nanog*, however the expression of *brachyury*, a mesoderm-specific marker, was elevated in these cells.

Blastocyst injection experiments showed that E-cadherin-null ES cells did not contribute to chimaeras, thus are not pluripotent (Larue et al., 1994). I received the E-cadherin-null cells from Prof. Rolf Kemler (MPI, Freiburg) and compared their appearance in culture to β -cat $^{\Delta/\Delta}$ plakoglobin siRNA treated ES cells (Fig. 39). Although E-cadherin $^{-/-}$ ES cells expressed *nanog* as described, morphologically cell-cell contacts were still present between some cells, occasionally forming compact ES cell colonies. Moreover, very recent study demonstrated that the same E-cadherin $^{-/-}$ ES cell line has an ability to form embryoid bodies (EBs) (Moore et al., 2009). According to the previously mentioned statement that ES cells have adherens junctions and desmosomes, I would hypothesize that E-cadherin $^{-/-}$ ES cells are not absolutely cell-adhesion defective, as E-cadherin present only in adherens junctions and deletion of E-cadherin does not affect desmosomes. This could explain why ES cells lacking E-cadherin express self-renewal markers and are able to form EBs upon LIF withdrawal.

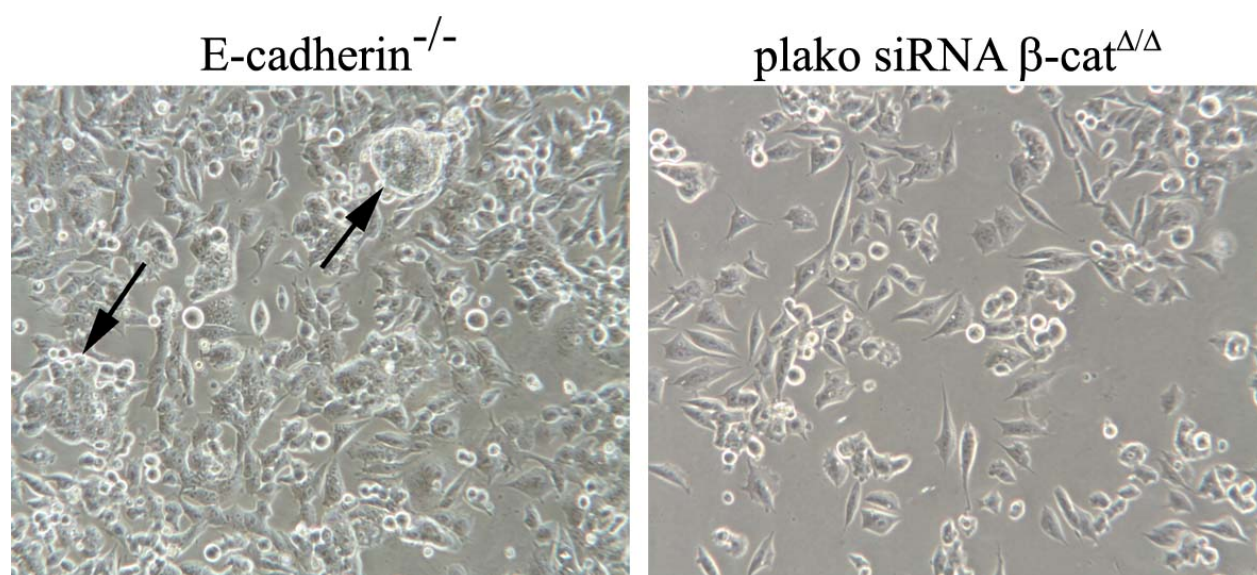


Figure 39 – Comparison of E-cadherin $^{-/-}$ and plakoglobin siRNA-transfected β -cat $^{\Delta/\Delta}$ ES cells

Arrows indicate compact colonies of E-cadherin $^{-/-}$ ES cells. Pictures were taken with 10x magnification.

2. β -catenin in early mouse development

2.1. *β -catenin-deficient ES cells as a model for developmental studies*

The formation of the primary body axis during early embryogenesis is controlled in many organisms by canonical Wnt signaling pathway (Sokol, 1999). In particular, knockouts of different components of this pathway contributed to our understanding of the mechanisms controlling early developmental events. *β -catenin* mutant mice were independently generated by two groups (Haegel et al., 1995; Huelsken et al., 2000). In both cases ablation of *β -catenin* resulted in a failure to position distal visceral endoderm at the anterior side of the embryo at E6,0 and a lack of primitive streak and mesoderm formation at E6,5. Additional studies, employing the generation of chimeric embryos from mutant and WT cells, suggest, that *β -catenin* acts in the epiblast, where it regulates the expression of other secreted molecules that direct positioning of visceral endoderm at this developmental stage (Huelsken et al., 2000; Lickert et al., 2002). Notably, the ectodermal cell layer was properly formed in *β -catenin* mutant embryo, however after E7,0 apoptosis became apparent and the embryos failed to develop beyond that developmental stage.

My *in vitro* and *in vivo* differentiation experiments, using β -cat ^{Δ/Δ} ES cells, showed, that the differentiation defects, observed in *β -catenin* mutant mice, could in principle be recapitulated. I was able to show that β -cat ^{Δ/Δ} ES cells failed to differentiate into mesoderm and endoderm, while differentiation into ectodermal layer was initiated. Moreover, β -cat ^{Δ/Δ} ES cells failed to form cyst-like EBs, displayed cell-cell adhesion defects and showed increased apoptosis, starting at day 5 of EB differentiation, correlating with onset of the endogenous down-regulation of plakoglobin. These defects correlate nicely with the onset of Wnt/ *β -catenin* signaling activity in the mouse embryo, arguing about the significance of Wnt/ *β -catenin* signaling in the primitive streak, but not in the ectoderm. It was demonstrated by monitoring the expression of the TCF/Lef-LacZ transgene (Maretto et al., 2003; Mohamed et al., 2004) or by non-phosphorylated *β -catenin* localization (Mohamed et al., 2004). The onset of transgene and non-phosphorylated *β -catenin* expression was first detected in the extraembryonic visceral endoderm of the early egg cylinder stage embryo (E5,5). Later it is restricted first to the posterior epiblast (E6,0) and then to the primitive streak (E6,25-7,0) and the node (E7,0-) (Mohamed et al., 2004). However, using different transgenic TCF/LEF reporter line it was

demonstrated that activity of the reporter can be detected not only in the primitive streak, but also found in the proximal epiblast region at E6,5 (Maretto et al., 2003).

2.2. Role of the β -catenin C-terminus in Wnt signaling

One of the important problems in developmental biology that has not yet been tackled is to distinguish between cell-cell adhesion and signaling function of β -catenin during early mouse development. In order to address this problem I generated β -cat ^{$\Delta\Delta$} ES cells, expressing murine full-length (WT; amino acids 1-781) and human Δ C (Δ amino acids 696-781) β -catenin transgenes, integrated into Rosa26 locus. There is evidence suggesting that the C-terminus of β -catenin has important functions. It was proposed that different conformations of β -catenin exist in the cell participating either in cell adhesion or transcription (Gottardi and Gumbiner, 2004). Here the C-terminus of β -catenin plays an important role it can fold back onto the armadillo repeat region thereby competing with β -catenin binding to the cytoplasmic domain of E-cadherin and favoring interaction with TCF/LEF transcription factors (Castano et al., 2002; Cox et al., 1999; Piedra et al., 2001). Moreover, the C-terminus of β -catenin contains the transactivation domain, which has been reported to interact with multiple transcriptional co-activators including CBP/p300 and TRRAP/GCN5. Deletion of the β -catenin C-terminus results in reduced binding efficiency to TCF/LEF transcription factors, but does not interfere with binding to E-cadherin (Sustmann et al., 2008). A very recent study proposed a model, where the C-terminal region of β -catenin promotes β -catenin stability by restricting its association with Axin, a key scaffold component of β -catenin destruction complex (Mo et al., 2009). All together, the published data based mostly on biochemical analysis suggests that the C-terminus of β -catenin plays an important role in regulation of β -catenin participation in Wnt signaling. However, its biological role is still not well characterized.

In order to address the activity of the Δ C β -catenin variant in Wnt signaling, I had generated different stable lines, which expressed either the WT, as a control, or the Δ C form of β -catenin either under the control of the CAG promoter or from the Rosa26 locus. Both isoforms localized to the membrane and moreover this led to a reduction in total plakoglobin levels, arguing that they could participate in adherens junctions. Upon stimulation with a Gsk3 inhibitor CHIR, TOPFLASH reporter activity was increased in the WT rescued β -cat ^{$\Delta\Delta$} ES cells, but no activity above the baseline was detectable in Δ C rescued β -cat ^{$\Delta\Delta$} ES cells. Thus, I was able to show that in β -catenin-deficient background the C-terminal truncated form of β -

catenin is indeed defective in canonical Wnt signaling mediated by TCF/LEF. However, we cannot rule out that the C-terminal truncated form of β -catenin might still possess the ability to activate target genes in a TCF/LEF-independent fashion, through interaction with other transcription factors. This possibility will have to be examined in the future, using, for example, reporters for alternative target genes.

2.3. Differentiation potentials of adhesion-rescued R26 Δ C ES cells

Knowing that the Δ C variant of β -catenin was signaling defective, but could participate in cell adhesion, enabled me to address its biological function during early development, performing EB differentiation assays with the R26WT and R26 Δ C ES cells. This revealed that, in particular, the Δ C variant of β -catenin was able to fully rescue the adhesion defect and apoptosis, which I had observed in the absence of β -catenin. Moreover, my analysis revealed that, by rescuing adhesion and apoptosis, the expression of endoderm lineage markers and ectoderm differentiation was restored as well.

With regard to the endoderm lineage, expression of *foxa2* and *mixl1* was initiated in the control R26WT, but most importantly also in the R26 Δ C EBs. The conserved forkhead transcription factor Foxa2 has been shown to be expressed in visceral endoderm, during gastrulation in the node, and later in the notochord and midline neural plate giving rise to all axial tissues of the embryo (Ruiz i Altaba et al., 1993). The homeobox transcription factor Mixl1 is required for mesendoderm specification and definitive endoderm formation (Hart et al., 2002). Besides Foxa2 and Mixl1, the expression of other visceral endoderm markers, such as the transcription factor Gata6 (Morrisey et al., 1998) and the Cerberus 1 homologue (Cer1) (Yamamoto et al., 2004), was also restored. These results suggest that the cell-cell adhesion function of β -catenin is important for endoderm layer formation during early mouse development.

Knowledge from developmental studies points to the importance of β -catenin in the neurogenesis. *β -catenin* mutant mice can initiate formation of the ectodermal layer, but it fails to develop further to neuroectoderm due to massive apoptosis (Haegel et al., 1995; Huelsken et al., 2000). Conditional ablation of β -catenin using Wnt1-Cre line results in severe malformations in brain development and increased apoptosis in brain structures (Brault et al., 2001). I could observe similar effects in EB differentiation of β -cat ^{$\Delta\Delta$} ES cells. Thus, ectodermal-specific markers were induced, but EBs lacking β -catenin did not develop beyond day 5, probably due to increased apoptosis. However, neurons and also astrocytes could occasionally be observed in teratoma formation assay, arguing that β -catenin is not necessary

for neuroectoderm formation, but for cellular survival. The role of Wnt/ β -catenin signaling in neuroectoderm formation and the formation of neurons is controversial in the literature, based on the *in vitro* experiments, employing mainly ES cell differentiation. Thus, enhancement of Wnt/ β -catenin signaling promotes neuronal differentiation of ES cells (Otero et al., 2004; Papadimou et al., 2009). On the other hand, using PC-12 cell model of neuronal differentiation, TCF/ β -catenin mediated transcription was proposed to limit neuronal differentiation (Teo et al., 2005). In addition, data from developmental studies in mouse and frog clearly show that Wnt/ β -catenin signaling has to be suppressed in order to allow the formation of the anterior neural ectoderm (Marikawa, 2006). Using R26 Δ C ES cells I wanted to decipher the cell-adhesion function of β -catenin during neurogenesis. The identification of neurons positive for β 3-tubulin in R26WT and R26 Δ C 16 days old EBs, which were not present in the β -cat $^{\Delta/\Delta}$ EBs suggests, that the ectodermal layer could propagate further, contributing to the formation of neurons upon rescue of the cell-adhesion defects and apoptosis. This notion is further supported by my findings from the teratoma formation assay. These results are also consistent with recently published studies, indicating the importance of cell-cell interactions for neuroectodermal specifications and lineage commitment (Parekkadan et al., 2008; Sun et al., 2008).

All together, my data show that there is an important role for the cell-cell adhesion function of β -catenin for the development of certain germ layers and that the defects observed in β -catenin mutant embryos are probably not exclusively due to the signaling function of β -catenin. The working model, presented in the Fig. 40, demonstrates that, using correlation of EB differentiation to the early embryonic development. However, it is still not clear whether R26 Δ C ES cells can contribute to the primitive streak and mesoderm. Future experiments, using injection of these cells into a host blastocyst should clarify that point.

Still one of the unresolved issues, that might put some of my results into question, is based on the observation, that the teratomas, formed from R26WT ES cells, did not develop the full complement of differentiated structures, as seen in teratomas, derived from β -cat $^{fl/fl}$ ES cells. This might be explained in several ways: firstly the WT β -catenin transgene was expressed at lower than endogenous levels from the Rosa26 locus; Secondly, the endogenous β -catenin expression might be regulated by transcription factors, binding to tissue-specific enhancers, and this regulatory network would, of course, be not maintained, when the transgene was expressed from the Rosa 26 locus.

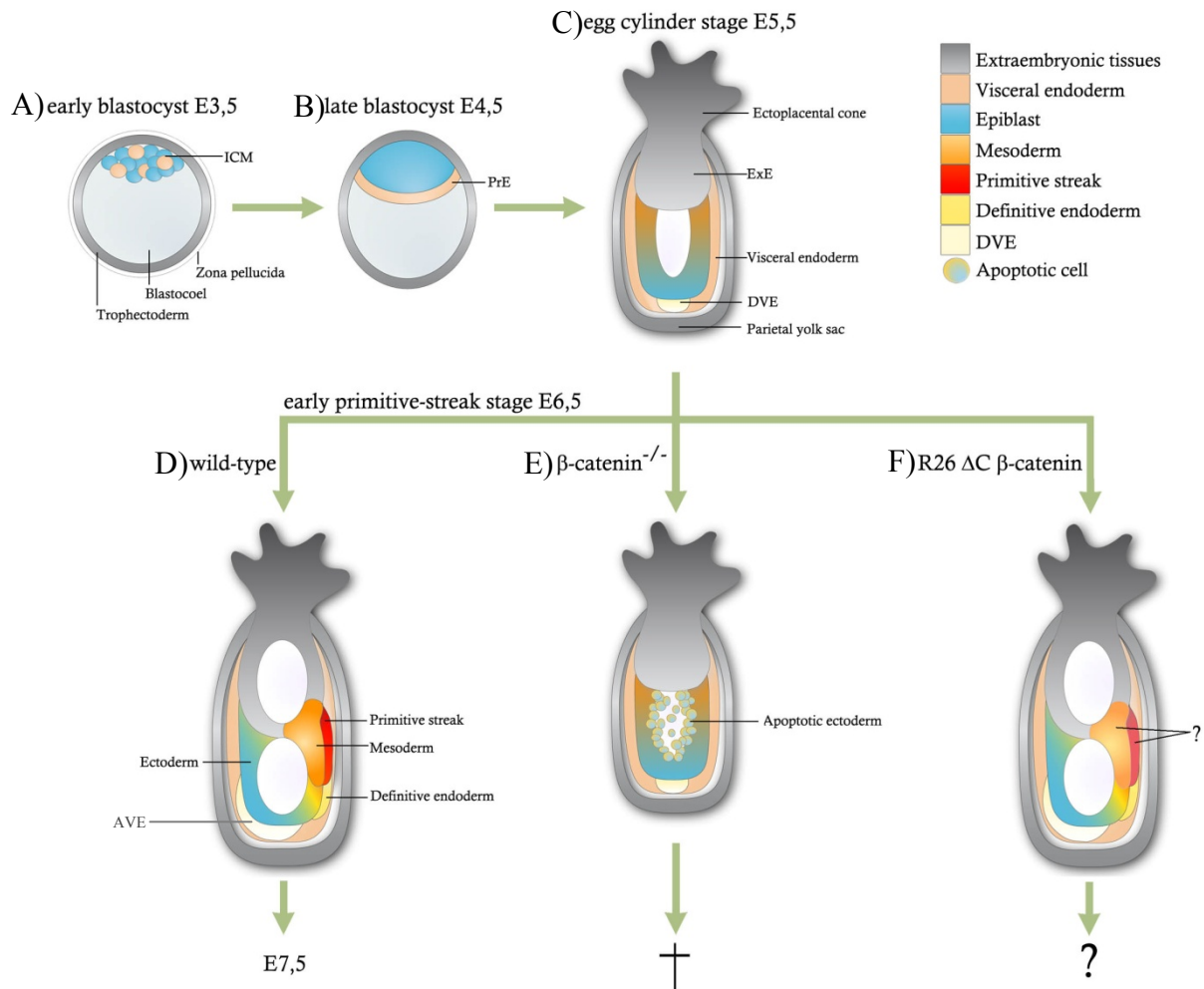


Figure 40 – Working model

The model shows the pre-implantation (A, B) and post-implantation mouse development (C, D, E, F). Wnt/ β -catenin signaling is not active during preimplantation development (A, B); however β -catenin is expressed in all developing tissues before and after implantation. The differences become apparent from the egg cylinder stage (C) onwards. In WT embryo (D) epiblast (blue) undergoes asymmetric rearrangements culminating with the primitive streak formation (orange) on the posterior side of the epiblast that gives rise to the mesoderm and definitive endoderm. Ectoderm forms at its anterior side and DVE migrates to the anterior side forming AVE. The β -catenin^{-/-} embryo (E) stops to develop at the egg cylinder stage, DVE does not migrate to the anterior side and apoptosis becomes apparent in the ectoderm. R26 Δ C embryo (F) should form definitive endoderm and ectoderm might develop further. However, it is not clear whether the primitive streak and mesoderm develop, and DVE migrates in the rescued embryo. DVE, definitive visceral endoderm; AVE, anterior visceral endoderm; ExE, extraembryonic ectoderm; PrE, primitive endoderm

3. Future directions

My analysis under self-renewal conditions points at a potential role for β -catenin and plakoglobin mediated cell-cell adhesion for proper maintenance of mouse ES cell pluripotency. However, additional studies are needed to confirm this observation. In particular, the generation of β -catenin^{fl/fl}; plakoglobin^{-/-} ES cells would be a helpful tool for further investigations, as transient knock-down of plakoglobin by siRNA does not allow to study long-term defects. Moreover, in order to rule out the existence of other types of cell junctions in mouse ES cells and to prove that, indeed, adherens junctions and desmosomes are the only cell junctions present, further characterization at the ultrastructural level, using antibody labeling specific for different adhesion-related molecules, would be necessary.

With regard to my findings, concerning the rescue of germ layers upon rescuing presumably exclusively the cell adhesion function of β -catenin, it would be necessary to perform *in vivo* blastocyst injection experiments with these lines to confirm the *in vitro* findings. This could be done by either labeling the respective ES cell lines with, for example, GFP or LacZ or by injecting the ES-cell lines into host blastocyst, that express a marker ubiquitously, thus allowing to discriminate between injected and host cells in their contribution to developing embryonic structures. Ultimately, it would be necessary to generate conditional ΔC β -catenin mice in order to study the cell-cell adhesion function of β -catenin in different tissues during embryonic or adult mouse development.

Last, but not least, it would be necessary to examine the expression of endogenous targets of Wnt/ β -catenin signaling in R26 ΔC rescued ES cells to confirm our findings, using the artificial TOPFLASH reporter. Furthermore, TCF/LEF independent β -catenin regulated targets should be analyzed to rule out that ΔC β -catenin has still some TCF/LEF independent signaling function.

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Supplementary information

Supplementary Table 4 - Top 300 Wnt3a activated genes identified by cDNA microarray analysis

Unigene_ID	Symbol	Log fold change	Fold change	adjPvalue
Mm.9086	Slc16a1	2.90	7.48	0.00464293
Mm.33764	Ivns1abp	2.64	6.24	0.00246058
Mm.31911	Ercc6l	2.43	5.40	0.00305295
Mm.4105	Lmnb1	2.43	5.40	0.00601027
Mm.245119	Lanc13	2.40	5.29	0.00293802
Mm.277024	Copb1	2.40	5.26	0.00101472
Mm.61526	Hmgcs1	2.36	5.14	0.00382273
Mm.274086	Kif18a	2.35	5.09	0.00076503
Mm.291029	Skiv2l2	2.33	5.05	0.00085122
Mm.133560	Ddx10	2.32	4.98	0.00244651
	Ccdc139	2.27	4.81	0.00081044
Mm.248779	Pop1	2.24	4.72	0.00239357
Mm.274086	Kif18a	2.23	4.68	0.00060955
	Gclc	2.21	4.63	0.00059003
Mm.239865	Hspa4	2.21	4.62	0.00557398
Mm.132208	Ipo11	2.20	4.59	0.00267387
	Opa1	2.18	4.53	0.00216536
Mm.4606	Bcat1	2.17	4.51	0.00147099
Mm.54126	Pnpla8	2.15	4.44	0.00227039
Mm.416918	4930413M19Rik	2.14	4.42	0.00300272
Mm.278471	Mphosph1	2.13	4.39	1.8661E-06
Mm.292001	Prpf6	2.13	4.38	0.00318447
Mm.291233		2.12	4.35	0.00263802
	Mthfd1	2.11	4.33	0.00390583
Mm.261453	Ect2	2.10	4.27	0.00638884
	Phip	2.08	4.22	0.00532291
	Opa1	2.07	4.20	0.00289975
Mm.278471	Mphosph1	2.07	4.19	4.7766E-07
Mm.289441	Cldn1	2.04	4.11	0.00260808
Mm.46397	Vps53	2.03	4.10	0.00287193
	Socs6	2.03	4.09	0.02083752
Mm.311585	Polh	2.03	4.07	0.00010807
Mm.4649	Cenpc1	2.02	4.05	0.00082971
	Opa1	2.01	4.02	0.00148957
Mm.38813	9030612M13Rik	2.00	4.00	0.00093302
	Otud4	1.99	3.97	0.00268413
Mm.36785	Aggf1	1.98	3.94	0.00226005
Mm.38816	Wdr36	1.97	3.92	0.00263092
	Dclre1c	1.97	3.91	4.7041E-05
Mm.200497	Hadha	1.96	3.88	0.00418915
	Rrn3	1.94	3.85	0.0035357
	Ccdc139	1.94	3.84	0.00118793

	Ccdc139	1.94	3.84	0.00092048
	Eml4	1.94	3.83	0.0009345
Mm.176695	Trim59	1.93	3.82	0.00461971
Mm.331007	Ndufc1	1.93	3.81	0.00229888
	Tmem181	1.93	3.81	2.2992E-06
Mm.2185	Bub1	1.93	3.81	0.00235023
	Papd1	1.92	3.80	0.00424986
Mm.36785	Aggf1	1.92	3.79	0.00235023
Mm.389595	Vmn2r-ps14	1.92	3.79	0.00458214
Mm.251548	Topors	1.92	3.79	0.00152598
	Nedd4	1.92	3.79	0.00046247
Mm.196638	Cdc23	1.91	3.76	0.0062297
	Zfp60	1.91	3.75	0.00050074
	Ipo7	1.90	3.74	0.00279563
	Wdr48	1.90	3.73	0.00152058
	Cpsf2	1.90	3.73	0.00239294
	Matr3	1.90	3.72	0.00244601
Mm.176695	Trim59	1.90	3.72	0.00552203
Mm.22339	Vwf	1.90	3.72	0.0015547
Mm.30660	Prpf4	1.89	3.72	0.00055843
Mm.36291	Heatr3	1.89	3.71	0.00377752
Mm.243849	Sycp1	1.89	3.69	1.0215E-05
Mm.299955	Lypla1	1.87	3.65	0.00488397
Mm.149071	Mre11a	1.85	3.62	0.00347772
Mm.426052	Afg3l2	1.85	3.60	0.00839698
Mm.28221	Utp15	1.84	3.59	0.00348573
	Gtpbp4	1.84	3.59	0.00728206
	Zmym1	1.84	3.58	0.00389193
Mm.40802	Rbm28	1.84	3.58	0.00068735
Mm.40292	Nol14	1.84	3.58	0.00234327
Mm.21629	Abcf2	1.84	3.57	0.01603707
Mm.3110	Ktn1	1.84	3.57	0.00070214
	Gtdc1	1.84	3.57	0.00360946
Mm.25298	Elp2	1.84	3.57	0.00968757
Mm.378921	Gja1	1.83	3.56	0.00021564
	Ppp1cb	1.83	3.56	0.0024201
Mm.132208	Ipo11	1.83	3.55	0.00110182
Mm.304899	Gstcd	1.82	3.54	0.00124369
	Arhgap12	1.82	3.54	0.00900016
Mm.315909	Hnrph2	1.82	3.54	0.00088799
Mm.21299	AW549877	1.82	3.53	0.00042774
	C920006C10Rik	1.82	3.52	0.00102409
Mm.265615	Wdhd1	1.81	3.51	0.00612643
Mm.28366	Ide	1.81	3.51	0.0008018
	Eif3c	1.81	3.50	0.00068573
	Yes1	1.81	3.50	0.00385311
	Atad1	1.81	3.50	0.00191982
	Zmym1	1.81	3.50	0.00017711

Mm.30088	Clpx	1.80	3.48	0.00514916
Mm.102903		1.80	3.48	0.00854619
	2610018G03Rik	1.80	3.47	0.00472144
	Mapre1	1.79	3.46	0.00278793
	Zcchc7	1.79	3.45	0.00157996
Mm.32009	5830406J20Rik	1.79	3.45	0.0031452
Mm.338175	Ints7	1.78	3.44	0.00071855
Mm.102970	Sbf2	1.78	3.44	0.00255353
Mm.227274	Prc1	1.78	3.44	0.00934741
Mm.293811	Rb1cc1	1.78	3.43	0.00012165
Mm.303534	Dr1	1.77	3.42	0.00134408
	Strbp	1.77	3.41	0.01122501
Mm.34606	Krr1	1.77	3.40	0.00264817
	Ifi203	1.77	3.40	0.01336614
Mm.282700	Hars2	1.76	3.39	0.00264703
Mm.291928	Ctnnb1	1.76	3.39	0.00041324
	Phtf2	1.76	3.38	0.00928041
Mm.286061	Tars	1.76	3.38	0.0011551
Mm.40292	Nol14	1.75	3.37	0.00128992
Mm.380307	Nfasc	1.75	3.37	0.00147134
	Atrx	1.75	3.37	7.5969E-05
Mm.10027	Prpf4b	1.75	3.36	0.00015469
Mm.371585	Suc1g2	1.75	3.35	0.00433728
Mm.40292	Nol14	1.74	3.35	0.00049629
	Opa1	1.74	3.35	0.00194607
Mm.30264	Chfr	1.74	3.34	0.00152598
Mm.2238	Eif3a	1.74	3.34	0.00046217
	Vcp	1.74	3.34	0.00236143
Mm.292109	Casc4	1.74	3.34	0.00286549
Mm.209419	Hspa9	1.74	3.34	0.00717005
Mm.200497	Hadha	1.74	3.33	0.00454667
	A430062P19Rik	1.74	3.33	0.00297018
	Plaa	1.74	3.33	0.00252746
	Mdm4	1.73	3.32	0.01637867
	D230025D16Rik	1.73	3.32	0.00344089
	Kit	1.73	3.31	0.00883776
Mm.142872	Hnrpk	1.72	3.30	0.00143581
	Dsp	1.72	3.30	0.00110186
Mm.36785	Aggf1	1.72	3.29	0.00279563
Mm.259638	4933421E11Rik	1.72	3.29	0.00127523
Mm.22480	Dmtf1	1.72	3.29	0.00811389
Mm.219675	Gfm2	1.71	3.28	0.00047379
Mm.251255	Ddx1	1.71	3.27	0.00282537
Mm.389595	Vmn2r-ps14	1.71	3.27	0.00244419
Mm.267061	Ddx6	1.71	3.26	1.5379E-05
Mm.291029	Skiv2l2	1.70	3.25	0.0005998
Mm.71710	Axin2	1.70	3.25	0.00068437
Mm.34606	Krr1	1.70	3.25	0.00081411

	Dtl	1.70	3.24	0.0014403
	Fasn	1.69	3.23	0.00019324
Mm.1904	Ttk	1.69	3.23	0.00053807
	2810405J04Rik	1.69	3.23	0.00442262
Mm.439768	Fgf14	1.69	3.23	0.00209416
Mm.219684	Usp53	1.69	3.22	0.00025697
	Rngtt	1.69	3.22	0.0011089
	Hnrnpr	1.69	3.22	0.00239357
	Dock2	1.69	3.22	0.00214788
	Atad1	1.68	3.21	0.00124369
	E2f2	1.68	3.21	0.00124369
	Arl15	1.68	3.20	0.00287338
Mm.66853	Iws1	1.67	3.19	0.00173972
Mm.25794	Fbx15	1.67	3.19	0.00251847
Mm.247566	Meis2	1.67	3.19	0.00077775
	Rnd3	1.67	3.19	0.00139284
Mm.284654	Wdr37	1.67	3.18	0.00068735
Mm.271986	Pcmt2	1.67	3.18	0.00166474
	Tmem181	1.67	3.18	0.00013704
Mm.463007	2900073C17Rik	1.67	3.18	0.02297919
Mm.87389	Wdr35	1.67	3.17	0.00422119
Mm.390978	A930001N09Rik	1.67	3.17	0.00657119
	Pfkip	1.66	3.17	0.01098636
Mm.276279	Zfp667	1.66	3.17	0.00022379
	Atrx	1.66	3.17	9.4745E-05
	Bclaf1	1.65	3.15	0.01108978
	Hmgcr	1.65	3.15	0.0009897
Mm.188979	9530056K15Rik	1.65	3.14	0.01679349
Mm.25527	Hbs1l	1.65	3.14	0.00144553
Mm.356580	Aftph	1.65	3.13	0.00021535
	Wdr3	1.64	3.13	0.00142507
		1.64	3.13	0.010608
Mm.24593	4833439L19Rik	1.64	3.12	0.01396053
	Pi4k2b	1.64	3.11	0.00341587
	Nfib	1.64	3.11	0.01047721
Mm.38927	Ppil4	1.64	3.11	0.00138532
	Exod1	1.64	3.11	0.00174312
Mm.133560	Ddx10	1.63	3.10	0.00118549
Mm.21848	Bzw1	1.63	3.09	0.00017464
	Ap1g1	1.63	3.09	0.00507989
	Terf2	1.62	3.08	0.01930874
	Dars	1.62	3.08	0.00075126
	Ccdc88a	1.62	3.08	0.00032835
Mm.67366	Zfp75	1.62	3.08	0.00222876
Mm.294503	Trmt11	1.62	3.07	0.0025469
Mm.271178	Arl8b	1.62	3.07	0.00197415
	Rnf6	1.62	3.07	0.00103874
Mm.3131	Dld	1.62	3.07	0.00042822

Mm.61526	Hmgcs1	1.62	3.07	0.00054299
Mm.689	Acvr1	1.62	3.07	0.0018538
Mm.12568	Nup107	1.61	3.05	0.00381945
	Ccne2	1.61	3.05	0.00024525
	Cep57	1.61	3.05	0.0020724
	Trim23	1.61	3.05	0.00301432
Mm.28152	BC055324	1.61	3.05	0.00284053
Mm.4375	Fto	1.61	3.04	0.00268413
	1700047O18Rik	1.61	3.04	0.00508761
	Ktelc1	1.60	3.04	0.00251168
	Sfrs1	1.60	3.03	0.00544874
Mm.290704	C330007P06Rik	1.60	3.03	0.00251168
Mm.403916		1.60	3.03	0.00275377
Mm.30088	Clpx	1.60	3.03	0.00195461
Mm.390681	P2ry5	1.59	3.02	0.00060955
	Ccne2	1.59	3.02	0.0002024
Mm.282743	Ikbkap	1.59	3.01	0.00161842
	Hnrpf	1.59	3.01	0.01037536
	Atp2c1	1.59	3.01	0.00104151
	Zcchc7	1.59	3.00	0.01236954
Mm.31911	Ercc6l	1.59	3.00	0.00059118
Mm.183042	Impa1	1.58	3.00	0.00017711
Mm.207345	Zfp449	1.58	3.00	0.0025469
Mm.301585	Tbc1d24	1.58	3.00	0.00091959
Mm.221499	Disp2	1.58	3.00	0.01227483
Mm.290791	Ndufs1	1.58	2.99	0.00216009
Mm.16753	Chek1	1.58	2.99	0.0002024
Mm.22448	Ckap2	1.58	2.99	0.00217175
	C230085J04Rik	1.58	2.99	0.00115651
Mm.183102	Actr3	1.58	2.99	0.00163235
	Srd5a1	1.58	2.99	0.00138738
Mm.29515	Pxk	1.58	2.98	0.00274117
Mm.255858	Ctr9	1.57	2.97	0.00236041
Mm.383091	Kif4	1.57	2.97	0.00417878
Mm.87773	Hsp90b1	1.57	2.97	0.00507989
Mm.38927	Ppil4	1.57	2.97	0.00046247
	Smek2	1.57	2.96	0.00012777
Mm.276109	Cwf19l1	1.57	2.96	0.00481453
Mm.143719	Dll4	1.57	2.96	0.00108036
	Piga	1.57	2.96	5.6447E-05
	C430003P19Rik	1.56	2.96	0.00051901
Mm.7709	Scnn1b	1.56	2.96	0.00191013
Mm.274086	Kif18a	1.56	2.95	0.00188096
	Hook3	1.56	2.95	0.00894639
Mm.326089	Top3b	1.56	2.95	0.02021386
	Fem1c	1.56	2.95	0.00109614
Mm.41447	Chd1l	1.56	2.94	0.00868475
	Suz12	1.56	2.94	0.00298939

	D030022P06Rik	1.56	2.94	0.02645496
Mm.443996	9030612E09Rik	1.55	2.94	0.0026614
Mm.4606	Bcat1	1.55	2.94	0.00101289
Mm.335237	Zwilch	1.55	2.93	0.01089622
Mm.303534	Dr1	1.55	2.93	0.00121184
	Atrx	1.55	2.93	7.4313E-05
	Slc25a13	1.55	2.93	0.00299202
	Tpx2	1.55	2.92	0.0029598
Mm.255044	Pank3	1.55	2.92	0.02325396
Mm.25794	Fbxl5	1.54	2.92	0.00251764
	Ptcd3	1.54	2.91	0.00810648
	Eif3c	1.54	2.91	0.00827024
Mm.28524	D6Wsu116e	1.54	2.90	0.000248
Mm.329194	Lycat	1.54	2.90	0.00463499
Mm.25548	Kpna3	1.54	2.90	0.00087915
Mm.275354	Qtrtd1	1.53	2.90	0.00163612
	Pbx1	1.53	2.90	0.00281179
Mm.154078	Gm1964	1.53	2.89	3.9787E-05
	Wdr3	1.53	2.89	0.00207322
Mm.100450	Sptlc3	1.53	2.89	0.0036876
	Trpc1	1.53	2.88	0.01554793
	Mtm1	1.53	2.88	0.00581665
	Serinc3	1.53	2.88	0.00569264
	C430048L16Rik	1.53	2.88	0.00081501
	2900084C01Rik	1.53	2.88	0.01198282
	Stam	1.53	2.88	0.00085522
Mm.196638	Cdc23	1.53	2.88	0.0019755
	Alkbh8	1.53	2.88	0.00097473
Mm.271986	Pcmt2	1.52	2.88	0.00287338
	B230312C02Rik	1.52	2.87	0.00838793
Mm.9086	Slc16a1	1.52	2.87	0.00463022
Mm.20522	Lancl1	1.52	2.87	0.00050227
Mm.186329	Trpa1	1.52	2.87	0.00056293
Mm.390681	P2ry5	1.52	2.87	0.00032815
Mm.10100	Trpc4	1.52	2.86	3.1976E-05
Mm.443222	Pot1a	1.52	2.86	0.00058993
Mm.20294	Sephs2	1.52	2.86	0.00880138
Mm.290341	Tmem50b	1.51	2.86	0.00611202
Mm.21649	Nkrf	1.51	2.85	0.00288582
Mm.290602	4930453O09Rik	1.51	2.85	0.00059118
	Nek2	1.51	2.85	0.00157499
Mm.378921	Gja1	1.51	2.85	0.00042244
	EG622129	1.51	2.85	0.0022908
Mm.271178	Arl8b	1.51	2.85	0.00360498
Mm.284944	Hsd17b13	1.51	2.85	0.0039017
Mm.137021	Tcfap2b	1.51	2.84	0.00060595
	Cul5	1.51	2.84	4.9877E-05
	Lrrc40	1.51	2.84	0.00667631

Mm.100125	Sh3bgrl2	1.51	2.84	0.00011725
	AI314180	1.50	2.84	0.00469518
	Pscdbp	1.50	2.84	0.00117969
	Fyttd1	1.50	2.83	0.00138372
Mm.65316	Nos1ap	1.50	2.83	0.00585472
Mm.41715	Gpbp1l1	1.50	2.83	0.00284791
Mm.273769	D030074E01Rik	1.50	2.83	0.00332841
Mm.390681	P2ry5	1.50	2.83	0.00104881
Mm.1904	Ttk	1.50	2.83	0.0005968
	Dars	1.50	2.83	0.00046247
	AF529169	1.50	2.83	0.00053611

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Manuscript

Title: Mice lacking the orphan receptor Ror1 have distinct skeletal abnormalities and are growth retarded

Natalia Lyashenko¹, Martina Weissenböck¹, Amnon Sharir², Reinhold G. Erben³, Yasuhiro Minami⁴, Christine Hartmann^{1*}

¹ Institute of Molecular Pathology, Dr. Bohrgasse 7, 1030 Vienna, Austria

² Department of Molecular Genetics, Weizmann Institute of Science, Rehovot 76100, Israel

³ Department of Biomedical Sciences, University of Veterinary Medicine, 1210 Vienna, Austria

⁴ Division of Cell Physiology, Department of Physiology and Cell Biology, Graduate School of Medicine, Kobe University, Kobe, Japan

*corresponding author

email: hartmann@imp.ac.at

phone: +43-1-797303390

Abstract

Ror1 is a member of the Ror-family receptor tyrosine kinases. Ror1 is broadly expressed in various tissues and organs during mouse embryonic development, however, so far little is known about its function. The closely related family member Ror2 was shown to play a crucial role in skeletogenesis and has been shown to act as a co-receptor for Wnt5a mediating non-canonical Wnt-signaling. Previously it has been shown that during embryonic development Ror1 acts in part redundant with Ror2 in the skeletal and cardiovascular systems. In this study we report that loss of the orphan receptor Ror1 results in a variety of phenotypic defects within the skeletal and urogenital systems and that *Ror1* mutant mice display a postnatal growth retardation phenotype.

Introduction

Ror1 is one of the two vertebrate Ror-family members of orphan receptor-tyrosine kinases (Green et al., 2008; Minami et al., 2009; Yoda et al., 2003). Like its closely related family member Ror2, Ror1 contains extracellular a cysteine rich domain (CRD) similar to the CRD domain of the Wnt-receptor frizzled (Saldanha et al., 1998). For Ror2 binding to Wnt-ligands as well as to the BMP/TGF β -family member, Gdf5, has been demonstrated (Liu et al., 2008; Oishi et al., 2003; Sammar et al., 2004). Ror2 activity can mediate non-canonical Wnt-signaling through interaction with Wnt5a, and modulates the canonical Wnt-signaling pathway through differential interaction with the Frizzled receptor (He et al., 2008; Hikasa et al., 2002; Li et al., 2008; Mikels and Nusse, 2006). Ror1 has been recently demonstrated to be capable of binding the Wnt5a ligand as well, however, if this binding results in functional activity is currently not known (Fukuda et al., 2008). Both *Ror* genes are widely expressed during embryonic development, in limbs, teeth, heart, lung, liver, gut, and hippocampal neurons (Al-Shawi et al., 2001; Matsuda et al., 2001; Oishi et al., 1999; Paganoni and Ferreira, 2003; Rodriguez-Niedenfuhr et al., 2004; Schwabe et al., 2004). Mutations in *ROR2* in humans cause autosomal dominant brachydactyly type B (BDB) and are associated with autosomal recessive form of Robinow syndrome (RS) (Afzal et al., 2000; Hamamy et al., 2006; Oldridge et al., 2000; Schwabe et al., 2000; van Bokhoven et al., 2000). Loss of *Ror2* in mice results in phenotypic changes resembling RS (DeChiara et al., 2000; Schwabe et al., 2004; Takeuchi et al., 2000). The point mutation Ror2-W749X, linked to human BDB, behaves as a recessive mutation in the mouse causing brachydactyly and models recessive RS (Raz et al., 2008). In addition, ROR family member have been implicated in tumor formation in humans. ROR1 has been found to be overexpressed in patients with acute and chronic lymphoblastic leukemia (Daneshmanesh et al., 2008; Shabani et al., 2007; Shabani et al., 2008) and was found to act as a survival kinase in HeLa cervical carcinoma cells (MacKeigan et al., 2005). ROR2 has been found to be overexpressed in squamous cell carcinoma and to regulate osteosarcoma cell invasiveness (Enomoto et al., 2009; Kobayashi et al., 2009). Previously it was reported that loss of *Ror1* in mice results in perinatal lethality due to respiratory defects, but that these mice lack any abnormalities in skeletogenesis (Nomi et al., 2001). Here, we have re-examined the *Ror1*-ablated mutants and found that the mice have subtle skeletal defects at birth; they showed fusions of the sternbrae, a cleft in the basisphenoid bone, and abnormal development of the cervical vertebral element C2. Homozygous mice survived in our facility and displayed abnormal synchondrosis in the cranial base, postnatal growth retardation and age-related

skeletal changes. Furthermore, we observed additional phenotypic defects in *Ror1*^{-/-} mutants, such as female infertility probably due to an imperforated hymen, kidney defects and occasionally enlarged seminal vesicles in *Ror1*^{-/-} males.

Material and Methods

Mouse husbandry

The generation of the *Ror1* mutant allele has been previously described (Nomi et al., 2001). The *Ror1* strain was maintained in a C57Bl/6 background. Mutants were generated by intercrosses of heterozygous mice, or through breeding of homozygous males with heterozygous females to increase the number of mutant offspring. Genotyping of mice was performed by PCR using the following primer pairs: for detection of the 1.1kb wild-type allele the WT_for-primer 5'GGCAACAAATGGCAAGAAAGTGGTGTC and WT_rev-primer 5'GAAATGGAATCCTTAGACTCCGTTATC were used. For detection of the 1.1kb mutant allele, the WT_for-primer was used in combination with the neo-primer 5'ATCGCCTTCTATCGCCTTCTTGACGAG.

Skeletal preparations, microCT and bone densitometry

For the alcian blue /alizarin red staining of newborns, postnatal and adult mouse skeletons the mice were sacrificed, skinned, eviscerated, fixed in 95% ethanol and stained according to (McLeod, 1980). Harvested organs were visually inspected for abnormalities. Three dimensional medium-resolution images were obtained from the skulls, spine, ribs and digits of *Ror1*^{-/-} and control mice (*Ror1*^{+/-}; *Ror1*^{+/+}) using microcomputed tomography (eXplore locus SP, GE Healthcare, London, Ontario, Canada). Scans were taken at 28 µm isotropic resolution and 720 projections were acquired over an angular range of 360°. Images were reconstructed and thresholded to distinguish bone voxels with MicroView software version 5.2.2 (GE Healthcare). One threshold was chosen for all specimens (or as previously described Amarilio et al., 2008). Volumetric bone mineral density (BMD) of the femurs was measured by peripheral quantitative computed tomography (pQCT) using a XCT Research M+ pQCT machine (Stratec Medizintechnik, Pforzheim, Germany) as described (Schneider et al., J Bone Miner Res 24:455-67, 2009). One slice (0.2 mm thick) in the mid-diaphysis of the femur, and 3 slices in the distal femoral metaphysis located 1.5, 2, and 2.5 mm proximal to the articular surface of the knee joint were measured. BMD values of the distal femoral metaphysis were calculated as the mean over 3

slices. A voxel size of 0.070 mm and a threshold of 600 mg/cm³ were used for calculation of BMD. .

Histology and section in situ hybridizations

For histology and section in situ hybridizations material from embryos and pups was dissected, washed in PBS and fixed overnight in 4% PFA/PBS, dehydrated to 70% ethanol, and processed using a standard program of the Tissue-Tek VIP5 Vacuum Infiltration Processor (Sakura). Processed tissue was embedded in paraffin and sectioned at 5µm. Hematoxylin/eosin stainings were performed using standard protocols. Alcian blue / van Kossa staining on sections were performed as following: tissue was rehydrated, washed twice in deionized water, exposed for 60 minutes in 2% silver-nitrate solution to a 60 watt lamp, washed three times in deionized water, incubated for 2 minutes in 1% acetic acid, stained with alcian blue solution (pH 2.2) for 15 minutes, washed in 1% acetic acid, dehydrated into 75% ethanol, counterstained for 30 seconds with eosin, destained with 100% ethanol and xylene and mounted using DPX (Fluka). Section in situ hybridizations were done as previously described (Murtaugh et al., 1999).

Immunohistochemistry, Western blot and Elisa for IGF1 serum levels

Immunohistochemistry for the following hormone precursors, GH (1:10000), TSHβ (1:2000), LHα (1:2000), LHβ (1:2000), ACTH (1:3000), was performed on paraffin sections of the pituitaries from E17 embryos and P2 mice. Antibodies were obtained from the National Hormone and Pituitary Program and immunohistochemistry was performed using the Ventana. Briefly sections were dewaxed and rehydrated, antigen-retrieval was done using citrate buffer pH6.0. Western blots to determine GH protein levels were performed on 50 µg protein extracts from dissected pituitaries from P17 & P19 mice (α-GH at a dilution of 1:2000). IGF1 serum levels were determined in the plasma of *Ror1*^{-/-} and control mice at 3 months of age using an IGF-I Elisa assay following the manufacture instructions (Quantikine Kit, R&D systems).

Results

*Skeletal defects in *Ror1*^{-/-} embryos*

Ror1 mutant embryos displayed very distinct skeletal defects, which were apparent prior to birth. The sternum of *Ror1*^{-/-} embryos was shortened and showed fusion of caudal sternebrae (S) involving to a variable degree S1-S4 (Fig. 1A and Table 1). In addition the xyphoid process

was abnormally bifurcated and had two ossification centers (Fig. 1A). Defects in sternal development were already detectable at E15.5 where the two sternal bands were still separated from each other in the caudal region of *Ror1*^{-/-} embryos, while they were already almost completely fused along the entire length in the wild-type littermate controls (Fig. 1B). The sternal-costal junctions, demarcations between the end of the ribs and the sternal tissue, can be distinguished by their reduced reactivity with the proteoglycan staining reagent alcian blue and low levels of *Collagen 2 α 1* (*Col2 α 1*) transcript, appeared right at the sternal bands in the wild-type (see arrowheads in Fig. 1Ba, b), while they were located more laterally in the *Ror1* mutants (Fig. 1Ba', b'). In situ hybridizations for the prehypertrophic marker, *Ihh*, revealed that the onset of chondrocyte differentiation occurs normal in the *Ror1* mutant sterni (Fig. 1Bc, c'). However, in the caudal part the *Ihh* expression domains were not absolutely restricted to the intercostal segments (arrowheads in Fig. 1Bc'). At E17.5 the abnormal shape of the shape and location of the sternal-costal junctions was still present in the mutant sterni. The sternal bands had either begun to fuse abnormally, visible by the presence of alcian blue positive and *Col2 α 1* (not shown) expressing cells in the midline, or were still separated by a small band of none-chondrogenic cells (data not shown). However like in the wild-type, chondrocytes within the sternal bands had matured into hypertrophic chondrocytes, which expressed *Collagen 10 α 1* (*Col10 α 1*) and *Osteopontin* (*Opn*) and produced mineralized matrix in all mutants examined (Fig. 1Ca-c'). In contrast to the controls where this process was restricted to the intercostals regions, hypertrophic cells differentiated also at the ends of ribs in the mutants leading to the formation of continuous hypertrophic domains in the region of sternebrae 3-4 (Fig. 1Cb', c'). Concomitantly, *Ihh* expression was altered and expressed all around the hypertrophic regions (Fig. 1Cd, d'). The abnormal expression of maturation markers, such as *Ihh* and *Col10 α 1* suggests that the absence of functional *Ror1* affects the normal organization into a growth plate-like structure in the intercostals region, instead growth plate-like structures are now located at the end of the ribs. This defect was even more apparent at P0 where *Col10 α 1* and *Ihh* were expressed almost all around the fused sternebrae elements, including the regions at the ends of the ribs (data not shown).

In addition to the defects in the sternum we detected skeletal abnormalities in the cervical vertebral element C2 and the base of the skull in newborns (P0). The cervical element C2 appeared in most mutants wider and was split in some mutants (Fig. 2A). In the skulls of mutant newborns clefts were present in the posterior region of the basisphenoid bone and in the anterior borders of the exoccipitale bones in 100% of the mutants analyzed (n=9; Fig. 2B). At

P5 the clefts had developed into holes, which were still visible at later stages and in adults (Fig. 2C, D, and data not shown). Furthermore, we noticed in the cranium of P5 and P19 heads a bony bridge within the spheno-occipitale synchondrosis connecting the basioccipitale with the basisphenoid bone (arrows in Fig. 2C, D). This resulted in premature synostosis of the spheno-occipitale synchondrosis in the cranial base and an overall shortening of the skull with a more dome-shaped appearance in X-rays at the adult stage (Fig. 2E). At P5 it was also apparent, that the vomeral bones were hypoplastic (Fig. 2C). In the P17 and P19 mutant heads we noticed a hole in the exoccipitale bones, which probably was the result of the cleft that we had noticed in the newborn skulls (n= 5; Fig. 2D). The fusion of the basisphenoid and basioccipitale bone was also clearly visible on microCTs of aged mutant specimens (Fig. 2F).

Growth retardation of the *Ror1* mutant pups became apparent at P2 (see Fig. 4), associated with morphological and molecular changes in the growth plates of long bones (Fig. 3B), while there were no changes detectable at E17.5 or in newborns (Fig. 3A and data not shown). At P2 the prehypertrophic, *Ihh* expressing and the hypertrophic, *Col10a1* expressing zones of the mutant growth plates were shorter compared to wild-type or heterozygous littermate controls (Fig. 3B). In the wild-type growth plates at P2 only the last 3-4 rows of hypertrophic chondrocytes had mineralized matrix, while the hypertrophic chondrocytes towards the articular region were slightly smaller and had a non-mineralized matrix (Fig. 3B). Similarly, *osteopontin* was expressed in the last 3-4 rows of hypertrophic chondrocytes in the wild-type growth plate (Fig. 3B). In contrast, in the *Ror1* mutant growth plate only 3-4 rows of hypertrophic cells were present, which all expressed *osteopontin* and had a slightly hyper-mineralized matrix (Fig. 3B). Thus, loss of *Ror1* seems to affect the differentiation of a distinct subpopulation of hypertrophic cells and prehypertrophic chondrocytes. Examination of P5 specimens revealed a similar phenotype in the growth plate, with a hyper-mineralized matrix and reduced zones of *Ihh* and *ColX* expressing chondrocytes in the mutant (Fig. 3C). In addition, we noticed in the skeletal preparations as well as in the sections that the formation of the secondary ossification center was delayed in *Ror1* mutants (Fig. 3C and data not shown).

Ror1 mutants show postnatal growth retardation and have a reduced life expectancy

At postnatal day 2 *Ror1* mutant pups could easily be identified by their reduction in size and weight (Fig. 4A, B). Statistically significant reduction in the weight was apparent already at P1, however, the size reduction was less apparent at that age (Fig. 4A and data not shown). Within the first 6 postnatal days mutant pups reached only approximately 50% of the weight of

their heterozygous and wild-type littermates (Fig. 4A). The life expectancy of the mutants was reduced, only 60% of the mutants (32/53) survived until weaning (Fig. 4C). The reduced life expectancy might in part be due to the occasional kidney defects (see below) or physical weakness of the runt pups compared to their littermates. *Ror1*^{-/-} pups, which survived until after weaning stayed smaller and reached only 60-75% of the weight of their littermates (Fig. 4D, F). X-rays of 3 months old female mice suggested a decrease in bone density and in cortical thickness (Fig. 4D, see also inset). This finding prompted us to examine volumetric bone mineral density (BMD) and bone geometry by peripheral quantitative computed tomography. Femurs from *Ror1*-ablated male and female mice were characterized by reduced cross-sectional area, total BMD, and cortical thickness in the shaft and the metaphyseal region (Table 2). Cortical bone osteopenia at the femoral shaft was more pronounced in females than in males (Table 2) (waer mir da aber nicht so sicher, denn bei n = 2 kann man eigentlcih wenig sagen, varianz in der male-Gruppe ist hoch). Likewise, the reduction in weight and size was more prominent in females than in males at the age of 3 months (Fig. 4F and data not shown). Together, this suggests a possible involvement of sex hormones in the expressivity of the mutant phenotype. Mutant males aged for 13-18 months still showed severe weight reduction (32-60%), however, the difference in size was relatively small (5-10%) compared to their littermate controls (Table 3).

Abnormal ossifications develop in the spine of aged Ror1 mutants

In alizarin red / alcian blue stained skeletal preparations of aged mutant mice (15-18 months) we observed abnormal mineralizations in the intervertebral discs of the lower thoracic region (n=2/2; T10-T13; arrow heads in Fig. 5A) and occasionally within un-fused sternal synchondroses (data not shown). Abnormal ossifications within the axial skeleton could also be observed in microCTs of the lower thoracic spine of 15-18 months old male mice (n=2/3; arrows in Fig. 5B). In addition, we noticed that there were abnormal ossifications between the spine and the proximal ends of the ribs (white arrowheads in Fig. 5C). The reduction in cortical bone thickness was also visible in the microCTs of the ribs and vertebral bodies (Fig. 5C and data not shown).

IGFI serum levels are reduced in Ror1 mutants

Given that the hole in the basisphenoid bone of *Ror1*^{-/-} embryos was located directly below the pituitary gland (see Fig. 6A), we suspected that the postnatal growth retardation might be due to altered hormone production by the pituitary gland. Thus, we examined the specification of different hormone producing cells using immunohisto-chemical stainings for growth hormone (GH), thyroid-stimulating hormone beta (TSHβ), luteinizing hormone alpha and beta (LHα, β) and adrenocorticotropin (ACTH) on E17.5 and P2 sagittal sections. However, no obvious differences in hormone staining were detected between wild-type and *Ror1* mutant pituitaries at both stages of development (Fig. 6A and data not shown). Older pituitary glands of *Ror1* deficient animals were slightly hypoplastic and had a different shape compared to wild-type and heterozygous littermate controls (Fig. 6B and data not shown). Western blots on pituitary gland extracts of P17 and P19 old littermates revealed, however, no difference in GH levels (Fig. 6C). Although GH production seemed not to be affected, its possible that the levels of secreted GH might be altered in *Ror1*^{-/-} animals. Since GH synthesis and release from pituitary somatotrophs is pulsatile, the serum GH levels fluctuate accordingly. Thus, instead of measuring the serum GH levels we measured the serum levels of IGF-I, since they directly depend on GH levels. The IGF-I levels were indeed reduced in 3 months old male and female *Ror1* mutant animals (Fig. 6D).

Urogenital defects in Ror1 mutant animals

Approximately 90% of the *Ror1* mutant females had an abnormal vagina with an imperforated hymen and could therefore not reproduce (Fig. 7A). With age these *Ror1*^{-/-} females accumulated liquid and cellular material in their uteri resulting in a swelling of the uteri and abdomen (Fig. 7B, C). Almost all *Ror1* mutant males tested could reproduce normal, but 3/7 dissected *Ror1*^{-/-} males showed abnormal seminal vesicles that were either cystic or solid and unilateral enlarged (Fig. 7D, D'). Approximately 30% (5/17) of the *Ror1*^{-/-} mice had abnormal kidneys, where double kidneys and double ureters were present unilateral (Fig. 7E). All of the mutants with kidney defects had been found dead within the first week, with the exception of one male that was sacrificed at the age of 13 months that had a cystic right kidney (see Table 1). In addition to the urogenital defects, we noticed that the mutant animals had almost no subcutaneous fat and that the visceral fat pads associated with the urogenital system were also reduced in size (Fig. 7D, F).

Discussion

In contrast to Ror2, loss-of Ror1 activity resulted only in minor skeletal defects, affecting primarily the sternum, the basisphenoid bone and the axial element C2 during embryonic development. Ror1 and Ror2 have been implicated as co-receptors in Wnt-signaling and for Ror2 it was shown that it can modulate Gdf5/BMP receptor signaling (Green et al., 2008; Sammar et al., 2004). As such it is interesting to note that a similar phenotype in the basisphenoid bone has been observed in embryos double mutant for *Tcf4/Lef1* during development at E15.5 and in mutants for *Bmp7* or conditional mutants of the Tgf-beta receptor 2, *Tgfb β 2* ^{Δ Col2}, (Baffi et al., 2004; Brugmann et al., 2007; Jena et al., 1997). Furthermore, it is interesting to note that double mutants for the heparan sulfate 6-O-endosulfatase genes *Sulf1* and *Sulf2*, which alter sulfate modification of heparan molecules in the extracellular matrix, showed a similar phenotype in the basisphenoid bone (Holst et al., 2007; Ratzka et al., 2008). In these mutants signaling of several growth factors (including Wnts, hedgehogs and fibroblast growth factors) is probably affected. Mutants for *Gli3* ^{Δ 699/ Δ 699}, which encodes only a N-terminal truncated version of Gli3 solely acting as a repressor, also have a hole in the basisphenoid bone (Bose et al., 2002). Fusions of sternbrae or ectopic mineralization within the fibrocartilage separating the sternbrae have been reported also in *Sulf1/2* double mutants and in mutants for the TGF β /BMP family members, *Bmp5* and *Gdf5* (Holst et al., 2007; Ratzka et al., 2008; Storm and Kingsley, 1996). Thus given the phenotypic similarities it might be possible that in analogy to Ror2, Ror1 might not only interact with Wnt-ligands altering Wnt-signaling, but might also modulate TGF β /BMP signaling.

Besides the patterning defects in certain skeletal elements, we noticed postnatal alterations in the growth plates of the long bones of *Ror1* mutants. Here the zones of prehypertrophic and hypertrophic chondrocytes were reduced from postnatal day 2 onwards. Based on our analyses, it seemed that primarily the zone of immature hypertrophic chondrocytes, in which the cells do not have a mineralized matrix nor do they express osteopontin, is severely reduced or maybe even lost. This specific defect in chondrocyte maturation might contribute to the fact that the appearance of the secondary ossification centers was delayed as well. Currently, we have no explanation for the molecular nature of the specific defect. The previous study by Nomi and colleagues suggests that Ror1 has redundant functions with Ror2 during embryonic skeletal development (Nomi et al., 2001). However, the study gave no insights into the underlying mechanism explaining the further shortening of the skeletal elements particularly in the stylopode region. Ror2 seems to be regulating chondrocyte maturation primarily at the

level of prehypertrophic chondrocytes (Schwabe et al., 2004). Our analysis suggests that in contrast to *Ror2*, *Ror1* might be required at a slightly different step of chondrocyte maturation acting primarily in immature hypertrophic chondrocytes. This could explain the additional decrease in overall length and of the mineralized zone of the stylopode elements, humerus and femur, upon loss of *Ror1* in a *Ror2* mutant background. This would also explain why there was no significant effect on zeugopode elements such as ulna and radius upon additional removal of *Ror1* (Nomi et al., 2001). Here the differentiation of prehypertrophic and hypertrophic chondrocytes is already severely delayed in *Ror2* single mutants, while it still occurs in the stylopode at E15.5 (Schwabe et al., 2004).

In addition to the specific reduction in immature hypertrophic chondrocytes, the postnatal growth deficiency might in part be influenced by the reduction in IGF-I serum levels but is probably also influenced by sex hormones given that the differences in size are much more pronounced in females than in males. Whether the reduced IGF-I serum levels are related to altered growth hormone production due to the morphological alteration in the basisphenoid bone and the associated pituitary gland deformation or if they might be due to local *Ror1* activity in IGF-I producing organs is currently unclear. While *Ror1* is expressed fairly broadly in the organism it is not expressed in the liver, which is the major site of IGF-I production (Al-Shawi et al., 2001; Baskar et al., 2008). Concomitantly, liver-specific deletion of *Igf-1* does not affect growth of the appendicular skeleton (Sjogren et al., 2002; Yakar et al., 1999). Local IGF-I production in the growth plate of the long bones is probably affected in *Ror1* mutants, given the reduction in the hypertrophic zone that is the predominant region in which *Igf-1* is transcribed postnatally (Reinecke et al., 2000; Smink et al., 2002). IGF-I has been shown to stimulate hypertrophic chondrocyte differentiation (Mushtaq et al., 2004), thus a reduction in IGF-I producing hypertrophic chondrocytes might exaggerate the phenotype. Our phenotypic analysis suggests that *Ror1* activity is required for postnatal growth and hypertrophic chondrocyte differentiation and that the phenotype is possibly mediated in part via the GH/IGF-I system. Since *Ror1* is fairly broadly expressed it is currently unclear to what extent the growth plate phenotype is due to a requirement of *Ror1* activity particular in chondrocytes. In addition, it is well known that cortical bone mass and size is severely reduced in *IGF-I*-ablated mice (Moerth et al. Endocrinology 2007). Therefore, it remains to be determined whether the cortical bone osteopenia observed in *Ror1* mutants is caused by lack of *Ror1* and concomitantly reduced Wnt signaling in periosteal osteoblasts, or by an indirect effect through the GH/IGF-I axis.

Furthermore, our phenotypic analysis revealed a requirement for Ror1 in the urogenital system. The relatively low penetrance of the kidney defects makes it difficult to study the underlying mechanisms. Interestingly, we noticed in *Ror2* mutants a very similar kidney phenotype with double ureters, unilateral or bilateral double kidneys, cystic kidneys or even sporadically agenesis of one kidney in 30-40% of *Ror2* mutants (C. Hartmann, unpublished observation). Thus, it is possible that Ror1 and Ror2 act redundantly in kidney development, although no defect in kidney development was reported in the double mutant analysis (Nomi et al., 2001). It is possible that Ror1 and Ror2 modulate Wnt or TGF- β /BMP-signaling during kidney development as Wnt-signaling as well as TGF- β /BMP-signaling has been implicated at different aspects of kidney development (Martinez and Bertram, 2003; Pulkkinen et al., 2008).

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Figure Legends

Figure 1: Sternum defects in *Ror1* mutant mice. A) Schematic representation of the ossification centers in the sternum (colored in red), from rostral to caudal: manubrium, sternebrae (s) 1- 4, xyphoid process. Alcian blue / alizarin red stained sterni with ribs of postnatal day 5 (P5) old wild-type (WT) and two *Ror1* mutant animals. B) Sections from E15.5 old wild-type (Oliveira et al.) and mutant (a'-c') embryos, stained with alcian blue and eosin (a, a'), hybridized with probes for *Col2a1* (b, b') and *Ihh* (c, c'). Arrowheads in a-b' indicate the normal (a, b) and abnormal shape and location of the sternal-costal junctions. Arrowheads in c' indicate the fused expression domain across two intercostals segments. C) Sections from E17.5 old wild-type (a-d) and mutant (a'-d') embryos, stained with von Kossa / alcian blue (vK/AB), or hybridized with probes for *Col10a1* (b, b'), Osteopontin (*Osp*) (c, c') and *Ihh* (d, d').

Figure 2: Skull and vertebral defects in *Ror1* mutant mice. A) Cervical region of the axial skeleton of wild-type and *Ror1*^{-/-} newborns, showing a widening of the C2 element or a split C2 element with a dorsal floating piece (arrowheads). B) Schematic view of the basal cranial bones (bo, basioccipitale, bs, basisphenoid, ps, presphenoid, eo exoccipitale) and ventral view on alcian blue / alizarin red stained skulls of wild-type and *Ror1*^{-/-} newborns (PO), showing the cleft in the basisphenoid bone (arrow). The magnified regions on the right show the exoccipitale bone of wild-type (WT) and *Ror1*^{-/-} (M), with the arrowhead pointing at the cleft in the mutant. C) Dorsal view on the alcian blue / alizarin red stained base of a wild-type and mutant P5 old skull, after removal of the ventral flat bones. The arrow indicates the bony bridge between the basioccipitale and basisphenoid bone, the arrowhead points at the hole in the basisphenoid bone and the asterisks mark the vomeral bones. D) Ventral view on alcian blue / alizarin red stained skulls of wild-type and *Ror1*^{-/-} P19 old mice, with the arrow pointing at the bony bridge between the basioccipitale and basisphenoid bone. The magnified regions on the right show the exoccipitale bone of wild-type (WT) and *Ror1*^{-/-} (M), with the arrowhead pointing at the hole in the mutant. E) X-ray of 3 months old mice showing the round, dome-like shape of the mutant skull in comparison to the wild-type. F) MicroCT image of the cranial base of wild-type and mutant 18 months old animals, with the white arrowhead pointing at the synostosis of the basioccipitale and basisphenoid bones in the mutant.

Figure 3: Postnatal growth plate phenotypes of *Ror1* mutants showing the proximal end of the humerus of wild type and *Ror1*^{-/-} at P0, P2 and P5. A) P0 growth plate stained with von Kossa / alcian blue (AB) and hybridized with probes for *Ihh*, *Col10a1* and osteopontin (*Osp*). Note there is no difference between wild type and mutant at this stage. Black bars indicate the zone of hypertrophic chondrocytes in the von Kossa / alcian blue staining and the *Col10a1* positive zone. B) P2 growth plate stained with von Kossa / alcian blue (AB) and hybridized with probes for *Ihh*, *Col10a1* and osteopontin (*Osp*). Note the reduced size of the *Ihh* and *Col10a1* positive zones in the mutant compared to the wild type. White bars indicate the zone of *Col10a1* positive hypertrophic chondrocytes. C) P5 growth plate stained with von Kossa / alcian blue (AB) and hybridized with probes for *Ihh*, *Col10a1* and osteopontin (*Osp*), showing reduced zones of *Ihh* and *Col10a1* positive cells in the mutant. Alizarin red (AR) / alcian blue (AB) stained wild-type (left) and mutant (right) humeri at P5, showing reduced or absent secondary ossification centers in the mutant (arrowheads).

Figure 4: Postnatal growth retardation and reduced survival of *Ror1* mutant mice. A) Bar graph showing weight reduction of the *Ror1* mutants (n= 6-9) at postnatal days P1-P6 compared to their heterozygous (n= 8) and wild type (n= 8) littermates. B) Pictures of P2 old pups showing size reduction of mutants compared to heterozygous littermate. C) Kaplan Meier survival blot of *Ror1* mutants (red line) compared to heterozygous and wild type littermates (green line) until weaning in percent survival. D) X-ray picture of 3 months old *Ror1*^{-/-} (left) and *Ror1*^{+/+} (right) female littermates showing size reduction (white arrows point at the tip of the tail) and reduced bone density and cortical thickness of the mutant femur (M) compared to the control (WT) in the inset. E) Bar graphs showing weight reduction of mutant females and males at 3, 6, 8 and 14 weeks of age, compared to littermate controls.

Figure 5: Alterations in the aged skeleton. A) Spines of 18 months old *Ror1*^{+/+} and *Ror1*^{-/-} males stained with alizarin red / alcian blue, showing abnormal mineralizations in the intervertebral discs (white arrowheads) between thoracic segments T10-T13 of the mutant. B) MicroCTs of 18 months old *Ror1*^{+/+} and *Ror1*^{-/-} males showing abnormal mineralizations in the intervertebral discs (black arrows) of the mutant spine. C) MicroCTs of the same specimens showing abnormal mineralization in the costovertebral joint of the mutant (white arrowhead).

Figure 6: Analysis of the pituitary gland. A) Sagittal sections through the skull and the pituitary gland of wild-type and *Ror1*^{-/-} littermates at P0 stained with von Kossa / alcian blue

(AB) and at P2 stained with antibodies to hormones produced by specific subpopulations of the anterior lobe: growth hormone (GH), thyroid-stimulating hormone β subunit (TSH β), luteinizing hormone α (LH α) and β (LH β) subunits, and adrenocorticotrophic hormone (ACTH). B) Morphological appearance of pituitary gland at P17. C) Western blot for growth hormone (GH) and tubulin (tub) from P17 old males and females showing no significant differences in pituitary GH levels between mutants and littermate controls. D) Blot of IGF serum levels from 2-3 month old males and females, showing reduced IGF serum levels in *Ror1*^{-/-} males and females (red bars) compared to littermate controls.

Figure 7: Non-skeletal defects in *Ror1* mutant adult animals. A) Abnormal vagina in the *Ror1* mutant female (on the right). B) Swelling of the uteri in a *Ror1* mutant female (on the right). C) Dissected uteri of wild-type and *Ror1* mutant females. D) Abnormal seminal vesicles (sv) in *Ror1* mutant male (right side) and reduced urogenital fat pads (fp) attached to the testis (t). D') Fixed genital tract of a *Ror1* mutant male, showing enlargement of one of the seminal vesicles. E) Kidneys of wild-type (left) and *Ror1* mutant (right) newborn animals, showing unilateral double kidney in the *Ror1* mutant. F) P18 old skinned wild-type (top) and *Ror1* mutant (bottom) specimens, showing absence of subcutane white adipose tissue (arrows) in the mutant.

Table 1: Summery of the observed sternebrae fusion defects in mutant and control (Wt/Het) animals.

Table 2: Bone densitometry data of femurs of 3-4 months old mice.

Table 3: Comparison of weight and size in 13-18 months (M) old *Ror1* mutants and littermates.

Figures:

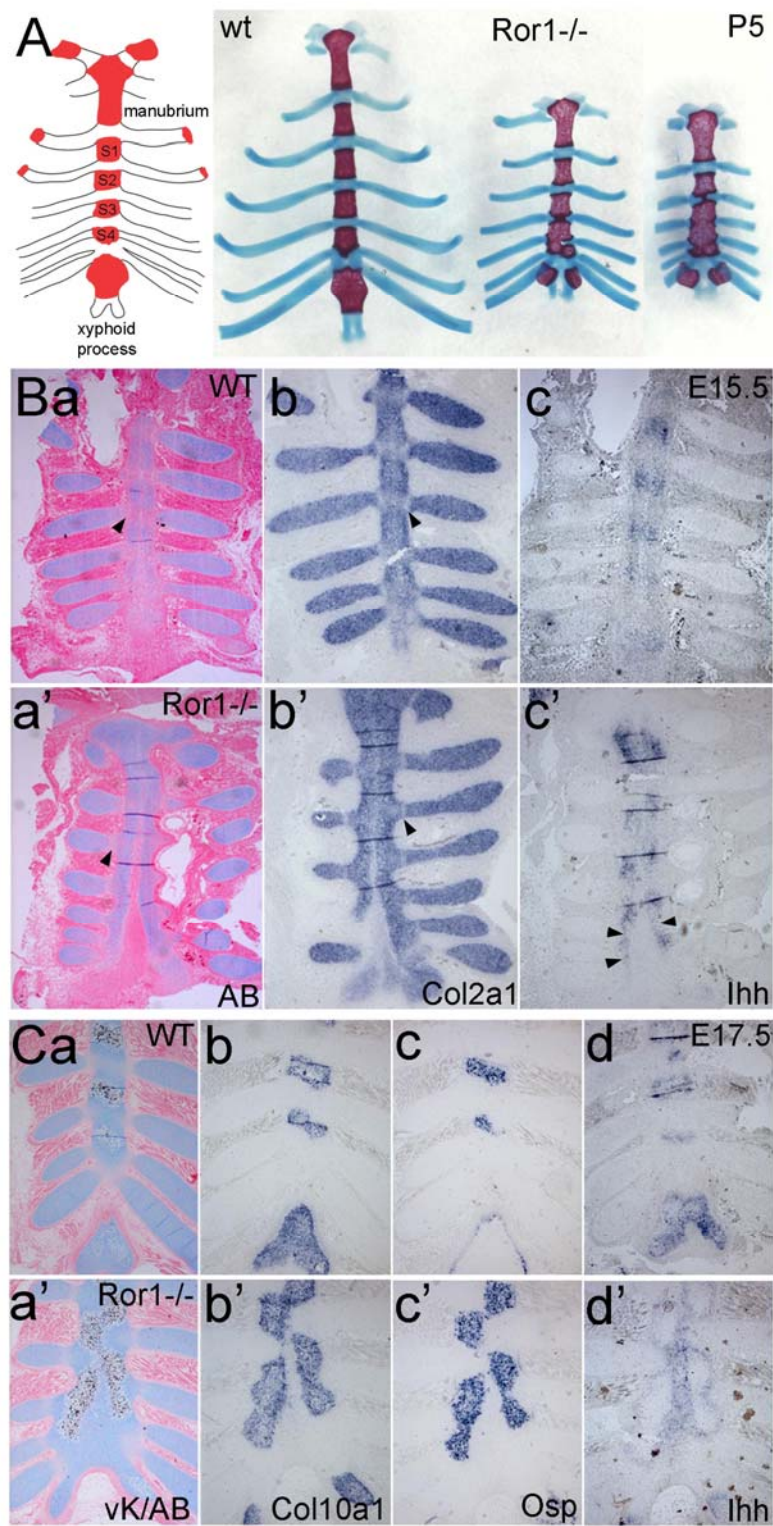


Figure 1

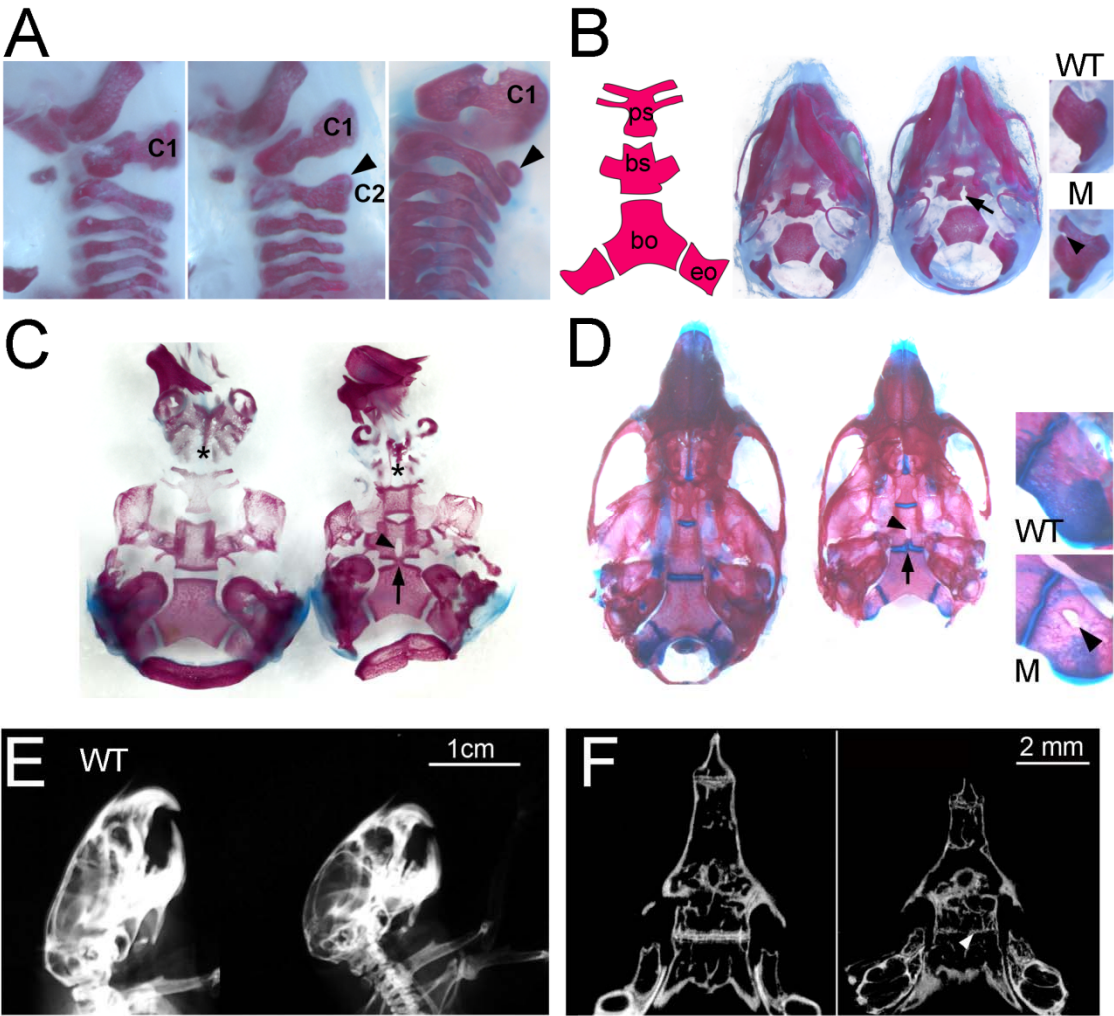


Figure 2

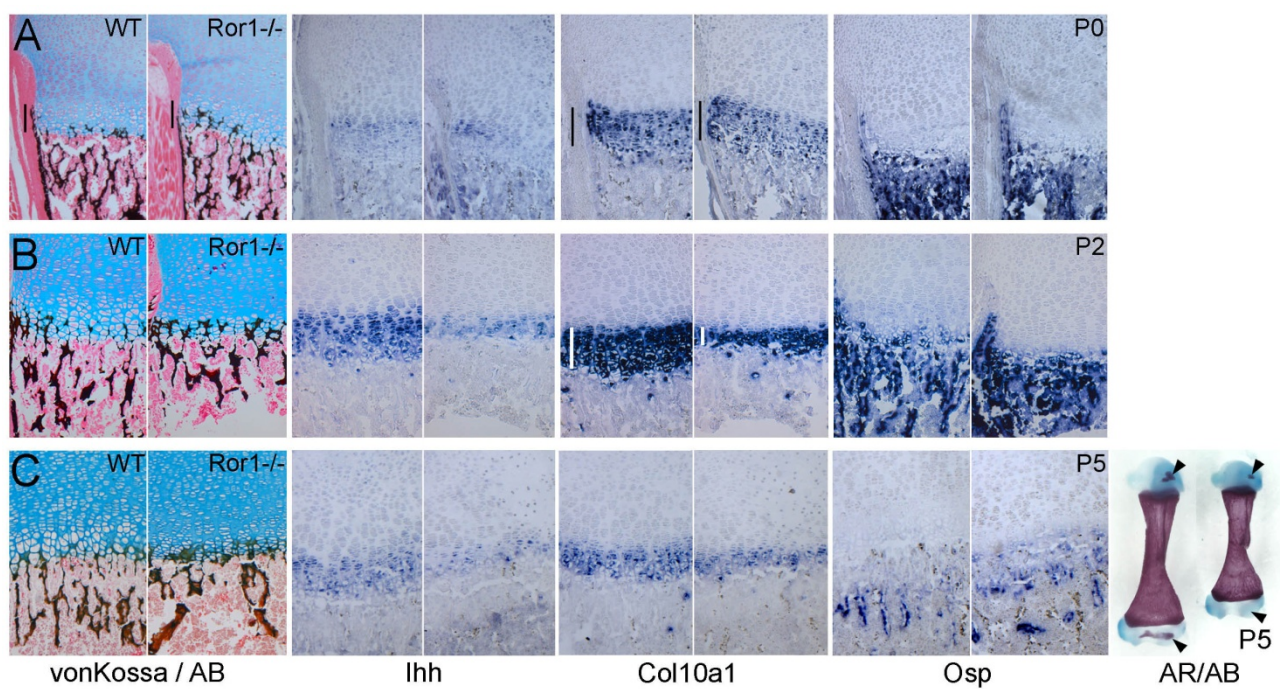


Figure 3

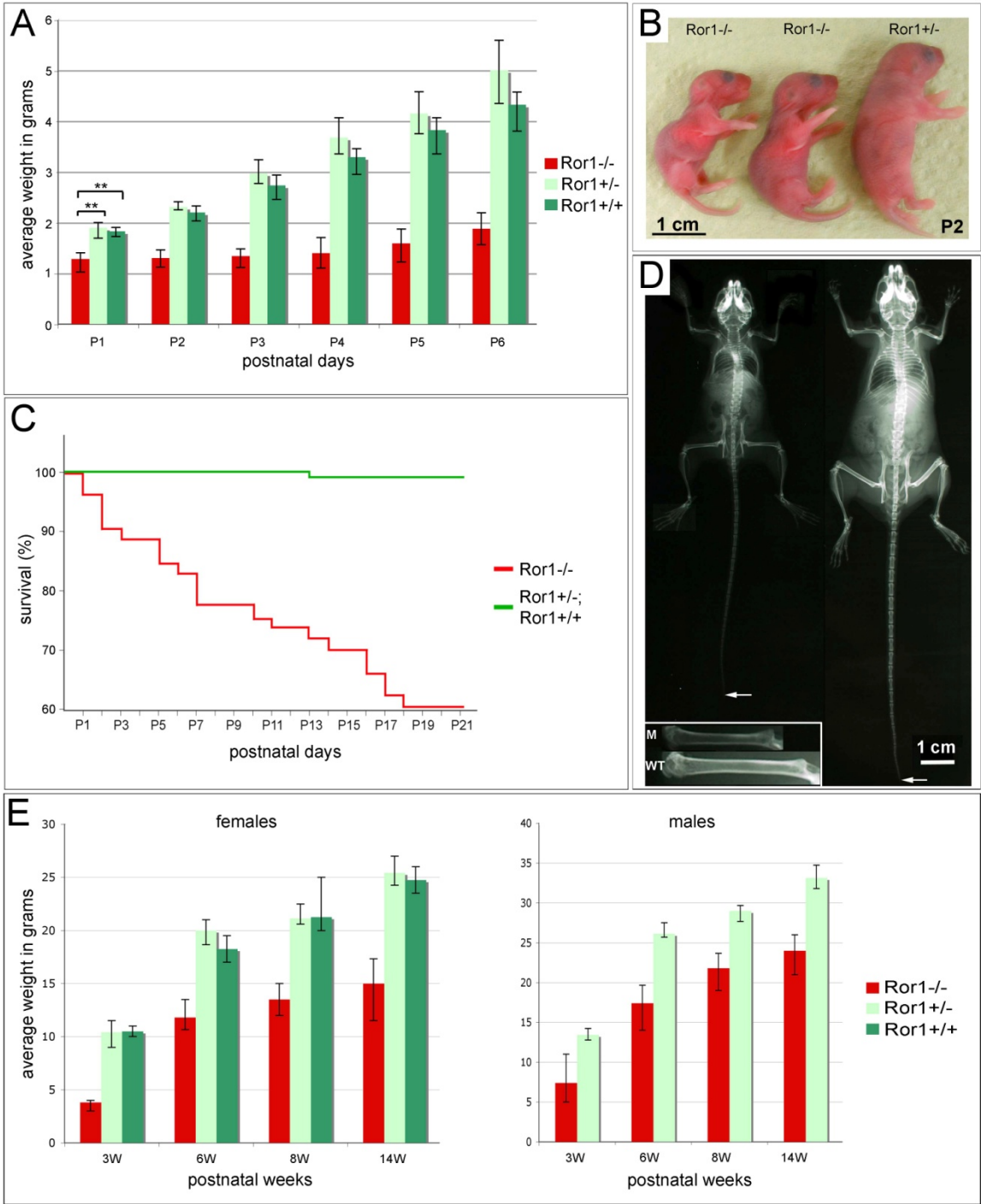


Figure 4

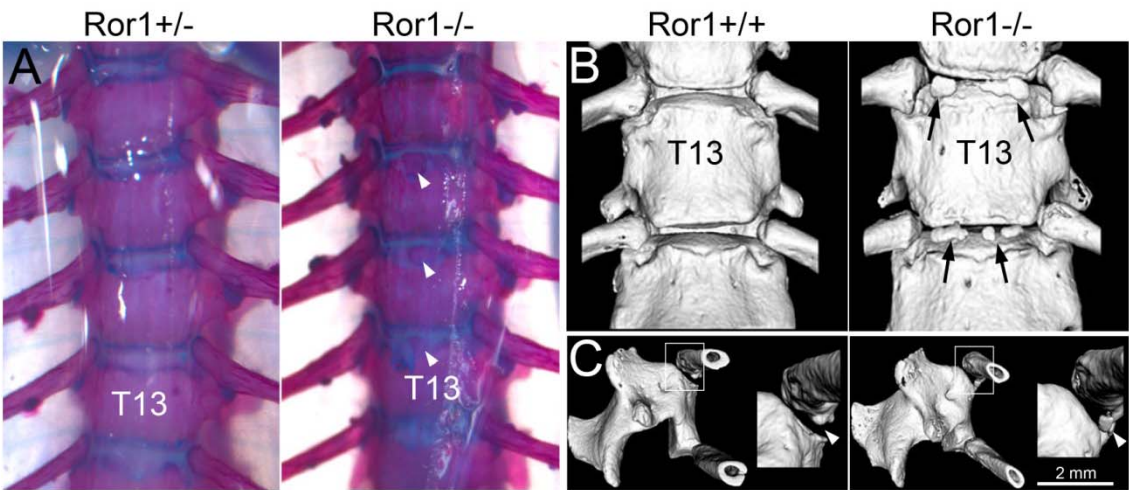


Figure 5

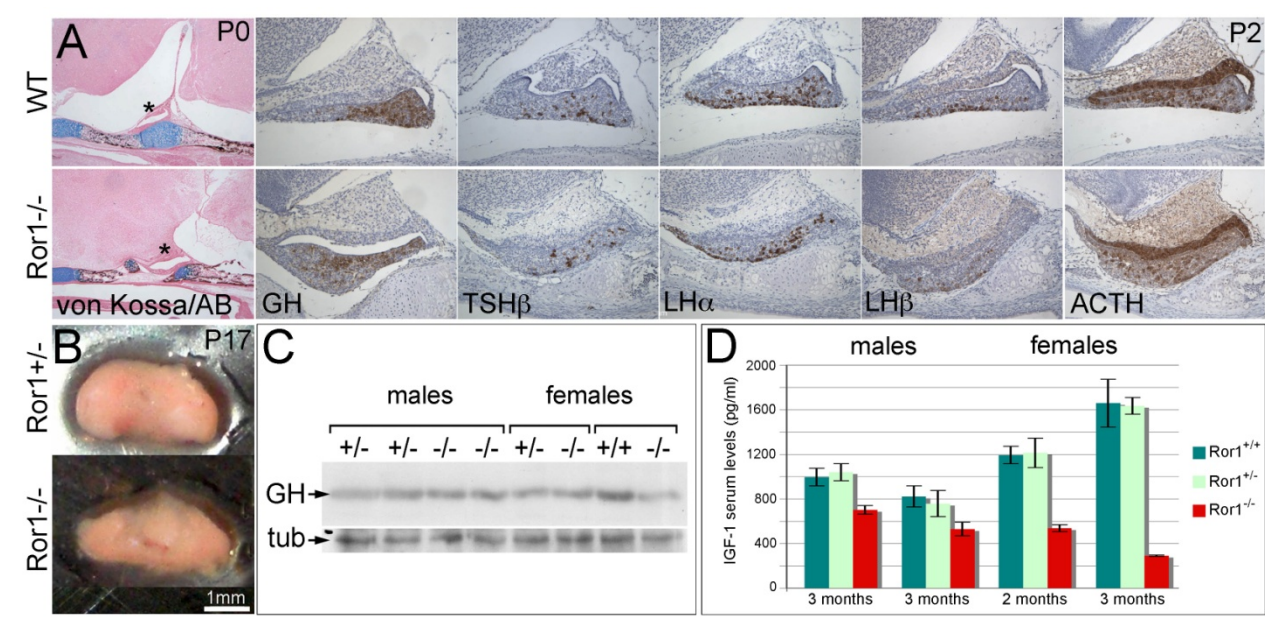


Figure 6

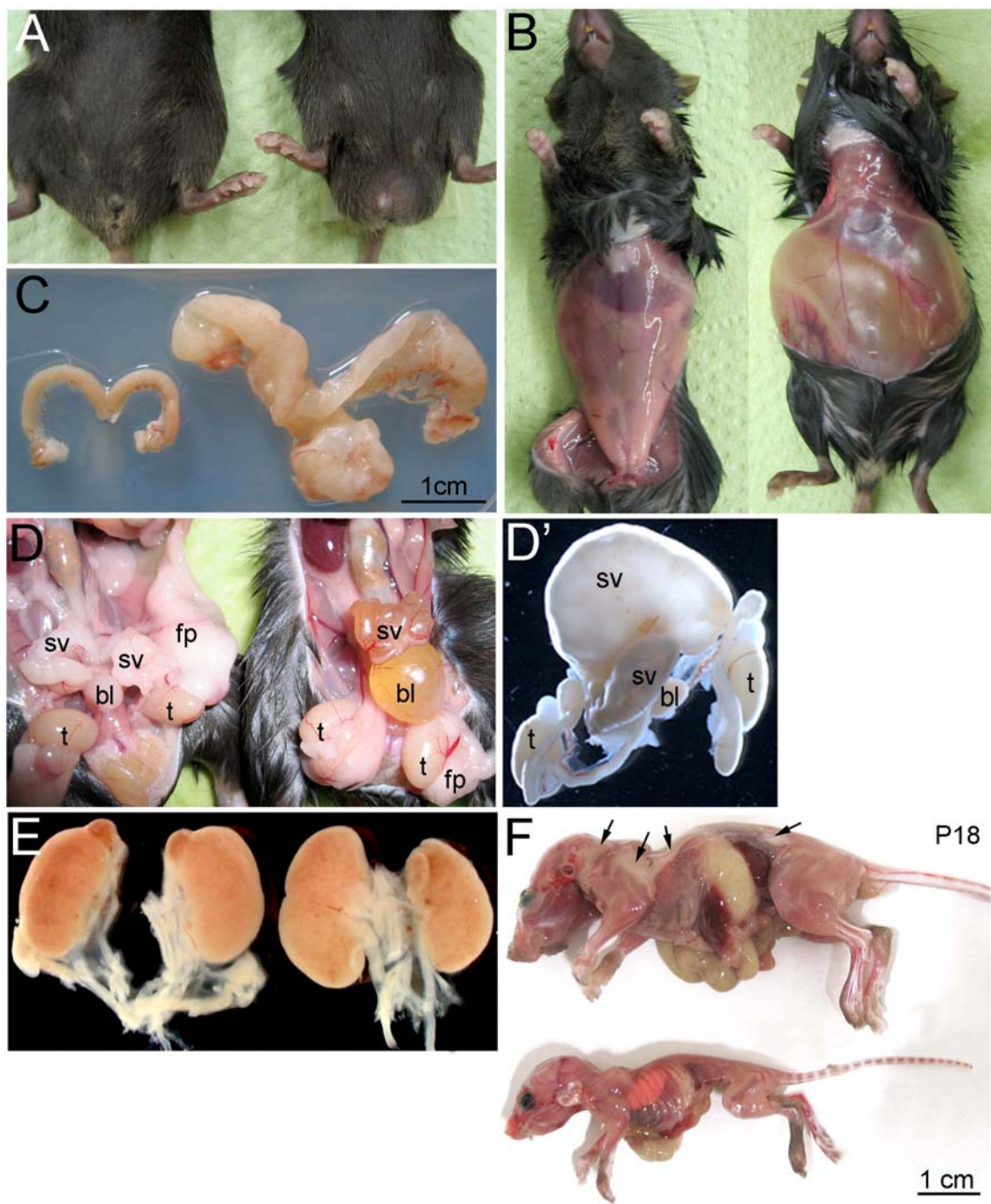


Figure 7

Tables:**Table 1:** Phenotypic variability of sternbrae fusions

Genotype	s4+s3	s4-s2	s4-s1	s4-manubrium
Wt/Het (n=108)*	0	0	0	0
Mut (n=35)	12	14	8	1

* Occasionally a slight misalignment of sternum halves was observed (n=3; e.g. WT in Fig. 1C)

Table 2: Bone densitometry data

Genotype	sex	age (wk)	Cross-sect. area (mm)	Total BMD (mg/cm ³)	Trab. BMD (mg/cm ³)	Cort. thickness (mm)
Femoral shaft						
Wt/Het (n=5)	F	15	1.78±0.09	713±37	-	0.230±0.009
Mut (n=2)	F	15	1.44±0.11	628±4	-	0.188±0.002
Het (n=2)	M	14	2.54±0.03	660±7	-	0.247±0.005
Mut (n=2)	M	14	1.94±0.08	645±84	-	0.219±0.014
Femoral metaphysis						
Wt/Het (n=5)	F	15	3.35±0.30	509±29	172±14	0.211±0.013
Mut (n=2)	F	15	2.75±0.51	420±6	153±12	0.149±0.014
Het (n=2)	M	14	4.20±0.02	510±47	228±25	0.226±0.032
Mut (n=2)	M	14	3.24±0.40	427±70	172±17	0.155±0.028

BMD = bone mineral density, Cort. = cortical, wk = weeks, F = female, M = male

Table 3: Weight and size of aged male animals

Genotype	age (M)	weight (g)	size (cm)
Het	18	46	18.2
Mut	18	29	17.5
Wt	16	52	18.7
Het	16	51	19
Mut	16	35	18
Wt	15	41	18.5
Het	15	42	18.5
Mut	15	23	16.5
Het	13	45	17.7
Mut*	13	18	16

*Cystic right kidney, left seminal vesicle enlarged

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Curriculum Vitae

Personal Information

Full Name	Miss Natalia Lyashenko
Date and place of birth	January 2 nd , 1983 Krasnoyarsk, Russian Federation
Nationality	Russian
E-mail	lyashenko@imp.ac.at
Phone	+43 (0) 650 593 49 61
Business Address	Institute of Molecular Pathology Dr. Bohr-gasse 7, 1030 Vienna, Austria

Work Experience

11/2005 – present	PhD Student – Vienna Biocenter PhD Programm, University of Vienna Laboratory of Formation and Patterning of Vertebrate Skeleton, Institute of Molecular Pathology – Vienna, Austria. PhD Thesis “ <i>Role of beta-catenin in mouse embryonic stem cells self-renewal and differentiation</i> ” Supervisor Dr. Christine Hartmann
04/2003 – 06/2005	Master Thesis Student Laboratory of Developmental Epigenetic, Institute of Cytology and Genetics Siberian Branch of Russian Academy of Sciences. Thesis Title “ <i>Establishment of in vitro system for studying nonrandom X-inactivation in vole (field mouse, Rodentia, Microtus)</i> ” Supervisor Dr. Suren M. Zakian

Education

09/2003 – 06/2005	MSc degree in Biology Novosibirsk State University, Faculty of Natural Sciences, Department of Cytology and Genetics – Novosibirsk, Russia. Special Subject Genetics/cell biology
09/1989 – 06/2000	Primary and High School Graduated with honors – Krasnoyarsk, Russia.

LIST OF PUBLICATIONS

2009 Lyashenko N.V., Migliorini D., Resch G., Biechele T., Grosschedl R., Hartmann C. Beta-catenin signaling versus cell-adhesion requirement for ES-cell pluripotency (manuscript in preparation)

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