The role of CD44 in Wnt/β-catenin signaling

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Abstract

The Wnt/β-catenin signaling pathway is one of the most extensively studied growth factor induced signaling pathways. The pathway is activated upon binding of Wnts to Frizzled and LRP5/6 and leads to cytosolic accumulation and nuclear translocation of the transcriptional co-activator β-catenin that activates TCF/LEF regulated gene transcription. The canonical Wnt-pathway was shown to play crucial roles during embryonic development as well as in tissue homeostasis at the adult stage. The activation of the Wnt-cascade needs to be tightly regulated as shown by the consequences of Wnt-signaling misregulation such as severe degenerative and metabolic diseases and cancer.

A huge flexibility prevails in the Wnt-regulated pathways due to the interplay between many regulators. Nineteen Wnt ligands and ten Frizzled receptors as well as co-receptors like LRP 5 or 6 have been described. In addition, proteoglycans such as syndecans or glypicans are able to fine-tune the signaling. One characteristic of the Wnt/β-Catenin signaling is its ability to control the expression of components of its own pathway in a feedback-control mechanism. One major Wnt-target gene is CD44, a family of transmembrane glycoproteins that plays a role in proliferation, differentiation, migration and survival.

My PhD work shows that CD44 is also a direct modulator of the Wnt-pathway. Downregulation of CD44 expression inhibits Wnt3a-induced activation and nuclear translocation of β-catenin and TCF/LEF regulated transcription. Conversely overexpression of CD44 increases Wnt-signaling. Epistasis experiments place CD44 function at the level of Wnt membrane receptors, specifically LRP6. Mechanistically, CD44 physically associates with LRP6 upon Wnt-induction and modulates LRP6 activation and membrane localization. The cytoplasmic domain of CD44 is instrumental for its function in the Wnt-pathway, suggesting that binding partners of CD44 such as ERM (ezrin-radixin-moesin) proteins are also involved. Indeed, interference with ezrin function has a drastic impact on β-catenin signaling and LRP6 membrane localization, indicating that binding of ERM proteins to the cytoskeleton plays a decisive role in the Wnt-pathway. In *Xenopus laevis*, downregulation of CD44 expression leads to a decrease in *tcf-4* and *engrailed-2* expression indicating an instrumental *in vivo* function of CD44 in the Wnt-pathway. This is confirmed by rescue experiments using both CD44 and β-catenin cDNA. Altogether, these findings identify CD44 as a novel and essential positive feedback-regulator of the Wnt receptor complex.

Zusammenfassung

Der Wnt/β-catenin Signalweg ist einer der am besten charakterisierten zellulären Signalwege. Die Aktivierung des Signalweges erfolgt durch Bindung von Wnt-Liganden an Membranrezeptoren der Frizzled-Familie sowie LRP5/6. Dies führt zu einer Stabilisierung und Anreicherung von β-catenin im Zytosol und als Folge zu dessen Translokation in den Zellkern. Im Zellkern fungiert β-catenin als transkriptioneller Ko-Aktivator für Transkriptionsfaktoren der TCF/LEF-Familie und induziert die Transkription TCF/LEF regulierter Gene. Der Wnt/β-catenin Signalweg ist nicht nur essentiel an der Embryonalentwicklung beteiligt, sondern spielt auch eine entscheidende Rolle bei der Gewebeerhaltung im adulten Organismus. Eine gestörte Regulation des Wnt-Signalweges ist die Ursache diverser degenerativer Erkrankungen, Stoffwechselerkrankungen und Krebs, was die Notwendigkeit einer genauen Regulation der Wnt-Signalwegaktivierung verdeutlicht. Die zugrundeliegenden Kontrollmechanismen des Wnt/β-catenin Signalweges sind sehr komplex. Allein neunzehn unterschiedliche Wnt-Liganden, zehn Frizzled-Rezeptoren sowie Ko-Rezeptoren wie LRP5/6 sind bisher bekannt. Hinzu kommen viele weitere extrazelluläre, transmembrane, membrangebundene und intrazelluläre Proteine die an der Regulation beteiligt sind. Einige Wnt-Zielgene sind zudem auch Regulatoren des Wnt-Signalweges, was auf einen Selbstregulations-Mechanismus hindeuted.

Ein bedeutendes Wnt-Zielgen ist CD44, ein transmembranes Glykoprotein das eine wichtige Rolle bei der Zellproliferation, -differenzierung, -migration und -erhaltung spielt.

Diese Arbeit zeigt, dass CD44 direkt an der Regulierung des Wnt-Signalweges beteiligt ist. Eine Inhibierung der CD44 Expression führt zu einer Hemmung der Wnt3a-induzierten Aktivierung von β-catenin und TCF/LEF regulierten Transkription. Umgekehrt führt eine Überexpression von CD44 zu einer verstärkten Aktivierung des Wnt-Signalweges. Epistasis Experimente zeigen, dass die Regulierung der Wnt-Signalwege durch CD44 auf Ebene der Wnt-Rezeptoren stattfindet. CD44 interagiert nicht nur mit dem Wnt-Ko-Rezeptor LRP6, sondern steuert auch dessen Aktivierung und Platzierung an der Zellmembran. Die intrazelluläre Domäne und deren Interaktion mit Proteinen der Ezrin/Radixin/Moesin-Familie, die CD44 mit dem Aktin-Zytoskelett verbinden, spielt hierbei eine entscheidende Rolle.

Darüber hinaus wird durch eine Inhbierung der Expression der Wnt-Zielgene *tcf-4* und *engrailed-2* in CD44-Morpholino injizierten *Xenopus laevis* Embryonen gezeigt, dass CD44 auch *in vivo* eine entscheidende Rolle bei der Regulierung des Wnt-Signalweges spielt. Zusammenfassend beschreibt diese Arbeit eine Rolle von CD44 als einen neuen und essentiellen positiven Feedback-Regulator des Wnt-Rezeptor Komplexes.

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Abbreviations

ABC	Active β-catenin
APC	Adenomatous polyposis
CAM	Cell adhesion molecule
cAMP	Cyclic adenosine monophosphate
CD44	Cluster of differentiation 44
Cdc42	Cell division control protein 42 homolog
CIA	Collagen induced arthritis
CK1	Casein kinase 1
CM	Conditioned media
CNS	Central nervous system
CRD	Cysteine rich domain
CRC	Colorectal cancer
CS	Chondroitin sulphate
CSC	Cancer stem cell
Dkk	Dickkopf
DN	Dominant negative
Dsh	Dishevelled
E	Embryonic day of development
ECM	Extracellular matrix
EGF	Epidermal growth factor
En-2	Engrailed-2
ER	Endoplasmatic reticulum
ErbB/EGFR	Epidermal growth factor
Erk	Extracellular-signal regulated kinases
ERM	Ezrin/radixin/roesin
EV	Empty vector
Ez	Ezrin
FAP	Familial adenomatous polyposis
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FITC	Fluorescein isothiocyanate
Fz	Frizzled
GFP	Green fluorescent protein
GSK3	Glycogen synthase kinase-3
GTP	Guanosine triphosphate
HA	Hyaluronan
HB-EGF	Heparin binding EGF-like factor
HGF	Hepatocyte growth factor
HSC	Hematopoietic stem cells
HSPG	Heparan sulphated proteoglycan
ICD	Intracellular domain
JNK	C-Jun N-terminal kinase
LGR	Leucine-rich repeat-containing G-protein coupled receptor
LEF	Lymphoid enhancer factor
LRP	Low density lipoprotein receptor related proteins
MAPK	Mitogen-activated protein kinase
MEK	MAPK/ERK kinase
Mesd	Mesoderm development
Mest/Peg1	Mesoderm-specific transcript/paternally expressed gene 1
Met	Mesenchymal-epithelial transition factor
MIN	Multiple intestinal neoplasia
MMP	Matrix metalloproteinase

MO	Morpholino
N-WASP	Neural Wiskott-Aldrich syndrome protein
PCNA	Proliferating cell nuclear antigen
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
Rho	Ras homolog gene family
Ror2	Receptor tyrosine kinase like orphan receptor 2
RSPO	R-Spondin
RTK	Receptor tyrosine kinase
RYK	Related to receptor tyrosine kinase
siRNA	Small interfering RNA
TCF	T-cell factor
TK	Thymidine kinase
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VSVG	Vesicular stomatitis Indiana virus G-protein
ZNRF3	Zinc and ring finger 3

1 Introduction

1.1 General introduction

The development of highly complex multicellular organisms originating from one single cell is one of the most fascinating events. From a biological point of view, embryogenesis and more globally development involves numerous cellular, molecular and biochemical processes. First, cells have to grow, divide and proliferate and then to differentiate to various cell types, which then have to be organized into specific tissues. To this end cells have to migrate along morphogen gradients from their place of origin to their specific place of destination. Furthermore, tissues have to be arranged into organs, organs are then part of organ systems and these organ systems finally give rise to functional organisms. At the adult stage, non-functional or dead cells have to be renewed or wounded tissues to be repaired in order to ensure the maintenance of the tissues and further of the whole organism.

The spatial and temporal regulation of all these events is crucial throughout development and adulthood and depending to a great extent on cell-cell communication. This communication is in part accomplished by the release of signaling molecules from one cell and subsequent reception of these molecules by other cells in autocrine, paracrine or endocrine ways. Hereby, the signaling molecules can diffuse inside or bind to membrane located receptors of the receiving cell, thereby inducing intracellular signaling cascades that finally change the gene expression profile and consequently the behavior of the cell. A tight regulation of the development processes is required and the consequences of a misregulation of these processes are tremendous and are reflected in developmental defects; malformations; degenerative diseases and cancer.

A major class of such extracellular signaling molecules, controlling the events described above, are proteins of the Wnt-family (reviewed in van Amerongen and Nusse, 2009). The binding of Wnt-proteins to various receptors at the cell membrane activates different intracellular cascades (reviewed in Kestler and Kuhl, 2008). The activation of these pathways is not only regulated by the binding of the ligands to their specific receptors, but also by several other co-regulators that can extracellularly or intracellularly modulate signaling events at each step of the signaling cascades. Many of these co-regulators are themselves target genes of Wnt-signaling, strongly suggesting that Wnt-signaling creates regulatory feedback mechanisms.

One protein that is a major target gene of Wnt-signaling is CD44 (Wielenga et al., 1999). CD44 is a protein that is well known for its co-regulatory functions in various signaling

pathways. Hereby, CD44 can not only influence binding of the ligands to the receptors, but also modify downstream signaling from the receptors to intracellular effector molecules.

Although it has often been proposed that CD44 might be involved in Wnt-signaling and even though it was found that CD44 is able to indirectly act on components of the Wnt-cascade, there is no evidence for direct regulation of Wnt-signaling. This PhD thesis addressed the question of, whether and how CD44 is involved in the regulation of Wnt/β-catenin signaling.

1.2 Wnt-proteins

The term Wnt describes a huge family of secreted hydrophobic glycoproteins that are highly conserved during evolution. Wnts act as short range rather than long range morphogens that activate specific intracellular signaling cascades according to which receptor and which Wnt ligand are involved. The name Wnt arose as a combination of the names of the segment polarity gene *wingless* (Wg) of *Drosophila melanogaster (D. melanogaster)* and the mouse oncogene *int-1*, which were discovered separately and later-on identified as one homologous gene (Rijsewijk et al., 1987). Whereas Wg is important for the number and polarity of the segments and furthermore crucial for proper wing and haltere development in *D. melanogaster* (Nusslein-Volhard and Wieschaus, 1980), the *int* genes were described as genes near the integration sites of the mouse mammary tumor virus (MMTV), and activation of their transcription was shown to cause formation of mammary carcinomas in mice (Nusse et al., 1984). Higher vertebrate genomes harbor multiple Wnt genes, with a total of 19 different *wnt*-genes in human, divided into 12 conserved Wnt-subfamilies and distributed over several chromosomes (reviewed in Clevers and Nusse, 2012).

Notably, multiple Wnt genes can be found in the ancient metazoan phylum Cnidaria (Guder et al., 2006), not only indicating that Wnts arose early in evolution, but also that Wnt signaling remained a crucial actor in organismal patterning throughout the animal kingdom (Kusserow et al., 2005). Already sponge genomes harbor Wnt genes, whereas single-cell organisms do not have any Wnt, indicating that the origin of Wnt signaling may have been crucial in the development of multicellular organisms (reviewed in Petersen and Reddien, 2009).

Wnt-proteins control important processes during development, like embryonic induction, cell fate specification and generation of cell polarity. They are furthermore important for body axis formation and convergent extension movements during embryogenesis (De Calisto et al., 2005; Huelsken et al., 2000; Moon and Kimelman, 1998; Solnica-Krezel, 1999).

In addition to their role in developmental processes, Wnts are also involved in homeostasis, tissue regeneration and maintenance of stem cells in adult organisms (reviewed in Reya and Clevers, 2005).

Characteristics of Wnt-proteins

Wnt-proteins are approximately 40 kDa in size and display a characteristic distribution of 22 cysteine residues. These cysteine residues form intracellular disulfide bonds, which are thought to be required for proper protein folding (Mason et al., 1992; Tanaka et al., 2000). Additional common features are a signal sequence for secretion, highly charged amino acid (aa) residues and several potential glycosylation sites (Papkoff, 1994; Smolich et al., 1993). Additional lipid modifications give Wnts their hydrophobic character and are required for efficient signaling as well as for Wnt-secretion (Franch-Marro et al., 2008; Kurayoshi et al., 2007; Willert et al., 2003). One of these lipid modifications is an attachment of a palmitoleic acid to a conserved serine, which is essential for the activity of Wnts (Schulte et al., 2005; Willert et al., 2003; Zhai et al., 2004). Most recently it was found that one of the two domains of Wnt8 that interact with the main Wnt-receptor Frizzled (Fz), contains a palmitoleic acid lipid, which projects into the binding pocket of the Fz-receptor. This finding strengthens the importance of the lipid modifications for signaling (Janda et al., 2012).

Canonical and non-canonical Wnt-proteins

From a historical point of view Wnt-proteins are commonly divided into two subgroups, canonical and non-canonical Wnt-proteins. Canonical Wnts like Wnt3a, Wnt1 and Wnt8 are defined by their ability to morphologically transform mouse C57MG mammary epithelial cells and to induce secondary body axes in early *Xenopus laevis (X. laevis)* embryos (Du et al., 1995; Wong et al., 1994). These Wnts mediate their signaling via the intracellular protein β-catenin dependent manner. Therefore this, canonical Wnt-signaling pathway is nowadays rather termed Wnt/β-catenin signaling.

Non-canonical Wnts like Wnt5a and Wnt11 neither transform C57MG cells nor induce secondary body axes in *X.laevis* and are known to activate several β-catenin independent pathways (Du et al., 1995; Heisenberg et al., 2000; Kilian et al., 2003; Veeman et al., 2003; Wong et al., 1994). However, further studies revealed that depending on the combination of Wnt-receptors present on the cell surface, Wnts earlier classified as non-canonical, are able to activate also the canonical Wnt/β-catenin pathway and that canonical Wnts, like Wnt3a, can also induce β-catenin independent signaling (He et al., 1997; Kishida et al., 2004; Mikels and Nusse, 2006).

1.3 Wnt-receptors

The ability of Wnts to activate the β-catenin dependent or independent pathways is rather determined by distinct sets of receptors and co-factors than by the presence of a particular Wnt-ligand. The binding of Wnt-ligands to their receptors Fz in combination with the binding to the co-receptors Low density lipoprotein receptor-related proteins 5 or 6 (LRP5/6) activates the β-catenin dependent Wnt-pathway. The binding to Fz in the absence of LRP5/6, or to receptors like Receptor tyrosine kinase like orphan receptor 2 (Ror2) or Related to receptor tyrosine kinase (Ryk) activates rather β-catenin independent, non-canonical Wnt-signaling. The receptors involved and the different Wnt-signaling pathways will be described in the next chapters.

1.3.1 Frizzled

The major and first identified receptor for Wnt was the seven pass transmembrane protein Fz (Bhanot et al., 1996). So far 10 different Fz-receptors, sharing 50-75% aa identity were identified in human. The size of Fz-receptors ranges from 500 – 700 aa with a sequence similarity of 50-75% (Huang and Klein, 2004). The N-terminal extracellular domain contains a cysteine rich domain (CRD) followed by a hydrophobic linker region (Wang et al., 1996). The CRD, necessary and sufficient to bind Wnt, consists of 125 aa with 10 conserved cysteine residues, forming disulfide bonds (Bhanot et al., 1996; Dann et al., 2001). Following the linker domain, the Fz-receptors contain seven hydrophobic domains that are predicted to form transmembrane α-helices and an intracellular carboxyterminal domain of variable length, which is not well conserved among the Fz-family members (Wang et al., 1996). The only motif within the intracellular domain that is highly conserved, is the KTXXXW-motif separated by two amino acids from the seventh hydrophobic domain. Fz-mutants with point mutations affecting any of these three conserved residues are defective in Wnt/β-catenin signaling, indicating that this motif is essential for the activation of the canonical Wnt-pathway (Umbhauer et al., 2000). Beside the canonical Wnt/β-catenin pathway, at least two other pathways, known as the Wnt/JNK (c-Jun N-terminal kinase) or planar cell polarity (PCP) pathway and the Wnt/calcium pathway are mediated by Fz-receptors (Adler, 2002; Veeman et al., 2003). The exact mechanism by which Fz-receptors actually transduce their signals upon ligand binding is largely unknown, but there is more and more evidence that Fz can bind to heterotrimeric G-proteins (Nichols et al., 2013) and that these are involved in the downstream signaling (Liu et al., 2001; Liu et al., 1999; Liu et al., 2005; Noordermeer et al., 1994; Yanagawa et al., 1995).

1.3.2 LRP5/6

In order to activate Wnt/β-catenin signaling Wnts require, in addition to Fz-receptors, the presence of a co-receptor called LRP5/6 (Pinson et al., 2000; Tamai et al., 2000). LRP5 or 6 are closely related type 1 transmembrane proteins and members of the low density lipoprotein receptor (LDLR) superfamily (Brown et al., 1998).

The extracellular LRP5/6 region contains repetitive patterns of three basic domains: YWTD type-β propeller domains, epidermal growth factor like (EGF-like) domains and LDLR type A domains (Brown et al., 1998). The proper folding of the YWTD domains by the chaperone Mesoderm Development (Mesd) is indispensable for LRP6 maturation and membrane localization (Hsieh et al., 2003). LRP5/6 can interact with Wnt-proteins. However, this binding is a low affinity binding compared to the Wnt/Fz-interaction and might only take place in a Wnt/Fz complex (Tamai et al., 2000). In contrast to Wnts, the Wnt/β-catenin signaling antagonists Dickkopf 1 (Dkk1) and Sclerostin (SOST) are high affinity ligands of LRP6 (Li et al., 2005; Mao et al., 2001a; Semenov et al., 2005; Semenov et al., 2001). These different ligands bind to different YWTD/EGF-like domains of LRP6 (Li et al., 2005; Mao et al., 2001a). In addition, LRP6 seems to harbor separate binding sites for different classes of Wnt-proteins, demonstrated by a recent study in which cells were treated with two different monoclonal LRP6 antibodies. These antibodies were raised against non-overlapping regions of LRP6 and surprisingly, inhibited signaling by some Wnt-proteins and increased signaling by others (Gong et al., 2010).

Interestingly, LRP6 lacking the extracellular domain constitutively activates β-catenin signaling, indicating that the extracellular domain has an autoinhibitory effect on the canonical Wnt cascade (Brennan et al., 2004). The cytoplasmic domain of LRP5/6 contains five P-P-(S/T)-P repeats, casein kinase 1 (CK1) sites -which are collectively called PPSPXS motifs- and upstream of these one S/T cluster (reviewed in Niehrs and Shen, 2010). These motifs are crucial for Wnt-signaling transduction and can be phosphorylated constitutively or in a Wnt-dependent manner, depending on the kinases involved (MacDonald et al., 2008; Wolf et al., 2008; Zeng et al., 2008). Especially, phosphorylation of S1490 by glycogen synthase kinase 3-β (GSK3-β) and T1479 by CK1-γ upon Wnt-induction, have been shown to be crucial for canonical Wnt-signaling transduction (Davidson et al., 2005; Zeng et al., 2005).

It has been proposed that binding of LRP5/6 to Wnt leads to formation of a ternary complex between Fz, Wnt and LRP5/6, necessary for Wnts to activate the β-catenin dependent pathway (Tamai et al., 2000). LRP5 or 6 contribute to the activation of the β-catenin signaling pathway via recruitment of Axin, a negative regulator of β-catenin signaling, to the plasma membrane, where Axin is inactivated and/or targeted for degradation (Fearon

and Cadigan, 2005; Mao et al., 2001b; Willert et al., 1999). Further studies revealed that Wnt-induction leads to the formation of LRP6-signalosomes at the membrane that do not only include Axin but also other Wnt-pathway components like Dishevelled (Dsh), GSK3 and Fz (see 1.4 Canonical or β-catenin dependent Wnt-signaling) (Bilic et al., 2007; Cong et al., 2004).

1.3.3 Ror2

Ror2 is a type-1 transmembrane receptor tyrosine kinase (RTK). The extracellular region of Ror2 contains an immunglobulin (Ig) domain and a cysteine rich domain (CRD), which is also found in Fz and a kringle domain. Intracellularly, Ror2 possesses a tyrosine kinase domain and a proline rich domain flanked by two Ser/Thr-rich domains (Masiakowski and Carroll, 1992).

Ror2 binds to Wnt5a and transduces the Wnt5a mediated signal via the serine/threonine kinase JNK (Hikasa et al., 2002; Oishi et al., 2003). The Wnt5a/Ror2 interaction is involved in convergent extension (CE) in *X. laevis* (Hikasa et al., 2002; Schambony and Wedlich, 2007). CE is the process by which the tissue of an embryo is restructured to converge along one axis and extend along a perpendicular axis by cellular movement. Furthermore, Ror2 mediates Wnt effects on cell polarity (Green et al., 2008; Yamamoto et al., 2008) and cell migration (Nishita et al., 2006; Nomachi et al., 2008).

Regarding canonical Wnt-signaling, the function of Ror2 is contradictory. On the one hand, Ror2 can antagonize canonical Wnt-signaling by sequestering Wnts and keeping them away from the Fz-receptors (Billiard et al., 2005; Green et al., 2007), or by mediating an inhibitory Wnt5a-signal, which acts downstream of β-catenin on the level of T-cell factor/lymphoid enhancer factor (TCF/LEF) regulated gene expression (Mikels and Nusse, 2006). On the other hand, in the presence of certain co-factors, Ror2 can also bind to canonical Wnt3a and potentiate Wnt/β-catenin signaling. However, this was only seen in the presence of specific co-factors, like the Fz-2 or Fz-6 or collagen triple-helix repeat-containing protein 1 (Cthrc1), a stabilizer for Wnt-ligand-receptor interaction (Billiard et al., 2005; Li et al., 2008; Yamamoto et al., 2008). Hence, it is very likely that the function of Ror2 in Wnt-signaling is rather defined by the presence of specific co-factors.

1.3.4 Ryk

Another receptor RTK which can bind to Wnt and mediate Wnt-signaling, is Ryk. Ryk signaling controls axon guidance and is additionally involved in neuronal differentiation (Bonkowsky et al., 1999; Lyu et al., 2008; Wouda et al., 2008). Ryk is functionally linked to Wnt/Fz-signaling components and can activate canonical as well as non-canonical Wnt-signaling depending on the cellular context (Kim et al., 2008a; Lu et al., 2004; Macheda et al., 2012). Downstream components of Ryk are still unknown although it is assumed that Ryk signals through recruitment and activation of members of the Src family of tyrosine kinases (van Amerongen et al., 2008).

1.4 Canonical or β-catenin dependent Wnt-signaling

1.4.1 The Wnt/β-catenin signaling pathway

The canonical or Wnt/β-catenin signaling cascade is the best characterized Wnt-signaling pathway. A key issue of this pathway is the level of free cytosolic β-catenin. In the absence of Wnt, this level is kept low by a multiprotein complex comprising the scaffold proteins adenomatous polyposis coli (APC) and Axin and the kinases GSK3-β and CK1-α. This complex constantly binds free cytosolic β-catenin. Upon binding, β-catenin is phosphorylated by CK1-α and GSK3-β, a modification that leads to its ubiquitylation by SKP1-cullin1-F-box ($SCF^{\beta\text{-}TrCP}$) E3 ligase. This step in turn leads to the degradation of β-catenin via the 26S proteasome (Fig.1 left) (Kimelman and Xu, 2006; Liu et al., 2002).

In a current and simplified model, canonical Wnt signaling is induced by binding of Wnt to its receptor Fz and its co-receptor LRP5/6 (Fig.1 right). In turn, the phosphoprotein Dsh gets phosphorylated and interacts with the destruction complex through the GSK3-β inhibitor GSK-3 binding protein/frequently rearranged in advanced T-cell lymphomas (GBP/FRAT). This prevents the phosphorylation of β-catenin and thus its degradation (Jonkers et al., 1997; Li et al., 1999b; Salic et al., 2000; Yost et al., 1998). Wnt binding to LRP leads to LRP phosphorylation by membrane bound CK1-γ and GSK3-β, creating docking sites for Axin (Davidson et al., 2005; Zeng et al., 2005). Thereby Axin is recruited to LRP5/6 and not anymore able to act as scaffold protein for the destruction complex, leading to disintegration of the complex. Hence, β-catenin is no longer phosphorylated and degraded and can accumulate in the cytoplasm.

In contrast to this model, more recent data show that Axin is not taken away from the β-catenin degradation complex by the recruitment to LRP6. The recruitment of Axin to LRP6 might rather bring the whole intact degradation complex to the membrane. Here, β-catenin is still phosphorylated within the complex but ubiquitination is blocked. Thereby, the complex

gets saturated by phospho-β-catenin and the newly synthesized β-catenin accumulates in the cytoplasm (Li et al., 2012).

However, in both models the degradation of β-catenin is stopped. Therefore, β-catenin accumulates in the cytosol and translocates to the nucleus. Within the nucleus, the transcriptional co-activator β-catenin interacts with transcription factors of the TCF/LEF family of HMG box transcription factors and induces their transcriptional activity (reviewed in Clevers, 2006; Logan and Nusse, 2004).

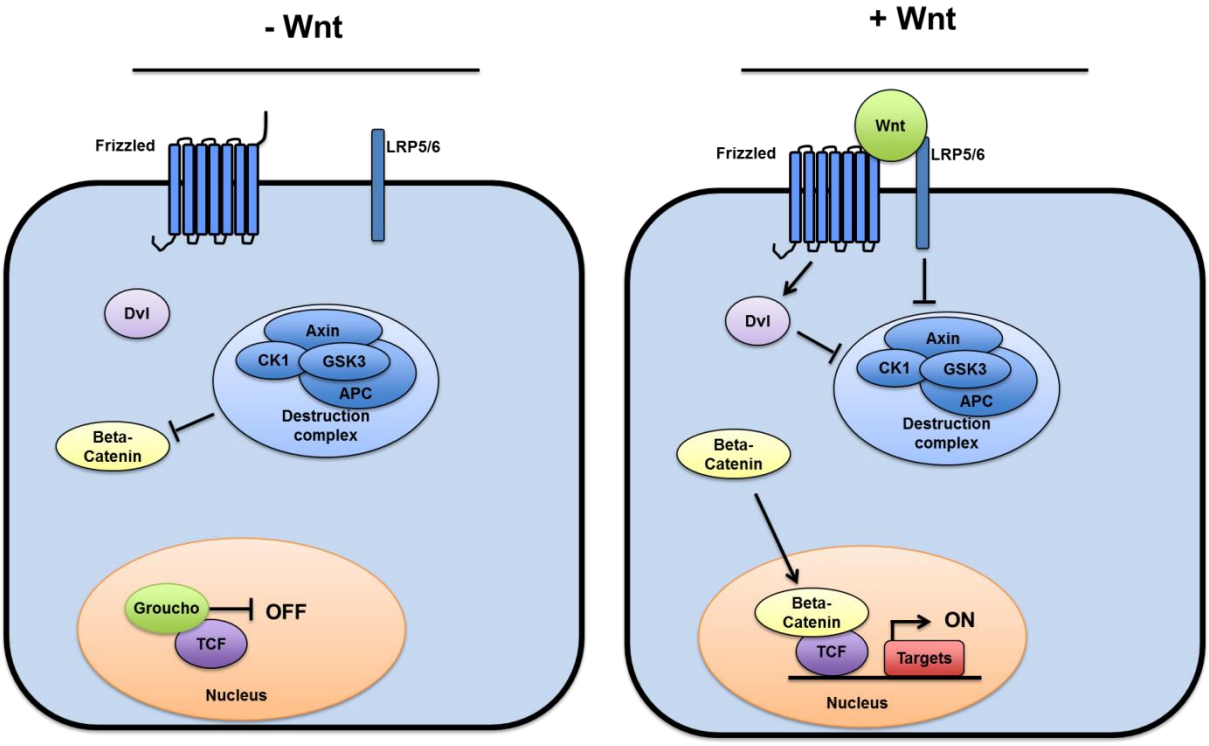

Fig. 1 A simplified view of Wnt-signaling.
In the absence of Wnt, β-catenin is targeted for degradation via the proteasome and TCF/LEF regulated gene transcription repressed by Groucho (left). Wnt binding to Fz and LRP5/6 prevents the degradation of β-catenin. β-catenin accumulates in the cytoplasm, translocates into the nucleus, where it activates TCF/LEF regulated gene transcription (right).

However, this simplified model is getting much more complex if additional components and modulating factors are taken into account. For example, in the Wnt-producing cells, the lipid modification of Wnts, a step that is crucial for the secretion and activity of Wnts, is regulated for example by porcupine/mom-1 (Kadowaki et al., 1996; Rocheleau et al., 1997). Another protein that was found to be indispensable for the secretion of multiple Wnt-proteins is Wntless (Banziger et al., 2006).

Once Wnt-proteins are secreted, they can be captured by secreted Frizzled-related proteins (sFRPs) or secreted proteins of the Wnt inhibitory factor (WIF) family, which compete with transmembrane Wnt-receptors for ligand binding and therefore act as Wnt

inhibitors (Bafico et al., 1999; Dennis et al., 1999; Hsieh et al., 1999; Leyns et al., 1997; Salic et al., 1997; Uren et al., 2000; Wang et al., 1997).

On the receiving cell a number of binding partners can modulate the ability of Wnts to mediate signaling. Emerging evidence suggests a role for heparan sulfate proteoglycan (HSPG) in Wnt-signaling. In Drosophila, the glypicans Dally and Dally like protein (Dlp) as well as glypican 3 in vertebrates are involved in Wnt signaling. These proteins might facilitate the presentation of Wnt to its receptor, the extracellular transportation or the stabilization of Wnt (Baeg et al., 2001; De Cat et al., 2003; Lin and Perrimon, 1999; Tsuda et al., 1999).

The transmembrane E3 ubiquitin ligases zinc and ring finger 3 (ZNRF3) and its homologue ring finger 43 (RNF43), act as negative regulators of canonical Wnt-signaling by promoting the turnover of Fz and LRP6 receptors from the cell surface (Hao et al., 2012; Koo et al., 2012). Furthermore, activation of the canonical Wnt-signaling pathway can be inhibited by members of the Dkk family, secreted glycoproteins that bind to LRP co-receptors. Dkk antagonizes Wnt action by blocking access to LRP and by mediating LRP endocytosis in cooperation with Kremen, a high affinity single-pass transmembrane receptor for Dkk (reviewed in Niehrs, 2006).

Secreted proteins of the R-Spondin family (RSPO) can prevent the removal or degradation of LRP6 and Fz from the plasma membrane. On the one hand, RSPO can antagonize ZNRF3-mediated downregulation of Fz, by inducing association between ZNRF3 and Leucine-rich repeat-containing G-protein coupled receptor (LGR4), which results in membrane clearance of ZNRF3 (Hao et al., 2012). On the other hand, RSPO can inhibit LRP6 endocytosis by binding to Kremen, thereby blocking the function of Dkk (Binnerts et al., 2007). RSPOs can also act as activating ligands by inducing LRP6 phosphorylation in collaboration with LGRs (Carmon et al., 2011; Wei et al., 2007).

In the cytoplasm Wnt signaling is mainly dependent on the phosphorylation status of β-catenin, as mentioned above. In resting cells, only low levels of free β-catenin can be detected in the cytoplasm and no β-catenin can be found in the nucleus. If β-catenin is not present in the nucleus, TCF/LEF proteins act as repressors for Wnt-target genes due to the binding of the transcriptional co-repressor Groucho (Cavallo et al., 1998; Roose et al., 1998).

Upon Wnt activation the cytosolic level of β-catenin drastically increases and β-catenin translocates to the nucleus. Once in the nucleus, β-catenin replaces the transcriptional co-repressor Groucho from TCF/LEF transcription factors and recruits transcriptional co-activators like cAMP response element binding protein (CBP/p300) or Brg-1, activating Wnt-target gene transcription (Barker et al., 2001; Hecht and Kemler, 2000; Takemaru and Moon, 2000).

These intra-nuclear events are again controlled by several proteins. For example, the β-catenin binding proteins Chibby and ICAT antagonize β-catenin activity by blocking the interaction of β-catenin to TCF/LEF or by mediating the dissociation of the transcriptional co-activator complex (Daniels and Weis, 2002; Graham et al., 2002; Tago et al., 2000). Also TCF/LEF-transcription factors are subjected to regulation. TCF/LEF phosphorylation by Nemo like kinase (NLK)/Nemo diminishes DNA-binding affinity of the β-catenin/TCF/LEF-complex, thereby affecting transcriptional regulation of Wnt-target genes (Ishitani et al., 2003b; Ishitani et al., 1999). In addition, expression of TCF-regulated genes can be repressed by the 14-3-3 protein partitioning-defective (Par5). Par5 can phosphorylate TCF and leading to the nuclear exportation of TCF (Lo et al., 2004; Meneghini et al., 1999).

Interestingly, Wnt-signaling is regulated by feedback-control mechanisms, demonstrated by the finding that the expression of several Wnt-signaling components are directly or indirectly regulated by Wnt-signaling itself (reviewed in Logan and Nusse, 2004). For example, Wnt-signaling can up- or downregulate the expression of several Fz-receptors as well as downregulate LRP expression (Cadigan et al., 1998; Muller et al., 1999; Sato et al., 1999; Wehrli et al., 2000; Willert et al., 2002). Also other components like the HSPG Dally (Baeg et al., 2001), the scaffold protein Axin2 (Jho et al., 2002), the Wnt inhibitor Dkk (Niida et al., 2004), as well as the kinase Nemo (Thorpe and Moon, 2004; Zeng and Verheyen, 2004) and the transcription factor LEF1 (Hovanes et al., 2001) are targets of Wnt-signaling.

In addition to this, canonical Wnt-signaling can be further regulated by distinct sets of Wnt-receptors on the cell surface and intracellularly by non-canonical Wnt-signaling pathways, which can potentiate or inhibit canonical Wnt-signaling (Green et al., 2008; van Amerongen and Nusse, 2009).

1.4.2 Biological functions of Wnt/β-catenin signaling

Wnt/β-catenin signaling plays key roles in numerous important developmental processes, regulates stem-cell self-renewal in adult stem and progenitor cells and is implicated in adult tissue homeostasis (reviewed in Clevers, 2006).

In development, Wnt/β-catenin signaling is i.a. involved in body axis formation in mice (Huelsken et al., 2000), xenopus (Moon and Kimelman, 1998) and zebrafish (Solnica-Krezel, 1999). Indeed, ectopic placement of canonical Wnt in xenopus eggs during early gastrulation gives rise to a secondary body axis and a second head, whereas inhibition of Wnt-signaling results in a lack of dorsal structures (Huelsken et al., 2000). An important function of Wnt-signaling in limb development is for example demonstrated by the inherited disease tetra-amelia, which is caused by mutations in the Wnt3 gene and characterized by the complete absence of all four limbs (Niemann et al., 2004). Canonical Wnt-signaling is also implicated in brain development shown for example by the loss of the hippocampus in mice lacking Wnt3a (Lee et al., 2000). Loss of canonical Wnts results also in defects in patterning of the paraxial mesoderm, defects in somitogenesis and defects in early gastrulation (reviewed in Logan and Nusse, 2004).

Wnt-signaling not only participates in developmental processes, it remains essential throughout life. Canonical Wnt-signaling is required for the establishment of the hair follicles (van Genderen et al., 1994) as well as for hair regrowth and hair morphogenesis (Enshell-Seijffers et al., 2010). Furthermore, Wnt-signaling is an important regulator of hematopoietic stem and progenitor cells (reviewed in Reya, 2003). For example, hyperactivation of canonical Wnt-signaling by loss of APC drastically increases hematopoietic stem cell (HSC) cycling, causing severe defects in multilineage hematopoiesis (Qian et al., 2008). Moreover, the level of Wnt-signaling activity can determine whether HSC self-renew or differentiate. Whereas moderate levels of Wnt activity contributes to HSC maintenance and increase hematopoietic reconstitution, abnormal activation of Wnt-signaling impairs HSC self-renewal and differentiation (reviewed in Rossi et al., 2012).

Mouse genetics strengthened the pivotal role of Wnt/β-catenin signaling in regulating bone homeostasis. Indeed, inhibition of the Wnt-pathway results in decreased bone mass and strength, whereas activation has opposite effects (reviewed in Baron and Kneissel, 2013).

Wnt/β-catenin signaling is the dominant force in controlling cell fate along the crypt-villus axis in the epithelium of the small intestine, the most rapidly self-renewing tissue in mammals (reviewed in Clevers, 2006). For example, abrogation of canonical Wnt-signaling by either removal of β-catenin or TCF4 or by overexpression of the Wnt inhibitor Dkk1 results in a complete loss of proliferation of intestinal progenitor cells at the bottom of the intestinal

crypts, with severe effects on stem cell maintenance and tissue homeostasis (Korinek et al., 1998; Kuhnert et al., 2004b; Pinto et al., 2003). Hence, it is not surprising that mutational activation of Wnt-signaling triggers hyperproliferation, resulting in adenomatous transformation of the intestinal epithelium. Indeed, constitutive activation of Wnt-signaling is the main reason for colon cancer in human (Korinek et al., 1997; Morin et al., 1997).

1.4.3 Wnt-signaling and disease

As described above Wnt/β-catenin signaling is vital for embryonic development and tissue homeostasis if its activity occurs in a physiological range and at the right place at the right time. Consequently, misregulation of Wnt-signaling is the cause for a variety of abnormalities, degenerative and metabolic diseases and cancer (reviewed in Clevers and Nusse, 2012; Logan and Nusse, 2004).

For example, loss of function mutations of LRP6 can be linked to early coronary disease and osteoporosis (Mani et al., 2007) and LoF of LRP5 to osteoporosis-pseudoglioma and eye vasculature defects (Little et al., 2002; Toomes et al., 2004). Also mutations in Fz-receptors cause eye defects. Loss of function of Fz-4 is implicated in familial exudative vitreoretinopathy, a genetic disorder affecting the growth and development of blood vessels in the retina, leading to visual impairment or in the worst case complete blindness (Robitaille et al., 2002). Mutations causing inactivation of another Fz-receptor, Fz-9, are found in patients with the Williams-syndrome, a neurodevelopmental disorder reflected in mental disability, developmental delay and heart defect (Wang et al., 1999). Loss of function mutations of Wnt-genes are linked to obesity (Wnt10b), the Fuhrmann syndrome (Wnt7a) and type 2 diabetes (Wnt5b) (Christodoulides et al., 2006; Kanazawa et al., 2004; Woods et al., 2006) Also gain of function mutations of TCF4 are associated with type 2 diabetes (Florez et al., 2006; Grant et al., 2006).

Moreover, mutations that promote constitutive activation of the Wnt/β-catenin pathway are the reason for many types of cancer. Indeed, misregulated Wnt/β-catenin signaling is involved in nearly all colorectal cancers (CRC) (reviewed in Lustig and Behrens, 2003). The predominant gene mutated in CRC is *Apc*. These mutations lead to inhibited β-catenin degradation and to constitutive active TCF/LEF regulated gene transcription. APC was identified as the germline mutation responsible for familial adenomatous polyposis (FAP)-patients (Vogelstein, 1990), an inherited condition characterized by the formation of numerous polyps in the epithelium of the large intestine. APC is also mutated in 80% and β-catenin in 10-15% of all sporadic colorectal carcinomas (Gayet et al., 2001; Ilyas et al., 1997; Sparks et al., 1998).

Wnt-signaling is also involved in liver tumorigenesis. Gain of function mutations of β-catenin were found in up to 70 % of all hepatoblastomas (HBs) and in ~25% of hepatocellular carcinomas (HCCs) (de La Coste et al., 1998; Koch et al., 1999). Also mutations of Axin and Axin2 are frequent (~10%) in HCCs and HBs (Satoh et al., 2000; Taniguchi et al., 2002). Moreover, about 40% of endometrial carcinomas show mutations in β-catenin and furthermore, at least 30% of prostate tumors display activation mutations of the Wnt-pathway (Gerstein et al., 2002; Mirabelli-Primdahl et al., 1999; Voeller et al., 1998).

However, the highest frequency of aberrant Wnt-signaling is detected in pilomatrixomas (95%), tumors that originate from hair follicles (Chan et al., 1999).

Furthermore, constitutive active Wnt-signaling is involved in several other types of cancers such as thyroid carcinomas, pancreatic cancer, renal cancer, skin cancer, gastric cancer and medulloblastomas (reviewed in Lustig and Behrens, 2003).

1.5 Non-canonical or β-catenin independent Wnt-signaling

Non-canonical Wnt-signaling pathways correspond to signals independent of β-catenin. These pathways can be induced by the binding of the so-called “non-canonical” Wnts such as Wnt5a and Wnt11 and can be classified into at least two different pathways.

1.5.1 Wnt/calcium signaling

The Wnt/calcium pathway is characterized by the Wnt induced release of intracellular calcium, which in turn leads to the activation of different cytoplasmic calcium-sensitive enzymes, such as protein kinase C (PKC), calcium-calmodulin-dependent kinase 2 (CamK2) and the phosphatase calcineurin, resulting in the activation of the transcription factor NF-AT (reviewed in Kestler and Kuhl, 2008).

Wnt/calcium signaling is involved in dorsoventral patterning of early *X. laevis* and zebrafish embryos (Kuhl et al., 2000; Saneyoshi et al., 2002; Westfall et al., 2003), tumor formation (Kremenevskaja et al., 2005; Liang et al., 2003; Weeraratna et al., 2002) and regulation of epithelial-mesenchymal transitions (Garriock and Krieg, 2007).

Interestingly, the Wnt/calcium pathway interacts with the Wnt/β-catenin pathway (Ishitani et al., 2003a). For example, Wnt/calcium signaling has been shown to activate Nemo like kinases (NLK) (Takada et al., 2007). These are able to phosphorylate TCF-transcription factors, thereby inhibiting canonical Wnt-signaling (see 1.4 canonical Wnt-signaling).

1.5.2 Wnt/JNK-signaling

In vertebrates the binding of Wnt5a to the RTK Ror2 activates Wnt/JNK-signaling (Oishi et al., 2003). The Wnt/JNK-pathway includes the activation of Dsh and further downstream, activation of rhoGTPases such as rhoA, rac and cdc42. These GTPases activate downstream mediators like rho kinase (ROK) or c-Jun N-terminal kinase (JNK). JNK in turn phosphorylates c-Jun and the activating transcription factor-2 (ATF-2), which heterodimerize in order to stimulate cAMP response element (CRE) regulated transcription (reviewed in Kestler and Kuhl, 2008).

Wnt/JNK-signaling regulates convergent extension movements during gastrulation or neurulation and cell migration of neural crest cells (De Calisto et al., 2005). This pathway is also described as the planar cell polarity PCP-pathway in *D. melanogaster*, which regulates the polarity of epithelial cells within the plane of epithelium, for example the orientation of wing hairs and the organization of ommatidia in the fly eye (Strutt, 2003).

1.6 CD44

The term CD44 designates a family of transmembrane glycoproteins that belong to the cell adhesion molecules (CAMs). CD44 proteins are ubiquitously expressed on vertebrate cells. They were first discovered in 1980 as antigens recognized by antibodies raised against human leukocytes (Dalchau et al., 1980). Like many other cluster of differentiation antigens, several research groups discovered the CD44 proteins individually and described the protein using a different name, like phagocyte glycoprotein 1 (Pgp1) (Hughes et al., 1983), Hermes antigen (Jalkanen et al., 1986), Class 3 extracellular matrix receptor (ECMR3) (Carter and Wayner, 1988), homing cell adhesion molecule (HCAM) (Goldstein et al., 1989) and HUTCH-1 (Gallatin et al., 1989). Sequencing studies and immunodetection-studies revealed that all these molecules were in fact identical. The International Workshop on Human Leukocyte Differentiation Antigens ascribed the cluster of differentiation number 44 to the protein, which was then called CD44.

1.6.1 CD44 expression

CD44 proteins appear in numerous isoforms, which are all encoded by one single gene of approximately 50 kb length, localized on the short arm of chromosome 11 in humans (Goodfellow et al., 1982). The CD44 gene is highly conserved across species and contains 20 exons (Fig.2).

The extracellular part of CD44 is encoded by exons 1-17. Exon 1-5 and 16-20 are constant and present in all CD44 proteins. The hydrophobic transmembrane part is encoded by exon 18. Exons 19 and 20 code for the intracellular domain of CD44. The huge number of variant isoforms is generated by alternative splicing of exons 6-15, also called variant exons 1-10 (v1-v10). Insertion of these variant exons can occur in different combinations and takes place between the constant exons 5 and 16 of the extracellular region (Screaton et al., 1992). Variant exon v3 can occur in two variants, due to the presence of an additional splice acceptor site in exon v3. Exon v1 is not expressed in human cells since it contains a stop codon (Screaton et al., 1993; Screaton et al., 1992; Tolg et al., 1993).

Alternative splicing also takes place in the cytoplasmic domain of CD44 allowing either inclusion of exon 19 or 20. Exon 19 harbors an alternative stop codon and generates a short cytoplasmic region of 5aa. The inclusion of exon 20 is much more frequent and gives rise to 73 aa intracellular CD44 domain (Goldstein and Butcher, 1990; Goldstein et al., 1989; Thorne et al., 2004). The splicing of CD44 is regulated by splicing factors like ESRP1 or 2 (Warzecha et al., 2009) and SRp20 (Goncalves et al., 2008) and can be triggered by signaling events such as Ras/MAPK or the β-catenin signaling cascades (Cheng et al., 2006; Goncalves et al., 2008; Hofmann et al., 1993; Weg-Remers et al., 2001).

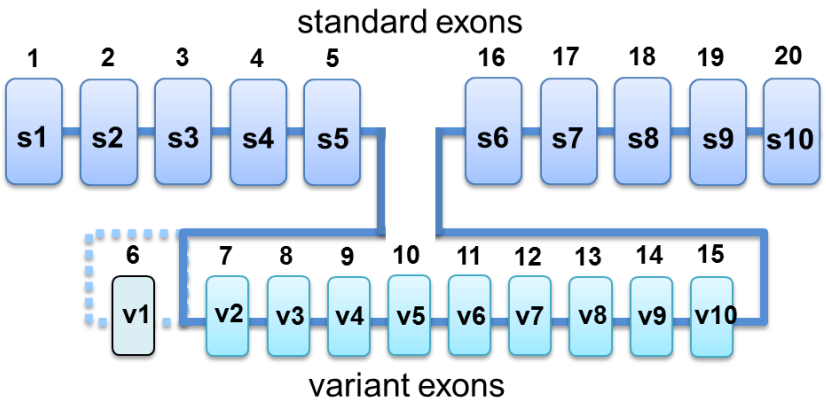

Fig. 2 Schematic diagram of the structure of the CD44 gene.
The standard exons (s1-s10) encode the ubiquitously expressed standard protein isoform CD44s. Combinations of the variant exons (v1-v10) can be alternatively spliced between s5 and s6 to encode variant protein isoforms, CD44v. Unlike the mouse gene, exon 6 (v1) of the human gene contains a stop codon and is not normally included in human CD44 mRNA (modified from Goodison et al., 1999).

The smallest isoform of CD44, CD44 standard (CD44s), is composed of 361 aas in human and lacks all variant exons in the extracellular domain. Whereas CD44s is ubiquitously expressed, the expression of CD44 variant isoforms (CD44v) is restricted to specific tissues. The largest CD44v in human (CD44v2-v10), for example, is only expressed in keratinocytes (Bloor et al. 2001; Screaton et al. 1993). In the intestinal epithelium it was recently demonstrated that CD44v4-v10 is exclusively expressed in $Lgr5^{+}$ stem cells, which do not express CD44s. However, the more differentiated transit amplifying cells express CD44s and other shorter CD44v isoforms but not CD44v4-v10 (Zeilstra et al., 2013).

In addition to alternative splicing events that generate a huge number of different CD44 isoforms, the heterogeneity can even be further increased by posttranslational modifications, like N-and O-linked glycosylations, heparansulphation or chondroitinsulphation (see 1.6.2 The extracellular the extracellular domain of CD44). Therefore, although the predicted size of the CD44 core protein is 38 kDa, these modifications highly increase the molecular weight of CD44, which varies from 80 to 230 kDa (Goldstein et al., 1989; Lokeshwar and Bourguignon, 1991; Stamenkovic et al., 1989).

1.6.2 The extracellular domain of CD44

The aminoterminal (N-terminal) part of CD44 is encoded by exon 1 – 5 and contains 180 aas. This part is common to all CD44 isoforms and highly conserved in mammals (~85%). This region contains six cysteine residues, forming three intramolecular disulphide bonds, necessary for the correct folding of this domain and for the binding of the extracellular matrix (ECM) component hyaluronan (HA), the major ligand of CD44 (Banerji et al., 1998; Goldstein et al., 1989). Within the N-terminus, exons 2 and 3 encode a link-homology hyaluronan binding domain that shows high similarities to domains found in other HA binding proteins like cartilage link protein (Deak et al., 1986), proteoglycan core protein (Neame et al., 1986), aggrecan and tumor necrosis factor stimulated gene 6 (TSG6) (Bajorath et al., 1998; Kohda et al., 1996). This domain has been shown to be a binding site of HA (Peach et al., 1993; Thorne et al., 2004). The N-terminal region harbours also motifs for N-glycosylations, which can modulate the binding of HA to CD44 (Bartolazzi et al., 1996; English et al., 1998). However, the extracellular region of CD44 does not only interact with HA but also with other ECM components, as well as growth factors and enzymes contributing to the diversity of functions of CD44 (see 1.6.5.5 Molecular functions of CD44).

Exons 4 and 5, together with exon 16 and 17, encode the stem region of CD44s proximal to the membrane (reviewed in Thorne et al., 2004). This stem region can be elongated by insertion of variant exons between exons 5 and 16, thereby giving rise to the variant isoforms (Screaton et al., 1992; Tolg et al., 1993). The stem region comprises different sites of post-translational modifications, like glycosaminoglycan (GAG) modifications (e.g. chondroitin sulphation, n sulphation) and N-linked and O-linked glycosylation. In addition, variant exon 3 contains a motif for heparansulphation. The attachment of heparansulphate-moieties enable CD44v3 isoforms to act as heparansulphated-proteoglycans (HSPGs) and to bind heparin-binding proteins (Bennett et al., 1995; Jones et al., 2000; Lokeshwar et al., 1994; Sherman et al., 1998). All these posttranslational modifications are cell type specific and are able to modulate the function of CD44 in different ways (English et al., 1998).

The CD44 stem region also contains a cleavage site for metallo- and serine-proteases. Cleavage results in soluble CD44, which can compete with ligand binding of membrane bound CD44 (Bazil and Strominger, 1994; Katoh et al., 1994; Yu et al., 1997). However, cleavage is not the only way to generate soluble CD44. Yu and Toole (Yu and Toole, 1996) describe an additional exon between exon v9 and v10 containing stop codons that, upon inclusion, would give rise to translated CD44 proteins which are truncated prior to the transmembrane domain and secreted as a soluble protein.

1.6.3 The transmembrane domain of CD44

The transmembrane domain of CD44 comprises of 23 hydrophobic amino acids and a cysteine residue, which undergo fatty acid acylation, especially palmitoylation. These modifications play roles in clustering of CD44, HA-binding (maybe due to clustering) and intracellular ankyrin binding (Bourguignon et al., 1991; Liu and Sy, 1996). In particular, acylation of the cysteine residue in this domain is involved in localization of CD44 in lipid rafts that is required for HA internalization and turnover/recycling of CD44 (Thankamony and Knudson, 2006). Within the transmembrane domain there are two sites for intramembranous cleavage. A cleavage within these sites by presenilin-1/γ-secretase results in secretion of an analogous CD44β-like peptide and an intracellular cytoplasmic domain of CD44 (CD44-ICD). Whereas the function of the CD44 peptide is not known, the released CD44-ICD can translocate in to the nucleus where it can activate transcription of genes via phorbol ester response elements (reviewed in Okamoto et al., 2001; Thorne et al., 2004).

1.6.4 The cytoplasmic domain of CD44

The cytoplasmic domain of CD44 is most often encoded by exon 20 and comprises of 73 aas. However, it was shown that inclusion of exon 19 by alternative splicing can generate CD44 isoforms with short 5 aa tails but little attention has been paid to these isoforms because of their very low abundancy. The 73 aa cytoplasmic domain contains several functional motifs, including a FERM (4.1, Ezrin, Radixin, Moesin) binding domain, an ankyrin binding site, a basolateral targeting domain, a PDZ-domain binding peptide and two phosphorylation sites at Ser325 and Ser316 (reviewed in Thorne et al., 2004).

The first protein, which was shown to interact with this domain is ankyrin, an adaptor protein with a spectrin-binding site that links transmembrane proteins to the actin-spectrin membrane skeleton (Bennett and Stenbuck, 1979; Kalomiris and Bourguignon, 1988). The interaction of CD44 with ankyrin is involved in HA-dependent cell adhesion, cell motility and proliferation (Lokeshwar et al., 1994; Singleton and Bourguignon, 2004; Zhu and Bourguignon, 2000).

CD44 can furthermore bind to Ezrin, Radixin and Moesin (ERM), members of the Band 4.1 superfamily. These proteins have actin binding sites and link CD44 to the actin-cytoskeleton (Legg and Isacke, 1998; Turunen et al., 1994). Merlin (Moesin-, Ezrin-, Radixin-like), an ERM related tumor suppressor protein lacking the actin binding site, was also shown to bind CD44 mediating contact inhibition of growth by binding to CD44 (Morrison et al., 2001). The binding of ERM proteins to CD44 is furthermore required for HGF and VEGF-A to promote downstream signaling from their corresponding receptors Mesenchymal-epithelial

transition factor (Met) and Vascular endothelial growth factor receptor 2 (VEGFR2) (Orian-Rousseau et al., 2007; Tremmel et al., 2009).

The binding of CD44 to its intracellular binding partners is dependent on the phosphorylation status of the conserved serine residues within the CD44 domain. In resting cells, Ser325 is constitutively phosphorylated, at least in part, by Ca2+/calmodulin dependent protein kinase 2 (CaMK2) (Lewis et al., 2001) that is involved in HA mediated cell migration (Peck and Isacke, 1998). A change in phosphorylation by dephosphorylation of Ser325 and phosphorylation of Ser291 by protein kinase C (PKC), impairs binding of CD44 to ERM proteins, therefore regulating ERM mediated linkage between CD44 and the actin-cytoskeleton (Legg et al., 2002). Also binding of ankyrin depends on the phosphorylation state of CD44 which is regulated by Rho kinase (ROK) (Bourguignon et al., 1999).

Neural Wiskott-Aldrich syndrome protein (N-WASP) is another protein that links CD44 to the actin-cytoskeleton. This occurs via Actin related protein2/3 (Arp2/3) and was shown to be involved in actin-polymerization and cell migration (Bourguignon et al., 2007c).

In addition to proteins that link CD44 to the cytoskeleton, the intracellular domain of CD44 complexes with other proteins like GTPases of the Rho-family and members of the Src-family, which was shown to be involved in in rearrangement of the actin-cytoskeleton with implications in tumor cell migration, invasion and progression (Bourguignon, 2008; Bourguignon et al., 2001).

1.6.5 Biological functions of CD44

Three decades of research on CD44 revealed a participation of CD44 in many important cellular responses like cell adhesion, proliferation and migration. Hence, CD44 is involved in and required for several physiological and pathological processes such as embryonic development (reviewed in Sherman et al., 1996), haematopoiesis (Miyake et al., 1990b), inflammation (Mikecz et al., 1999; Wittig et al., 2000), tissue repair (Kaya et al., 1997) and tumorigenesis (reviewed in Orian-Rousseau, 2010).

1.6.5.1 Physiological functions of CD44

CD44 isoforms are expressed in several tissues during embryogenesis. The main regions where CD44 is expressed correspond to areas of the heart and the somites where HA-mediated morphogenesis and organogenesis take place (Fenderson et al., 1993; Wheatley et al., 1993). In *X. laevis* CD44 expression is detected already in the presomitic mesoderm and later also in the somites. Here, a morpholino-mediated knockdown of CD44 caused altered migration of hypaxial muscle cells that originate from the somites and for this reason the morphants failed to develop a properly organized ventral musculature (Ori et al.,

2006). Furthermore, silencing CD44 expression in *X. laevis* interfered with cranial neural crest cell migration and caused craniofacial skeleton abnormalities (Casini et al., 2012).

CD44 isoforms play moreover an important role in limb development. Indeed, CD44v3-v10, expressed in the apical ectodermal layer of the limb, can interact via heparan-sulphate residues with the fibroblast growth factors FGF-8 and FGF-4 and present them to their corresponding receptors at the membrane of the underlying mesenchymal cells. This process is necessary for inducing proliferative signals, required for the correct outgrowth of the limbs (Sherman et al., 1998).

Interference studies with CD44 specific antibodies revealed a role of CD44 in ontogeny. In pregnant rats, i.v. injection of antibodies recognizing epitopes common to all CD44 isoforms causes delays in delivery and numerous intrauterine abortions and injection of antibodies recognizing epitopes on CD44v6 isoforms interfered with development of the rats further then E16-18 (days after fertilization) (Zoller et al., 1997).

CD44 is required for differentiation of haematopoietic stem and progenitor cells of the myeloid and lymphoid lineage. Treatment with antibodies recognizing all CD44 isoforms blocked the development of progenitor cells and the generation of lymphoid and myeloid cells in long-term bone marrow cultures (LTBMC) (Khaldoyanidi et al., 1996; Miyake et al., 1990a). Interestingly, treatment of LTBMC with antibodies against CD44v4 and CD44v6 stimulates myelopoiesis, whereas treatment with CD44v6 also induces lymphopoiesis (Khaldoyanidi et al., 2002; Rossbach et al., 1996).

CD44 is also involved in activation and maturation of T- and B-lymphocytes (Arch et al., 1992; Huet et al., 1989) and in the extravasation of lymphocytes from blood vessels into surrounding tissue (DeGrendele et al., 1996). Homing of lymphocytes, namely the return from the blood vasculature to the lymphatic tissues is also affected by CD44 (O'Neill, 1989; Shimizu and Shaw, 1991).

1.6.5.2 CD44 in inflammation and autoimmune diseases

CD44 and its interaction with HA plays crucial roles in inflammation and autoimmune diseases. Leukocytes increase the expression of CD44 during inflammation (Foster et al., 1998) and - at the inflammation site - microvascular endothelial showed increased expression of HA after induction with pro-inflammatory cytokines like IL-1β or TNF-α. This suggests a role of HA/CD44 interaction in extravasation of immune cells (Mohamadzadeh et al., 1998).

Furthermore, the interaction between HA and CD44 might be a critical factor for inflammation in rheumatoid arthritis. Treatment with an antibody blocking the interaction between HA and CD44 reduces the inflammation, whereas treatment with an antibody that increases the binding of HA to CD44 increases inflammation (Mikecz et al., 1999).

The formation of brain lesions in multiple sclerosis and allergic encephalomyelitis, caused by T-cell crossing of the blood-brain-barrier, is reduced by treatment with CD44 antibodies (Brocke et al., 1999). In addition, HA binding to CD44 on CNS vascular endothelial cells is critical for extravasation of activated T-cells into the CNS, a possible mechanism involved in autoimmune encephalomyelitis (Winkler et al., 2012). Moreover, there are evidences for the impact of CD44 in other autoimmune- and inflammatory diseases, like the inflammatory bowel diseases (Hoffmann et al., 2007; Pfister et al., 2001; Wittig et al., 2000), skin inflammations (Camp et al., 1993) or the IL-2 (interleukin-2) induced vascular leak syndrome (Rafi-Janajreh et al., 1999).

1.6.5.3 CD44 in cancer

CD44 isoforms are expressed on numerous primary human tumors originating from cells of the skin, breast, colon and brain (Dall et al., 1995; Hart et al., 1991; Naor et al., 1997; Reeder et al., 1998). Especially, the expression of specific isoforms often correlates with advanced stages and a poor prognosis of the cancer, for instances CD44v6 expression in colorectal cancer or CD44v6, v7 and v8 in cervical cancer (Kainz et al., 1995; Naor et al., 1997; Sleeman et al., 1997; Wielenga et al., 1993). Thus, CD44 isoforms are potential prognostic markers for several types of cancer, e.g. breast or colon cancer and melanomas (Kaufmann et al., 1995; Simon et al., 1996; Wielenga et al., 1998).

As CD44 is highly expressed in various cancer types, it is not surprising that an involvement of CD44 was linked to several critical steps in tumorigenesis, such as tumor initiation, tumor progression as well as in the metastasis of tumor cells (Bourguignon, 2012; Seiter et al., 1993; Takeuchi et al., 1995; Zeilstra et al., 2008). CD44 is also involved in recurrence (Hirata et al., 2013; Toma et al., 1999) or chemo- and radiotherapy resistance (Ohashi et al., 2007; Tamada et al., 2012).

A crucial role of CD44 in tumor initiation was for example demonstrated in $Apc^{MIN/+}$ mice, a mouse model of the human FAP-syndrome, characterized by the development of multiple adenomatous polyps in the colon caused by a mutation in the *apc* gene. The crossing of these mice with $Cd44^{-/-}$ mice resulted in decreased formation of aberrant crypts and a drastic reduction in the number of intestinal adenomas, when compared to $Cd44^{+/+}/Apc^{MIN/+}$ mice (Zeilstra et al., 2008).

The tumor growth promoting properties of CD44 were for example shown by transfection of mouse melanoma cells with CD44s, that led to an increase of tumor size and weight (Bartolazzi et al., 1994).

Studies in which $p53^{+/tml}$ mice (p53 deficient mice with targeted mutation 1 in the p53 gene) were crossed with CD44 knockout mice, also demonstrated the striking impact of

CD44 on tumor progression. In *Cd44*$^{-/-}$*/p53*$^{+/tml}$ mice, only benign tumors could be detected, whereas control mice showed metastases (Weber et al., 2002).

The causative role of CD44 in metastasis, the life-threatening step in cancer, was demonstrated i.a. by introduction of CD44 in non-metastatic cells, converting them to metastasising ones. Indeed, introduction of CD44v4-v7 in non-metastatic rat pancreatic cancer cells resulted in formation of metastases. This effect was blocked by treatment with specific antibodies directed against CD44v6 (Gunthert et al., 1991; Seiter et al., 1993).

Furthermore, a CD44 negative Burkitt lymphoma cell line, which is unable to form metastases, became metastatic after transfection of CD44s (Sy et al., 1991). Yae and colleagues (Yae et al., 2012) showed recently that expression of CD44 variants enhanced formation of metastasis in a mouse model for breast cancer. Orthotopically transplanted CD44v positive 4T1 breast cancer cells caused metastasis of these cells to the lung, whereas CD44v negative 4T1 cells did not.

However, although specific expression of CD44 variants usually correlates with tumor progression and metastasis, the opposite was also shown in some cancers where CD44 exerted tumor suppressive functions. For example, CD44v8-v10 expression in human B-cell lymphoma cells impairs tumor formation in mice (Sy et al., 1991). Furthermore, transformed fibroblasts showed drastically reduced tumor growth upon transfection with CD44s (Schmitts et al 1997). In oral squamous cell carcinomas, the tumors expressing lower levels of CD44v6 exhibited more frequent regional lymph node metastases (Kunishi et al., 1997). In addition, decreased expression of CD44s is involved in the progression of prostatic cancer to a metastatic state and increased expression of CD44s was able to reduce metastasis formation of a prostate carcinoma cell line (Gao et al., 1997). Moreover, there is evidence that downregulation of CD44 contributes to cancers such as squamous cell carcinomas, neuroblastomas and prostate cancer and here, a lack of CD44 also correlates with poor prognosis and metsastasis (De Marzo et al., 1998; Sato et al., 2000; Shtivelman and Bishop, 1991). This might be due to a loss of function of CD44 as cell adhesion molecule and may be related to the detachment of potential metastatic cells from the primary tumor (reviewed in Naor et al., 2002).

1.6.5.4 Deletion of CD44 in mice

CD44 germline knockout mice

Targeted deletion of single genes in animals provides information about the *in vivo* functions of the protein encoded by these genes. CD44 total knockout mice were generated by germline deletion of different sequences of the CD44 gene (Protin et al., 1999; Schmits et al., 1997). Although, the diversity of functions of CD44 molecules suggested that a deletion of the CD44 gene in mice might have drastic consequences on development, these *Cd44*$^{-/-}$ mice were born viable, fertile, with the normal mendelian ratio and developed to a large extend normally. Several mild abnormalities were detected, for example in the haematopoietic system. Indeed, the tissue distribution of myeloid progenitor cells between bone marrow and spleen was altered (Schmits et al., 1997). Furthermore, lymphocyte homing to the lymph nodes and the thymus was hampered, whereas lymphocyte development seemed not to be affected (Protin et al., 1999). Moreover, some defects caused by CD44 deletion were shown in pregnant mice, where the maintenance of lactation was impaired and uterine involution accelerated (Yu et al., 2002). Lastly, loss of CD44 in the germline knockout mice resulted in decreased HA-mediated keratinocyte differentiation and lipid synthesis/secretion, which in turn affected epidermal permeability barrier function (Bourguignon et al., 2006).

More drastic phenotypes were found when *Cd44*$^{-/-}$ mice were challenged. Induced lung injury lead to death of *Cd44*$^{-/-}$ mice, characterized by impaired clearance of apoptotic neutrophils and persistent accumulation of pro-inflammatory HA-fragments. This phenotype could be reversed by injection of CD44-positive leukocytes demonstrating an important role of CD44 in inhibiting lung inflammation (Teder et al., 2002). Another study showed that infection with *Cryptosporidium parvum*, a parasite of the intestinal tract, resulted in enhanced granuloma response in CD44 knock-out mice, suggesting defects in T-cell function (Schmits et al., 1997). Moreover, vascularization during tumor growth and wound healing was impaired in the *Cd44*$^{-/-}$ -background in a particular KO mouse (Cao et al., 2006). However, the importance of CD44 as a corceptor for Met (see 1.6.5.5 Molecular functions) was demonstrated when *Cd44*$^{-/-}$ mice were crossed with *Met*$^{+/-}$ mice (Matzke et al., 2007). Two thirds of the mice bearing only one Met allele in the CD44 null background died at birth from breathing defects. These defects could be explained both by an alteration of the phrenic nerve and by a reduced activity of the pre-Bötzinger complex, a region of the brain that controls respiration.

Further insight in the role of CD44 for tumor progression is given in studies where *Cd44*$^{-/-}$ mice were crossed with animals containing mutations in tumor suppressor genes, e.g.

p53+/tm1 or *Apc*$^{MIN/+}$ where CD44 was shown to be responsible for metastasis and tumor initiation, respectively (see 1.6.5.3 CD44 in cancer).

Conditional CD44 knockout in keratinocytes

In contrast to the CD44 total knock-out mice, the “conditional” knock-out mice showed more drastic effects. In transgenic mice, expressing an antisense CD44 cDNA under control of the bovine keratin-5 promoter, CD44 expression was inhibited in all layers of the epidermis and outer root sheath of hair follicles from E11.5 (Kaya et al., 1997). These mice developed a thick skin with reduced elasticity and increased rigidity. Furthermore, they showed an accumulation of HA in the superficial epidermis and a loss of HA in the interkeratinocyte spaces. Altogether, the removal of Cd44 in the skin resulted in a delayed wound healing, delayed hair regrowth and reduced response to carcinogens.

The striking differences between the classical total and the “conditional” CD44 knockout mice suggest that a loss of CD44 at different timepoints in embryonic development has different consequences. The weak phenotype observed in Cd44 germline knock-out mice as compared to strong phenotypes in the “antisense knock-out mice” might be explained by a substitution of CD44 by other proteins. This substitution might be possible at the beginning of development but not when CD44 is removed at later time points e.g. when the K5 promoter gets active.

This theory proved to be true since a protein substituting for CD44, namely intercellular adhesion molecule 1 (ICAM-1) was identified in the germline knockout mice. In the absence of CD44v6, an essential co-receptor for c-Met (see 1.6.5.5 Molecular functions), ICAM-1 still allows c-Met receptor activation *in vivo* and *in vitro* and replaced the CD44 co-receptor function for c-Met in liver regeneration upon partial hepatectomy (Olaku et al., 2011). Another protein that can act as a substitute for CD44 is Receptor for HA-Mediated Motility (Rhamm), which is, similar to CD44, a receptor for HA (Hardwick et al., 1992). Indeed, although treatment with antibodies against CD44 reduced collagen-induced arthritis (CIA) in wildtype mice (Nedvetzki et al., 1999), CIA is enhanced in the CD44 germline knockout mice. This is most probably a result of enhanced expression of Rhamm. This might compensate for the loss of CD44 and triggers the inflammatory response during CIA in the absence of CD44 (Nedvetzki et al., 2004b).

1.6.5.5 Molecular functions

CD44 a receptor for extracellular matrix components

The best characterized binding partner of CD44 is the ECM component HA, a hydrophilic linear polysaccharide consisting of repeating subunits of D-Glucuronic acid and N-acetyl-D-glucosamine linked together via alternating β-1,4- and β-1,-3 glycosidic bonds (Aruffo et al., 1990). CD44 proteins bind to HA via their amino-terminal domain, containing hyaluronan-binding motifs, which are found in the 'link domain' (amino-acids 32-123) and also outside of the link-domain (amino acids 150-158) (Naor et al., 1997). This binding motif is $B(X_7)B$ (B represents the basic amino acids arg or lys, X is any amino acid) and is found in all HA-binding motif of HA binding proteins (Yang et al., 1994). CD44 mediates HA-dependent cell adhesion in a number of different cell types including fibroblasts and leucocytes, as demonstrated by hyaluronidase treatment or antibodies directed against the HA binding domain of CD44, both leading to an inhibition of adhesion (Green et al., 1988; Pessac and Defendi, 1972; Underhill and Dorfman, 1978; Underhill and Toole, 1981). HA/CD44 binding leads also to cell migration, this was shown by experiments in which HA induced cell migration could be blocked by treatment with antibodies against CD44 (Koochekpour et al., 1995; Naor et al., 1997).

The binding affinity of CD44 to HA can be altered by inclusion of variant exons. For example CD44v4-v7 shows a higher affinity than CD44s (Sleeman 1996, Sleeman 1997). Additionally, the binding affinity can be modulated by changes in the glycosylations of the extracellular CD44 domain and by phosphorylation of serine residues in the intracellular domain (Naor et al., 1997; Skelton et al., 1998). Interestingly, CD44 is involved in mediating the internalization of HA in variant cell types (reviewed in Thorne et al., 2004) and this uptake could be connected to physiological aspects in wound healing and immune response (Kaya et al., 1997; Nedvetzki et al., 2004a; Teder et al., 2002). The interaction between CD44 and HA has been shown to modulate several cellular signaling events like β-catenin signaling, ErbB2-, PI3K/Akt-, RhoGTPase- or CXCR4-signaling (Bourguignon, 2008; Bourguignon et al., 2007c; Fuchs et al., 2013; Misra et al., 2008).

In addition to HA, CD44 can also interact with other ECM components like collagen, laminin and fibronectin, but till now no evidence for physiological functions of these interactions were provided (Borland et al., 1998; Naor et al., 1997). Furthermore CD44 can bind to osteopontin, in order to promote cell survival in Chinese hamster ovary cells (CHO-cells) via activation PI3K/Akt-signaling (Lin and Yang-Yen, 2001).

CD44 provides a platform for matrix metalloproteinases

CD44 can interact with matrix metalloproteinases (MMPs) and therby act as platform where MMPs are brought in close vicinity to their substrates. For example, CD44 recruits MMP-9 that can on the one hand activate Transforming growth factor β (TGF-β) by proteolytic cleavage in order to promote angiogenesis (Yu and Stamenkovic, 2000). On the other hand, the recruitment of MMP-9 by CD44 leads to proteolytic degradation of Collagen IV, thereby promoting tumor cell invasion (Yu and Stamenkovic, 1999). Furthermore, MMP-7 uses CD44 for the cleavage of heparin-binding epidermal growth factor (HB-EGF) precursors which can bind to the heparan sulphate moiety of variant exon 3. This cleavage results in activation of HB-EGF/ErbB4-signaling that in turn induces cell survival and tissue remodelling (Yu et al., 2002).

CD44 as a co-receptor for receptor tyrosine kinases

RTKs are cell surface receptors for numerous growth factors. Upon ligand binding, RTKs dimerize, get activated and induce intracellular signaling cascades via their tyrosine kinase domain. However, more and more evidence show that the only presence of the ligand is often not sufficient for the activation of the receptor. Additional factors are required for a proper activation and these factors are in most instances cell adhesion molecules (CAM) (Orian-Rousseau and Ponta, 2008).

CD44 is one of these CAMs that contribute to the activation of various RTKs. Hereby, the co-regulatory function of CD44 is very diverse and often dependent on specific CD44 isoforms. CD44s, for example, was shown to trigger dimerization and activation of ErbB2 and ErbB3 (members of the EGF-receptor family) upon neuregulin induction with implications on Schwann cell differentiation and survival (Bourguignon et al., 1997; Sherman et al., 2000). In addition the binding of HA to CD44s can trigger the activation of ErbB2 in ovarian or colorectal cancer cell lines (Misra et al 2008, Bourguignon 1997 and 2007). Heparan sulphated CD44v3 isoforms are required for the activation of HB-EGF by MMP7 and the presentation of activated HB-EGF to its receptor ErbB4 (Bennett et al., 1995; Yu et al., 2002). In addition, CD44v3 isoforms are also able to bind FGF in order to present the ligand to its receptor FGFR which is necessary for proper limb development (Sherman et al., 1998).

However, the best-characterized co-regulatory function of CD44 isoforms is the collaboration between CD44v6 and the HGF-receptor mesenchymal epithelial transitioning factor (c-Met). Here, it was demonstrated that the CD44v6 isoform is specifically required for Met-signaling (Orian-Rousseau et al., 2002). The co-receptor function of CD44 in HGF/Met-

signaling is two-fold. Whereas inclusion of variant exon 6 in the extracellular domain is crucial for the initial activation of the receptor, the cytoplasmic domain of CD44 and its linkage to the actin-cytoskeleton via ERM proteins is required for the formation of a signalosome complex that allows downstream signaling from the receptor to its intracellular effector molecules (Orian-Rousseau et al., 2002; Orian-Rousseau et al., 2007). Mutational analysis by alanine-linker scan mutations of the v6-exon revealed that three specific amino acids in this exon are crucial for this function. Moreover, treatment of cells that express CD44v6 and Met with peptides covering this region can efficiently block Met activation and HGF-induced cell scattering and migration (Matzke et al., 2005). Hence, the co-receptor function of CD44v6 for HGF/Met-signaling is decisive for the HGF induced metastatic spreading of tumor cells from the primary tumor.

In addition to HGF/Met-signaling, a pivotal role of CD44v6 isoforms was also identified for activation of the RTK vascular endothelial growth factor receptor 2 (VEGFR-2). VEGFR-2 is the most prominent RTK involved in tumor angiogenesis, a fundamental step in the transition of tumors from a dormant state to a malignant one (Holmes et al., 2007). The mechanism of action of CD44v6 in VEGFR-2-signaling seems to be similar to the one described for Met. The same v6-specific peptides that block HGF/Met-signaling also interfere with vascular endothelial growth factor (VEGF-A) induced activation of VEGFR-2 and inhibited VEGFR-2 mediated angiogenesis (Tremmel et al., 2009) Furthermore, like in HGF/Met-signaling VEGF-A mediated activation of downstream effectors requires the linkage of CD44 to the actin-cytoskeleton via ERM-proteins (Tremmel et al., 2009).

1.7 Correlation between CD44 and Wnt-signaling

The finding that CD44 overexpression is an early event in colorectal cancer, a disease characterized by aberrant β-catenin signaling, established the first link between CD44 and Wnt-signaling. Indeed, *Apc*$^{MIN/+}$ mice with hyperactivated TCF-regulated gene transcription showed increased expression of CD44s and CD44variants in aberrant crypt foci (ACF), the earliest detectable lesions of colorectal neoplasia, as well as in more advanced stages like adenoma or carcinoma (Wielenga et al., 1999). Furthermore, *Tcf4*$^{-/-}$ mice showed a complete absence of CD44 in the epithelium of the small intestine, indicating that CD44 expression is part of a genetic program controlled by β-catenin/TCF signaling (Wielenga et al., 1999). Also FAP patients that develop multiple intestinal adenomas as a result of a mutation in the *apc* gene, showed increased expression of CD44s and a strong overexpression of CD44v6 in ACF. Further studies revealed that adenoviral expression of Dkk1 in adult mice could repress intestinal CD44 expression indicating that CD44 is indeed a specific canonical Wnt target (Kuhnert et al., 2004b) and nowadays, CD44 gene expression

is used as readout of Wnt/β-catenin signaling activation like c-Myc or Axin2 (Han et al., 2012; Modder et al., 2011; Zhu et al., 2013).

Interestingly, CD44 has impact on the onset of β-catenin induced tumorigenesis, since loss of CD44 in *Apc*-mutant mice results in a decreased number of intestinal adenomas (Zeilstra et al., 2008). In addition, downregulation of CD44v6 by means of shRNA results in reduced adenoma number and outgrowth (Misra et al., 2009). In both cases, loss of CD44 resulted in increased apoptosis, indicating that CD44 is required for tumor cell survival. Hence, it is not surprising that CD44 overexpression, especially of CD44v6, is correlated with more advanced stages and poor prognosis in colorectal cancer (Wielenga et al., 1993; Wielenga et al., 1998).

Altogether, there is evidence that CD44 has great impact on Wnt-driven tumorigenesis as a Wnt-target gene. However, studies analyzing a role of CD44 as a modulator of Wnt-signaling have been missing.

The possibility that CD44 itself could be involved in Wnt-induced signaling is underscored by the finding, that HA/CD44 interaction can trigger β-catenin nuclear translocation and upregulation of TCF/LEF-regulated gene transcription, most likely indirectly via ErbB2-mediated PI3K/Akt-signaling or β-catenin acetylation (Bourguignon et al., 2007c). Most recently, it was described that silencing of CD44 by means of shRNA affects β-catenin expression and stability and suppresses nuclear translocation of β-catenin in chronic myeloid leukemia cells (Chang et al., 2013). However, in this study the cells were not induced by Wnt-ligands or any other activator of the canonical Wnt-pathway. As the activation and nuclear translocation of β-catenin can be mediated by several other pathways like ErbB2- or FGFR-signaling (Bourguignon et al., 2007c; Krejci et al., 2012; Misra et al., 2008) any conclusion about a specific requirement of CD44 for the activation of the Wnt/β-catenin signaling pathway is missing in this study. Furthermore, Chang and colleagues did not prove any *in vivo* function of CD44 in β-catenin signaling activation. The fact that CD44 can act as a HSPG or CSPG makes it even more likely that CD44 could be involved in Wnt-signaling, as it was shown that other HSPGs like glypicans or CSPGs can bind to Wnt-proteins and modulate signaling (Baeg et al., 2001; De Cat et al., 2003; Lin and Perrimon, 1999; Nadanaka et al., 2008; Tsuda et al., 1999).

The fact that CD44 isoforms are known to be involved in the regulation of several signaling pathways activated by RTKs or G-protein coupled receptors (GPCRs) is a further suggestive incentive to investigate a potential role of CD44 in Wnt-signaling. Moreover, several other Wnt target genes are involved in the regulation of Wnt-signaling, indicating that feedback-control is a key feature of canonical Wnt-signaling regulation. The regulatory role of CD44 in many other growth factor induced signaling pathways would fit to our suggestion

that CD44 contribute to the feedback-regulation of the Wnt-pathway. Figure 3 shows a schematic representation of CD44 and the factors that might be involved in a potential regulation of Wnt/β-catenin signaling.

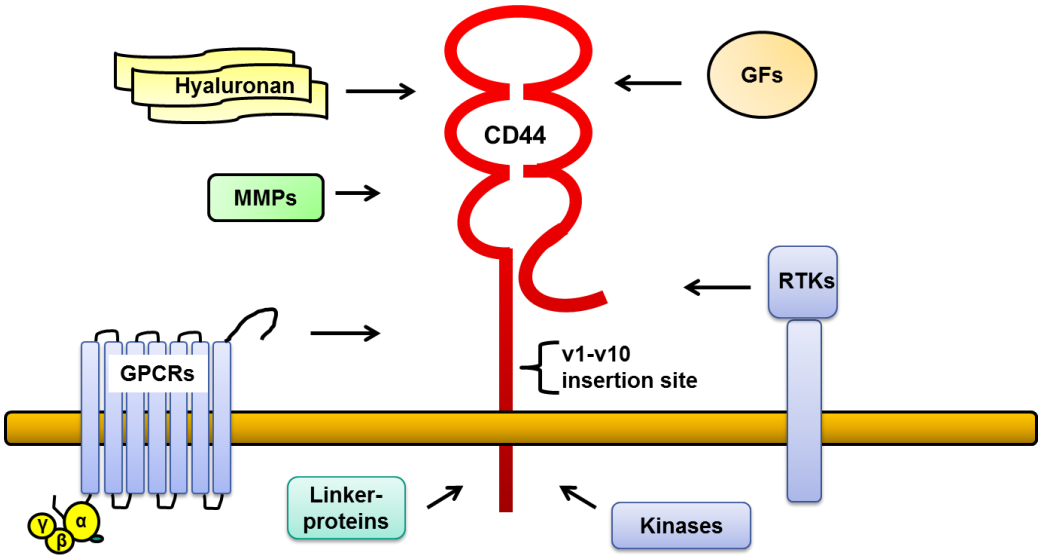

Fig. 3 Schematic representation of CD44.
The extracellular domain of CD44 can interact with components of the extracellular matrix (ECM), enzymes like matrix-metalloproteinases (MMP) and several growth factors. The extracellular stem region of CD44 can be elongated by insertion of different combinations of variant exons v1-v10. The intracellular domain can interact with several proteins like kinases or linker proteins, linking CD44 to the actin-cytoskeleton.

1.8 Aims

The discovery that the Wnt-target gene CD44 is involved in Wnt-driven tumorigenesis together with the findings that HA binding to CD44 can stimulate β-catenin activation and TCF/LEF or that CD44 is required for nuclear translocation of β-catenin strongly suggests a regulatory function of CD44 in Wnt/β-catenin signaling. The well-known function of CD44 as signaling modulator and the similarities of CD44 other regulators of Wnt-signaling makes it even more likely that CD44 is involved in Wnt-signaling. As also other Wnt-target genes are involved in the regulation of Wnt/β-catenin signaling implicates that feedback-regulation exists and that CD44 might take part in this feedback-regulation. Hints that CD44 might contribute to Wnt-induced β-catenin signaling were found during my Diploma thesis.

This work investigates whether CD44 is involved in the modulation of the canonical Wnt-pathway and addresses a possible function of CD44 as a feedback-regulator of Wnt/β-catenin signaling *in vitro* and *in vivo*. Furthermore, this PhD thesis examines in detail the molecular mechanism how CD44 might regulate the Wnt/β-catenin signaling pathway.

2 Materials and Methods

2.1 Material

2.1.1 Chemicals, reagents, media and additives

All chemicals used in this work were of highest purity grate. Water used for aqueous solutions was desalted.

Name	**Source**
2-Propanol	Merck, Darmstadt, Germany
2-Mercaptoethanol	Roth, Karlsruhe, Germany
Acetic acid	Merck, Darmstadt, Germany
Acrylamid/N,N'-Methylenbisacrylamid (37,5:1)	Roth, Karlsruhe, Germany
Agarose	Peqlab, Erlangen, Germany
Ampicillin	Roth, Karlsruhe, Germany
Ammonium peroxodisulphate (APS)	Roth, Karlsruhe, Germany
Anti-Digoxigenin-Fab fragments	Roche, Mannheim, Germany
Aprotinin	Sigma-Aldrich, Taufkirchen, Germany
Bacto Agar	Roth, Karlsruhe, Germany
Bacto Trypton	Roth, Karlsruhe, Germany
Blocking reagent	Roche, Mannheim, Germany
Bovine serum albumin (BSA)	PAA, Coelbe, Germany
Bromophenol blue	BioRad, München, Germany
Calcium acetate	Roth, Karlsruhe, Germany
4',6-diamidino-2-phenylindole (DAPI)	Life technologies, Darmstadt, Germany

Dimethylsulfoxide (DMSO)	Fluka, Neu-Ulm, Germany
Dithiothreitol (DTT)	Roth, Karlsruhe, Germany
Dulbecco´s modified Eagle´s medium (DMEM)	Invitrogen, Karlsruhe, Germany
Enhanced chemoluminescence western-blotting reagents	Thermo Scientific, Dreieich, Germany
Ethanol (EtOH)	Roth, Karlsruhe, Germany
Ethidiumbromide	Roth, Karlsruhe, Germany
Ethylenediaminetetraacetic acid (EDTA)	Roth, Karlsruhe, Germany
Fetal calf serum (FCS)	PAA, Coelbe, Germany
Fluorescence mounting medium	Dako, Hamburg, Germany
Gel extraction kit	PEQLAB, Erlangen, Germany
Glucose	Roth, Karlsruhe, Germany
Glycerol	Roth, Karlsruhe, Germany
Glycine	Roth, Karlsruhe, Germany
HEPES	Roth, Karlsruhe, Germany
Hydrochloric acid (HCl)	Merck, Darmstadt, Germany
Igepal	Sigma-Aldrich, Taufkirchen, Germany
Isopropanol	Roth, Karlsruhe, Germany
Leupeptin	Sigma-Aldrich, Taufkirchen, Germany
Luciferin	Biosynth AG, Staad, Schweiz
Magnesium chloride hexahydrate	Roth, Karlsruhe, Germany
Magnesium sulphate ($MgSO_4$)	Sigma-Aldrich, Taufkirchen, Germany

Methanol (MeOH)	Roth, Karlsruhe, Germany
NBT/BCIP stock solution	Roche, Mannheim, Germany
NeutrAvidin agarose beads	Thermo Scientific, Dreieich, Germany
Nonidet P-40 (NP-40)	Roth, Karlsruhe, Germany
Page Ruler Prestained Protein Ladder	Fermentas, St. Leon-Rot, Germany
Paraformaldehyde	Merck, Darmstadt, Germany
peqGold DNA ladder-mix 100-10000 bp	PEQLAB, Erlangen, Germany
pegGold Protein Marker IV	PEQLAB, Erlangen, Germany
Penicilin/Streptomycin	Invitrogen, Karlsruhe, Germany
Phenyl methanesulphonyl fluoride (PMSF)	Sigma-Aldrich,Taufkirchen, Germany
Phosphate buffered saline w/o $CaCl_2$ and $MgCl_2$ ($PBS^{-/-}$)	Invitrogen, Karlsruhe, Germany
Plasmid Maxi Kit	Qiagen, Hilden, Germany
PromoFectin	Promocell, Heidelberg, Germany
Protein A and G Agarose beads	Calbiochem, Bad Soden, Germany
Proteinase K	Sigma-Aldrich, Taufkirchen, Germany
Rotiphorese® Gel30: Acrylamide/ bis-acrylamide (30%/0,8%)	Roth, Karlsruhe, Germany
Sodium acetate (NaAc)	Roth, Karlsruhe, Germany
Sodiumdodecylsulphate (SDS)	Roth, Karlsruhe, Germany
Sodium chloride (NaCl)	Roth, Karlsruhe, Germany
Sodium Fluoride (NaF)	Roth, Karlsruhe, Germany

Sodium hydrogen carbonate ($NaHCO_3$)	Roth, Karlsruhe, Germany
Sodium hydroxide (NaOH)	Roth, Karlsruhe, Germany
Sodium dihydrogenphosphat (Na_3VO_4)	Roth, Karlsruhe, Germany
Sodium hydrogenphosphat (Na_2HPO_4)	Roth, Karlsruhe, Germany
Sodium orthovanadate (Na_3VO_4)	Sigma-Aldrich,Taufkirchen, Germany
Taq polymerase	Fermentas, St. Leon-Rot, Germany
Tetramethylethylenediamine (TEMED)	Roth, Karlsruhe, Germany
Tris-base	Roth, Karlsruhe, Germany
Tris-HCl	Roth, Karlsruhe, Germany
Triton-X-100	Roth, Karlsruhe, Germany
Trypsin 0.25% (w/v)-EDTA	Gibco/Invitrogen, Karlsruhe, Germany
Trypton/Pepton	Roth, Karlsruhe, Germany
Tween 20	Roth, Karlsruhe, Germany
Yeast extract	Roth, Karlsruhe, Germany

2.1.2 Hardware and consumables

Name	Source
Agarose gel electrophoresis chamber	Peqlab, Erlangen, Germany
Bacteria petri dishes	Greiner, Nürtingen, Germany
Bacteria incubator	Heraeus, Hanau, Germany
Camera: Axiocam HRc	Zeiss, Jena, Germany
Cell incubator	Thermo Scientific, Wilmington, DE, USA
Centrifuge Megafuge 1.0	Heraeus Instruments, Hanau, Germany
Cold light source for injections EK-1	Euromex, Arnheim, Netherlands
Confocal microscope SP5	Leica, Wetzlar, Germany
Cooling centrifuge J2-HS	Beckmann, Stuttgart, Germany
Developer	Kodak, New Haven, USA
Eagle eye	Peqlab, Erlangen, Germany
ECL Hyperfilm	Amersham, Freiburg, Germany
Electrophoresis chambers	Hoefer, Holliston, MA, USA
Freezer -80°C	New Brunswick, Edison, NJ, USA
Freezer -20°C	Liebherr, Ochsenhausen, Germany
Fridge	Liebherr, Ochsenhausen, Germany
Glass cover slips	Thermo-Fischer Scientific, Wilmington, DE, USA
Heatblock Thermomixer Comfort	Eppendorf, Hamburg, Germany
Immobilon-P (PVDF membrane)	Millipore, Bedford, USA
Luminescence counter 1420	PerkinElmer, Rodgau, Germany

Microinjector	H. Saur Laborbedarf, Reutlingen, Germany
Microscope Axiovert 40c	Zeiss, Jena, Germany
NanoDrop Photometer	Thermo Scientific, Wilmington, DE, USA
Needles	Braun, Melsungen, Germany
PCR reaction tubes	Stein, Wilferdingen, Germany
Pipetboy Costar Stripettor	Corning, Tokyo, Japan
Pipettes	Gilson, Paris, France
Pipette tips	Stein Labortechnik, Remchingen, Germany
Power supply	Hoefer, Holliston, MA, USA
Reaction tubes	Eppendorf, Hamburg, Germany
Shaker	Fröbel Labortechnik, Lindau, Germany
Stereomicroscope M 10	Leica, Wetzlar, Germany
Stereomicroscope MIC 1630 ZS	Euromex, Arnheim, Netherlands
Sterile filters	Roth, Karlsruhe, Germany
Syringes	Braun, Melsungen, Germany
Table-Centrifuge	Eppendorf, Hamburg, Germany
Tissue culture plastics	Greiner, Nürtingen, Germany
Thermo Cycler MJ Research	Scientiffic Support, Hayward, CA, USA
Vortex blender	Janke & Kunkel, Staufen, Germany
SDS-PAGE/Westernblot chamber	BioRad, München, Germany
Whatman 3MM paper	Whatman, Part of GE Healthcare, Munich, Germany

2.1.3 Software

Name	Description	Source
Axiovision	Program for photodocumentation	Zeiss, Jena, Germany
Adobe Photoshop CS4	Program for image editing	Adobe systems, San Jose, CA, USA
ImageJ	Program for image editing	NIH, Bethesda, Maryland USA
LAS AF	Program for photodocumentation	Leica, Wetzlar, Germany
Openlab	Program for photodocumentation	PerkinElmer, Rodgau, Germany

2.1.4 Enzymes

Name	Source
DNase I	Ambion Ltd, Warrington, UK
Restriction enzymes	Fermentas, St.Leon-Rot, Germany
Taq-Polymerase	Fermentas, St.Leon-Rot, Germany
Reverse transcriptase	Fermentas, St.Leon-Rot, Germany

2.1.5 Antibodies

2.1.5.1 Primary Antibodies

The following antibodies were used for immunoblotting and immunoprecipitation studies.

Name	**Isotype**	**Specificity**	**Source**
1.1 ASML	Mouse IgG1	Detection of exon v6 encoding epitope from rat	Purified from ascites
Actin (I-19)	Goat polyclonal	Human, mouse, rat	Santa Cruz, Heidelberg, Germany
Active Beta-Catenin (clone 8E7)	Mouse monoclonal IgG1	Mouse, rat, human, Specific for the active form of β-Catenin, dephosphorylated at Ser37 and Thr41	Millipore, Bedford, USA
Beta-Catenin (Western-blot)	Mouse monoclonal IgG1	Human, mouse, rat, dog chicken β-Catenin	BD Transduction Laboratories
Beta-Catenin (D10A8) (Immunfluorescence)	Rabbit	Human, mouse, rat, monkey	Cell signaling, Beverly, England
ERK1/2 (K-23)	Rabbit polyclonal	Mouse, rat, human ERK1/2	Santa Cruz, Heidelberg, Germany
Ezrin (3C12)	Mouse monoclonal	Recognizing aa 362-585 of human Ezrin	Santa Cruz, Heidelberg, Germany

ANTI-Flag-antibody clone M2	Mouse monoclonal	Monoclonal anti-Flag antibody recognizing Flag-peptides	Sigma-Aldrich, Taufkirchen, Germany
Hermes 3	Mouse monoclonal	All human CD44 isoforms	Gift from Sirpa Jalkanen, Turku, Finnland
LRP6 (C5C7)	Rabbit monoclonal	Detection of endogenous levels of human LRP6, recognizing residues surrounding Met 1409	Cell signaling, Beverly, England
LRP6 (T1479)	Rabbit polyclonal	Detection of endogenous levels of human LRP6, recognizing residues surrounding Thr 1479	Gift from Gary Davidson, Karlsruhe, Germany
Moesin (E10)	Mouse monoclonal	Specific for an epitope mapping between aa 497-526 at the C-terminus of human Moesin	Santa Cruz, Heidelberg, Germany
PCNA (PC10)	Mouse monoclonal	Mouse, rat, human PCNA p36 protein	Santa Cruz, Heidelberg, Germany
Phospho-LRP6 (Ser1490)	Rabbit polyclonal	Human	Cell signaling, Beverly, England
Phospho-p44/42 MAP kinase	Rabbit polyclonal	Mouse, rat, human phosphorylated ERK1/2	Cell signaling, Beverly, England

Radixin	Goat polyclonal	Raised against a peptide mapping the C-terminus of human Radixin	Santa Cruz, Heidelberg, Germany
Anti-VSV-G	Rabbit polyclonal	Detection of VSV-G tagged recombinant proteins	Stressgen, Ann arbor, Michigan, USA

2.1.5.2 Secondary antibodies and bead conjugates

Name	**Source**
Goat anti-rabbit HRP-conjugated	Dako, Hamburg, Germany
Goat anti-mouse HRP-conjugated	Dako, Hamburg, Germany
Rabbit anti-goat HRP-conjugated	Dako, Hamburg, Germany
Protein G agarose beads	Merck, Darmstadt, Germany
Mouse-IgG sepharose bead conjugates	Cell signaling, Beverly, England
Alexa488-streptavidin conjugates	Life technologies, Darmstadt, Germany
Donkey anti-rabbit-Alexa 546	Life technologies, Darmstadt, Germany

2.1.6 Blocking reagents, Hyaluronan, Hyaluronidase and HA binding proteins

Name	**Description**	**Source**
Bovine Testis Hyaluronidase	Enzyme that degrades hyaluronan	Sigma-Aldrich, Taufkirchen, Germany
BU52	Antibody that blocks interaction between CD44 and HA (Avigdor et al., 2004).	Pierce, Rockford, USA

IgG-isotype control	IgG control used for the blocking studies with BU52	Natutec, Frankfurt, Germany
Hyaluronan (Healon)	Hyaluronan used to induce CD44/HA interaction	Abbott Medical Optics, Santa Ana, USA
Hyaluronan-binding protein (HA-bp)	Biotinylated HA-bp for detection with streptavidin conjugates	Merck, Darmstadt, Germany
Ly294002	Chemical PI3K-inhibitor	Cell signaling, Beverly, England
U0126	Chemical MEK-inhibitor	Promega, Mannheim, Germany

2.1.7 Marker

Name	**Source**
ER Marker (ER-TrackerTM Red)	Invitrogen, Karlsruhe, Germany
PageRuler prestained protein ladder	Fermentas, St.Leon-Rot, Germany
GeneRuler DNA ladder mix	Fermentas, St.Leon-Rot, Germany

2.1.8 Kits

Name	**Source**
mMESSAGE mMACHINE Transkription Kit	Ambion, Darmstadt, Germany
DIG RNA Labeling Kit (SP6/T7)	Roche, Mannheim, Germany
QIAGEN Plasmid Maxi purification kit	Qiagen, Hilden, Germany
peqGold Gel extraction kit	Peqlab, Erlangen, Germany

Nucleospin RNA L purification kit	Macherey-Nagel, Düren, Germany

2.1.9 Plasmid constructs

The following plasmids were used for cloning, transfection in eukaryotic cells, injections in *X. laevis* or as template for RNA probes used for *in situ* hybridization.

2.1.9.1 Empty vectors (EV)

Name	**Description**
PCDNA3.1 (+)	EV control for hCD44s and hCD44s-HAmut, ratCD44s and xCD44s overexpression studies
pGK	EV control for CD44v6 overexpression studies
pSV	EV control for pSV-Meta#1 and pSV-Meta-Stop overexpression studies
pcB6	EV control for Ezrin-DN overexpression studies
pEBG	EV control for CD44-Cyt and CD44-CytΔEz-BD overexpression studies

2.9.1.2 Overexpression constructs

Name	**Description**
hCD44s	Sequence of the human CD44 standard isoform cloned into pCDNA3.1(+)
rCD44s	Sequence of the rat CD44 standard isoform cloned into pCDNA3.1(+)
xCD44s	xCD44s (*X.laevis* hypothetical protein LOC443701) cloned into pCMV-Sport6 was a gift from M.Ori (University of Pisa, Italy)
pSV-Meta#1 (rCD44v4-v7)	Sequence of rat CD44v4-v7 cloned into pSV (Orian-Rousseau et al., 2002)
pSV-Meta-Stop	Sequence of rat CD44v4-v7 lacking the cytoplasmic tail region

(rCD44v4-v7ΔCyt)	cloned into pSV (Orian-Rousseau et al., 2002)
pGKs6 (rCD44v6)	Sequence of rat CD44v6 cloned into pGK (Orian-Rousseau et al., 2002).
CD44-Cyt	Sequence of CD44 cytoplasmic tail region cloned into pEBG (Legg and Isacke, 1998). Gift from C. Isacke (Breakthrough Breast Cancer Research Center, London, United Kingdom)
CD44-CytΔEzBD	Sequence of CD44 cytoplasmic tail region mutated in the Ezrin binding domain cloned into pEBG (Legg and Isacke, 1998). Gift from C. Isacke (Breakthrough Breast Cancer Research Center, London, United Kingdom)
hCD44s-HAmut	hCD44s mutated in the HA binding site was generated by a point mutation (R41A) in the HA binding domain as described in (Peach et al., 1993)
DN-Ezrin	Sequence of Ezrin-Wt without the last 29 aa encoding for the actin binding site was cloned into pCB6 (Algrain et al., 1993). Gift from Monique Arpin (Institut Pasteur Paris, France)
pCS2-hDsh-1	Sequence of human Dsh-1 was cloned into pCS2+ (Cruciat et al., 2013). Gift from Gary Davidson (KIT, Karlsruhe, Germany)
pCS2-LRP6	Sequence of human LRP6 cloned into pCS2+ (Tamai et al., 2000). Gift from Gary Davidson (KIT, Karlsruhe, Germany)
pCS2-flag-hLRP6	Sequence of a flag-tagged version of human LRP6 cloned into pCS2+ (Mao et al., 2002). Gift from Gary Davidson (KIT, Karlsruhe, Germany)
pCS2-hLRP6-eGFP	Sequence of an eGFP-tagged version of human LRP6 cloned into pCS2+ (Mao et al., 2002). Gift from Gary Davidson (KIT, Karlsruhe, Germany)
pCMV-Mesd	Sequence of mouse Mesd cloned into pCMV (Davidson et al., 2005). Gift from Gary Davidson (KIT, Karlsruhe, Germany)

pCS2-myc-mβ-Cat(S33A)	Sequence of a constitutive active myc-tagged version of mouse β-catenin point mutated at Serin 33 (S33A) cloned into pCS2+ (Aberle et al., 1997). Gift from Dietmar Gradl (KIT, Karlsruhe, Germany)
xRor2-mCherry	Sequence of mCherry-tagged *X. laevis* Ror2 (Feike et al., 2010). Gift from Dietmar Gradl (KIT, Karlsruhe, Germany)

2.9.1.3 Reporter constructs

Name	**Description**
M50 Super 8xTOPFlash	pTA-Luc vector with a luciferase gene under control of 7 TCF/LEF-binding sites, used as reporter of β-catenin mediated transcriptional activation was obtained from Addgene, Cambridge, Massachusetts, USA.
pRL-TK renilla	pRL-vector with a HSV-TK promotor driving Renilla luciferase used as vector for normalization was obtained from Promega, Mannheim, Germany

2.1.10 Recombinant proteins

Name	**Source**
Human recombinant Wnt3a	R&D systems, Wiesbaden, Germany

2.1.11 Oligonucleotides

2.1.11.1 Small interfering RNA (siRNA) and Morpholinos

Name	Description	Source
CD44pan-siRNA	For downregulation of all CD44 isoforms. Pool of three: 5´-CTG AAA TTA GGG CCC AAT T-3´ 5´-AAT GGT GCA TTT GGT GAA C-3´ 5´-CAG AAA CTC CAG ACC AGT T-3`	Qiagen, Hilden, Germany
Ezrin-siRNA	For downregulation of Ezrin. Pool of three : 5´-GGA ACA UCU CUU UCA AUG A-3´ 5´- CCA CGU CUG AGA AUC AAC A-3´ 5´-GAC UCU GUU UGC UUG UGU U-3`	Santa Cruz, Heidelberg, Germany
Control-siRNA	Non silencing siRNAs. Pool of three : 5`-UAA UGU AUU GGA ACG CAU AUU-3` 5`-AGG UAG UGU AAU CGC CUU GUU-3` 5`-UGC GCU AGG CCU CGG UUG CUU-3`	Eurofins MWG, Ebersberg, Germany
xCD44-Morpholino	5´-TAACAATCCACAGCATTGAGGCCAT-3´ (described in Ori et al., 2006).	Gene Tools, Philomath, USA
Control-Morpholino	Standard control morpholinos	Gift from Dietmar Gradl, KIT, Karlsruhe, Germany

2.1.11.2 RNA probes In situ hybridization (ISH)

Name	Description	Source
xEngrailed-2 (En-2)	Digoxygenin labelled En-2 RNA-probes for ISH to detect En-2 expression in *Xenopus laevis* embryos (described in Koenig et al.,	Gift from Dietmar Gradl, KIT, Karlsruhe, Germany

	2010).	
xTCF4 (TCF-4)	Digoxygenin labelled TCF-4 RNA-probes for ISH to detect TCF-4 expression in *Xenopus laevis* embryos (described in Koenig et al., 2010).	Gift from Dietmar Gradl, KIT, Karlsruhe, Germany

2.1.12 Transfection reagents

Name	**Source**
HiPerFect	Qiagen, Hilden, Germany
PromoFectin	PromoKine, Heidelberg, Germany

2.1.13 Cell lines

Cell line	**Description**	**Culture medium**	**Source**
HEK293T (CRL-1573)	Human embryonic kidney cells	DMEM + 10% FCS	American tissue culture collection, ATCC, Wesel, Germany
HeLa (CCL-2)	Human cervix epithelium adenocarcinoma cells	DMEM + 10% FCS	American tissue culture collection, ATCC, Wesel, Germany
L-cells (CRL-2648)	Mouse fibroblasts	DMEM + 10% FCS	American tissue culture collection, ATCC, Wesel, Germany
Wnt3a-L-cells (CRL-2647)	Mouse fibroblasts stably transfected with Wnt3a	DMEM + 10% FCS	American tissue culture collection, ATCC, Wesel, Germany

2.1.14 Bacterial strain

Name	**Description**
E.coli DH5α	Recombination defect suppressive strain for plasmid expression. Genotype: F-80d*lac*ZM15(*lac*ZYA*arg*F)U169*deo*R*rec*A1*end*A1*hsd*R17(r_K^- m_K^+) phoA*sup*E44thi-1*gyr*A96relA1

2.2 Methods

2.2.1 Cell culture methods

All cells were cultured under sterile and standard conditions (37°C, 95% humidity, 5% CO2) in a cell culture incubator. The cells were grown in sterile Cellstar® cell culture dishes (Greiner Bio-One) of different sizes according to the experimental setup. All cells were grown in DMEM (Gibco/Invitrogen) supplemented with 10% fetal calf serum (PAA, Pasching) and used up to passage 25. All cells, where passaged at least every third day or when they reached 90% confluency. All cell culture experiments were performed in a sterile clean bench.

2.2.1.1 Passaging cells

To passage cells, old growth medium was removed by aspiration, cells were washed once with PBS and Trypsin-solution (0.25% Trypsin) was added to the cells. Cells were incubated at 37°C until they started to detach from the wells. Trypsination was stopped by adding serum containing medium and cells were collected by centrifugation. After resuspending the cells in new growth medium cells were distributed in new tissue culture plates.

2.2.1.2 Seeding cells

Cells were trypsinized as described above, collected by centrifugation and resuspended in new growth medium. To obtain the number of cells per ml, 10 µl of cell suspension was transferred into a Neubauer counting chamber and counted by using a bright field microscope. After adjustment of the designated cell concentration by mixing cell suspension with culture medium, cells were distributed in tissue culture plates for the experiment.

2.2.1.3 Freezing cells

Cells were trypsinized as described above and collected by centrifugation. After resuspending the cell pellet in freezing medium (10% DMSO in FCS), cells were transferred into cryostatic vials. Vials were slowly frozen in an Isopropanol containing box at -80°C and transferred to liquid nitrogen.

2.2.1.4 Thawing cells

Cells stored in liquid nitrogen were thawed at 37°C in a waterbath and immediately mixed with pre-heated growth medium. To remove freezing medium, cells were collected by centrifugation, resuspended in growth medium and transferred into tissue culture plates. Growth medium was replaced with fresh medium after 24 hours.

2.2.1.5 Production of Wnt3a-conditioned medium

To obtain Wnt3a-conditioned medium (Wnt3a-CM), L-Wnt-3A cells were grown in DMEM+10% FCS for 4 days in 15 cm petri-dishes from a 1:10 split. Subsequently the medium was harvested, centrifuged at 4000 rpm and the supernatant was filtered through a 0.2 µm filter. The filtered supernatant was stored as stock solution at 4°C. The same procedure was performed with untransfected L-cells to obtain control-conditioned medium (Control-CM).

2.2.1.6 Induction of cells with Wnt3a

For induction of cells with recombinant Wnt3a (recWnt3a), the recWnt3a (dissolved in 0.1% BSA in PBS) was used at a final concentration of 100 ng/ml in DMEM + 10% FCS. For induction of cells with Wnt3a-CM, the Wnt3a-CM stock solution was used as a 1:4 dilution in DMEM + 10% FCS, which led to similar activation as 100 ng/ml recWnt3a. As control, cells were treated with a 1:4 dilution of Control-CM.

2.2.1.5 Transfections

2.2.1.5.1 DNA-Transfection of HEK293 and HeLa cells

For overexpression studies, cells were transfected with mammalian expression vectors containing the genes of interest by using PromoFectin transfection reagent. Transfection was performed according to the manufacturer´s protocol, but using only the half of the recommended amounts of DNA, transfection reagent and medium as this resulted in better cell survival. For example, in 96 well plates, cells were seeded at a concentration of 1.8×10^4 cells per well in 100 µl growth medium 6 h before transfection. For transfection of each well, 125 ng of vector DNA was diluted in 5 µl DMEM without serum. 0.25 µl PromoFectin were added to 5 µl DMEM without serum and mixed with the DNA solution. The mixture was incubated for 20 min at room temperature and 10 µl of transfection mixture was applied to each well. After distributing the transfection mixture in the wells by swirling the plate, cells were transferred to the incubator. Cells were lysed for measurements 48 h after transfection.

2.2.1.5.2 Transfection of siRNA

Small interfering RNAs (siRNAs) are double stranded RNA molecules, 20-25 nucleotids in length, which interfere with the expression of specific genes via the RNAi pathway. Transfection of siRNA directed against a specific gene leads finally to a transiently reduced level of the protein encoded by this gene.

siRNA-Transfection of HEK293 cells

Cells were seeded in 6 cm plates at a concentration of 5 x 10^5 cells per plate in 4 ml growth medium, 5 h before transfection and transferred to the incubator. HEK293 cells were transfected using HiPerFect transfection reagent according to the manufacturer´s protocol. Briefly, for each plate, 100 ρmol of siRNA were diluted in 100 µl DMEM without serum. 20 µl of HiPerFect were added to the diluted siRNA and the mix was incubated for 5-10 min at room temperature to allow the formation of transfection complexes. After incubation the transfection mix was added dropwise onto the cells. After distributing the transfection mixture by gently swirling the plate, cells were transferred to the incubator. 16 h post-transfection cells were transferred in 96 wells for luciferase reporter gene assay respectively Western-blot analysis. Cells were lysed for measurements approximately 70 h after siRNA transfection.

siRNA-Transfection of HeLa cells

HeLa cells were grown until 50-70 % confluency and transfected with 5 nM siRNA using HiPerFect transfection reagent according to the manufacturer´s instructions, at least 6h after seeding. Where necessary, cells were transfected with DNA (see above) at least 20 hrs after siRNA transfection. Cells were lysed and subjected to Western-blot analysis, respectively fixed for confocal fluorescence microscopy measurement, at least 48 hrs after siRNA transfection.

2.2.2 DNA-methods

2.2.2.1 Linearization of plasmid DNA

In order to obtain linearized plasmid DNA for generation of antisense RNA-probes for *in situ* hybridization (ish) respectively sense RNA for injection, cDNA-plasmids (listed in 1.9.4 and 1.9.3) were digested with the needed restriction enzymes. In a total volume of 30 µl, 3 µg of plasmid DNA was mixed with 3 µl 10x Fast Digest Green Buffer and 3 µl Not-I or Nco-I restriction enzyme in RNase-free water and incubated for 1 h at 37°C. Enzymes were

inactivated by heating for 5 min at 80°C. The digested DNA was directly loaded on an agarose gel, separated by size, isolated out of the gel and stored at -20°C.

2.2.2.2 Reverse transcriptase- polymerase chain reaction (RT-PCR)

For analysis of specific mRNA expression of cells, cells were lysed, RNA was purified and mRNA was transcribed into cDNA by reverse transcription as described in 2.3.1. To prove presence of a specific mRNA, cDNA was subjected to PCR with specific primer for the gene of interest. PCR was performed as follows:

In a total volume of 30 µl, cDNA template (5 µl of reverse transcriptase reaction) was mixed with 6 µl 5x PCR buffer, 0.5 µl dNTP´s (10 mM), 10 ρmol forward primer, 10 ρmol reverse primer and 0.25 µl Taq-polymerase (1 U/µl) in water. PCR was performed in a Thermocycler using the standard PCR program:

1 cycle:	1 minute at 95°C	(initial denaturation)
36 cycles:	30 seconds 95°C	(denaturation)
	1 minute 50°C	(primer annealing)
	2 minutes 72°C	(elongation)
1 cycle:	5 minutes 75°C	(final elongation)
1 cycle:	hold at 4°C	

Amplified DNA was loaded on an agarose gel, separated by electrophoresis and visualized by UV-irradiation.

2.2.2.3 Separation of DNA fragments via agarose gel electrophoresis

In order to separate DNA fragments according to their size, DNA was subjected to an electric field within a 1% agarose gel matrix. To produce a 1% agarose gel the corresponding amount of agarose was weighted and dissolved in TAE buffer. By heating the mix in the microwave, the agarose was melted. Ethidium bromide was added and the gel was casted in a casting chamber, equipped with a comb. While cooling down to room temperature the agarose gel became solid. Before loading the samples into the sample pockets, the gel was covered with TAE buffer and the samples were mixed with 10x loading buffer. For size determination of the DNA samples, a standard DNA-ladder (peqGOLD Ladder-Mix 100-10.000 bp) was loaded on the gel. Separation of DNA-fragments was achieved by applying an electric current with a voltage of 100 V. The separated DNA was visualized and compared to the standard DNA-ladder using UV-light.

TAE-buffer: 40 mM Tris pH 8.3; 2 mM EDTA; 40 mM sodium acetate

10 x DNA loading buffer: 15% Ficoll 400; 0.5 mM EDTA pH 8; 0.25% bromphenol blue; 0.25% xylene cyanol

2.2.2.4 Isolation of DNA fragments out of agarose gels

Bands containing DNA-fragments of interest were localized by UV irradiation and cut out manually with a scalpel. DNA was isolated out of the gel using the peqGOLD Gel extraction kit (Peqlab, Erlangen, Germany) according to the manufacturer´s instructions.

2.2.3 Transformation of chemically competent *E. coli* (DH5α)

Transformation of bacteria was done to obtain high amounts of plasmid DNA for further experiments. 100µl of chemically competent *E. coli* (DH5α) were thawed on ice and 0.1 µg of plasmid DNA was added. The mix was incubated on ice for 30 min and then the bacteria were subjected to heat shock treatment for 30 sec at 42°C in a heating block. The bacteria were placed on ice for 2 min and 1 ml LB-medium was added. The transformed bacteria were incubated at 37°C for 1 h before adding the selection antibiotic. For selection, the bacteria were plated onto LB agar plates that contained the selection antibiotic. The bacteria were allowed to grow for 24 h at 37°C.

Ampicillin stock solution: 100 mg/ml Ampicillin in ddH_2O

LB-Medium: 10 g Bacto Trypton; 5 g yeast extract; 10 g NaCl; ad 1 l H_2O; pH 7.5.

LB_{AMP}-Medium: 100 µg/ml Ampicillin-stock solution in LB-Medium.

LB_{AMP}-Agar-plates: 1.5% (w/v) Bacto agar in LB-Medium (autoclaved) containing 1 ml/l ampicillin stock solution.

2.2.3.1 Large scale plasmid DNA purification

After selection of transformed bacteria, positive clones were grown in 100 ml LB-medium containing a selection marker over night at 37°C. The preparation of the large scale plasmid DNA purification out of the bacteria was performed according to the manufacturer's protocol (Qiagen). The highly pure plasmid DNA was washed with 70% EtOH. Before dissolving the plasmid DNA in 1x TE-buffer, the EtOH was completely removed. Quantity and quality of the plasmid DNA was checked using a NanoDrop spectrophotometer (PeqLab, Erlangen).

2.2.3.2 Determination of DNA concentrations

In order to determine the DNA-concentration and purity of a DNA-solution, the optical density (OD) was measured at 260, 230 and 280nm using a NanoDrop photometer and the software ND-1000 (version 3.1.2). An OD260 of 1.0 corresponds to 50 µg/ml of pure double-stranded DNA. To evaluate the purity of the DNA-solution, the ratios OD260/OD280 and OD260/OD230 were calculated. A ratio of OD260/OD280 of 1.8 indicates that the solution is relatively free of protein contamination, whereas a ratio of OD260/OD230 above 1.6 indicates that the solution is free of organic chemicals and solvents.

2.2.4 RNA methods

2.2.4.1 RNA isolation and cDNA synthesis

To investigate expression of a specific in gene in cells by RT-PCR, RNA of these cells has to be isolated and transcribed into cDNA by reverse transcription.

4 x 10^7 cells were lysed and RNA was isolated and purified with the NucleoSpin RNA L kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. RNA concentration and purity were measured with a NanoDrop photometer. For denaturation, 0.5 µg RNA was diluted in 10 µl RNase free water and incubated at 70°C for 3 min, then quickly cooled down in ice water. RNA was then subjected to reverse transcription, which was performed with 20 U of AMV reverse transcriptase in 80 µl reactions containing 80 U RNasein, 400 ng of oligo d(T) primer and nucleotides. Following incubation for 45 min at 41°C reverse transcriptase was inactivated by heating at 70°C for 15 min. cDNA was stored at -80°C.

2.2.4.2 Preparation of *antisense* RNA for *in situ* hybridization

The labelled *antisense*-RNA probes for whole mount *in situ* hybridization were synthesized using the Digoxygenin RNA-Labelling Kit (Roche). The ribonucleotid-mix used for this purpose contains UTP labelled with Digoxygenin. In a total volume of 20 µl, 1 µg linearized DNA was incubated with 2 µl 10x NTP labelling mixture, 2 µl 10 x transcription buffer, 20 U RNase inhibitor and 40 U SP6- or T7-RNA polymerase for 2 h at 37°C. Following incubation template DNA was removed by adding 2 µl DNase and further incubation for 15 minutes at 37°C. The RNA was cleaned by passing it over a RNeasy column (Qiagen) and eluting with 30 µl of RNase-free water.

2.2.5 Protein methods

2.2.5.1 Preparation of cell lysates for protein analysis

In order to determine the expression respectively the activation of proteins by western-blot, cells were washed with cold PBS and lysed in SDS lysis buffer. Cells were transferred to a reaction tube and the lysate was sheared through a 24 gauge needle to reduce viscosity and allow better separation of the proteins. Denaturation of proteins was obtained by the presence of DTT in the lysis buffer and boiling samples for 5 minutes at 100°C. Samples were spun down for 5 min at 12000 rpm before loading the SDS-PAGE-gel.

SDS-lysis buffer: 125 mM TrisHCl pH 6.8; 4% SDS; 20% Glycerol; 0.01% bromphenol blue; 100 mM DTT

2.2.5.2 Separation of proteins by SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were electrophoretically separated on the basis of their molecular weight using the method of Laemmli (Laemmli, 1970). For separation, the charge of proteins was equalized by SDS which forms aggregates with positive charged structures of proteins and impairs a negative charge on the protein that is proportional to the mass of that protein (about 1.4 g SDS/g protein). The proteins were loaded on a gel, through which they migrate to the positive pole of an electric field. The gel is composed of two parts. The upper part, the stacking gel with a lower concentration of acrylamid (5%) is for focussing the proteins whereas the proteins are separated in the lower part of the gel, the resolving gel with a higher concentration of acrylamide (7.5%). The gel was built as follows. Two vertical glass plates with a distinct distance according to the size of the spacers serve as a mould. The components of the resolving gel were thoroughly mixed, poured into that mould and overlaid with 70 % ethanol. In the meantime the components of the stacking gel were mixed. After polymerization of the resolving gel, ethanol was removed and the stacking gel was filled into the mould and a comb was inserted to get spaces for loading the samples. After polymerization of the stacking gel, the gel was put into an electrophoresis chamber, reservoirs were filled with running buffer and the protein samples were loaded on the gel. Electrophoresis occured at a voltage of 100-140 V.

Running buffer: 25 mM Tris; 192 mM Glycine; 0.1% SDS

Stacking gel: 5% acrylamide; 0.1% SDS; 0.1% APS; 0.1% TEMED; 0.125 M, Tris pH 6.8

Resolving gel: 7.5% acrylamide; 0.1 % SDS; 0.1% APS; 0.06% TEMED; 0.375 M Tris pH 8,8

2.2.5.3 Western-blotting

The separated proteins within the polyacrylamide gel were transferred onto a PVDF-membrane using a semi-dry blotter with a constant voltage of 20 V for 2 h. For transfer, three layers of Whatman paper soaked in transfer buffer were placed onto the blotter. PVDF membrane was activated by incubating one minute in methanol and equilibrated in transfer buffer before applying on the Whatman paper. The acrylamid gel was placed onto the PVDF membrane, covered with three layers of transfer buffer soaked Whatman paper and the transfer was started. For preparing the different layers of Whatman paper and membrane it was each time ensured that no air bubbles were present. The proteins migrate in direction of the positive electric pole and adhere on the PVDF membrane, where they are accessible for antibody detection.

Transfer buffer: 20 mM Tris; 192 mM Glycine; 10% methanol

2.2.5.4 Immunodetection

To block unspecific binding of antibodies, the PVDF membrane with immobilized proteins was incubated in blocking buffer for one hour at room temperature. The blocked membrane was incubated with primary antibodies diluted in blocking buffer at concentrations recommended by the supplier overnight at 4°C. After washing the membrane three times for 5 min in TBS-T buffer, the membrane was incubated with diluted secondary antibodies coupled to HRP (horseradish peroxidase) for one hour at room temperature. Following incubation, the membrane was washed three times for 5 minutes in TBS-T buffer. Detection of specific protein signals was achieved using enhanced chemoluminescence (ECL) Western-blotting detection reagents and ECL hyperfilms as described in the manufacturer`s instructions. In order to reprobe the membrane with different antibodies, membrane bound antibodies were removed by incubation with stripping solution for 45 minutes at 50°C. The membrane can then be treated as described above to detect different proteins.

Blocking buffer: 5% BSA in TBS-T
TBS-T buffer: 20 mM Tris; 140 mM NaCl; 0.2% Tween 20; pH 7.6
Stripping solution: 62.5 mM TrisHCl pH 6.8; 2% SDS, 5 mM DTT

2.2.6 Developmental biology methods

2.2.7.1 Whole mount *in-situ* hybridization

The whole mount *in-situ* hybridization is a method used for detection of temporary and spatially expression profiles of single genes in the entire tissue of an organism. To detect mRNA expression of a gene, embryos are incubated with *antisense*-RNA labelled with Digoxigenin, which is complementary to the respective endogenous RNA. Following digestion of unbound single stranded RNA-probes, location of hybridized RNA-probes can be visualized by antibody staining against the antigen-labelled RNA. *Xenopus laevis* embryos were cultured in 0.1x MBSH until they obtained the designated stage of development. Embryos were then fixed in MEMFA for 1h at room temperature. Following fixation, embryos were transferred stepwise in methanol and stored at -20°C. Before hybridization, embryos have to be converted stepwise from alcoholic to aqueous medium, by incubating them 5 min in 75% methanol, 50% methanol, 25% methanol, respectively. Embryos were then washed four times in TBS-T for 5 min each time. After washing, embryos were permeabilized by incubation with Proteinase K solution for 15 min. To stop permeabilization, embryos were washed twice in 0.1 M triethanolamine, then another time for 5 min in 0.1 M triethanolamine containing acetic anhydride (2.5 µl/ml). Concentration of acetic anhydride was arisen to 5 µl/ml and incubation was continued for 5 min before washing the embryos two times for 5 min in TBS-T. Afterwards embryos were postfixed for 20 min in 4% (v/v) formaldehyde in TBS-T and then washed five times in TBS-T. Subsequently, embryos were incubated 10 min in 500 µl hybridization buffer at 60°C in a waterbath, then hybridization buffer was renewed and incubation continued for 6 h at 60°C for pre-hybridization. Digoxigenin-labelled RNA probe was taken up in hybridization buffer (0.5 µg/500 µl) and embryos were incubated in this solution over night at 60°C. Following hybridization, RNA probes were collected and stored at -20°C. Embryos were then washed in hybridization buffer one time for 10 min at 60°C, three times for 20 min in 2x SSC (60°C) each, then twice in 2x SSC containing RNase A (20 µg/ml) and RNase T1 (10 U/ml) for 30 min each at 37°C, two times for 20 min each in 2x SSC at room temperature and another two times for 30 min each in 0.2x SSC at 60°C.

To prepare embryos for incubation with antibody-solution, they were incubated in 1x MAB - which was renewed after 15 minutes - for 30 minutes. Embryos were then incubated for 30 min in 1x MAB containing 2% BMB and 1 h with MAB + 2% BMB + 20% serum- for blocking. Embryos were treated with alcaline phosphatase coupled antibodies against Digoxigenin for 4 h at room temperature and then over night in 1x MAB. Next day, embryos were washed twice for 5 min in 1x MAB at room temperature and then incubated in alcaline phosphatase buffer for 20 min in room temperature and another time 20 min at 4°C. Embryo staining was carried out in alcaline phosphate buffered NBT/BCIP-staining solution in

darkness at 4°C. As soon as the designated intensity of staining was reached, embryos were washed in 100% methanol to get rid of unspecific background staining. The stained embryos were converted to water in a stepwise descending concentration of methanol, fixed in MEMFA (1 h room temperature) and stored in 1x MEM at 4°C. Photodocumentation was performed using Axiocam HRc (Zeiss) fixed on a stereo-microscope.

Antibody solution:	0.05% (v/v) anti-Digoxygenin-AP Fab fragments in blocking-buffer
AP-buffer:	0.1 mM Tris-HCl pH 9.5; 0.05 mM MgCl2; 0.1 mM NaCl, 0.1% (v/v) Tween 20; DEPC-H2O
Blocking buffer:	1x BMB; 10% (v/v) horse serum; 0.05% (v/v) Tween 20; DEPC-H_2O
5x BMB:	10% (w/v) blocking reagent in 5x MAB
Hybridization buffer:	50% (v/v) Formamid deionized; 5x SSC; 2% (v/v) Torula RNA-solution; 0.1% (v/v) heparin solution; 1x Denhardt's; 0.1% (v/v) Tween 20; 0.1% (w/v) CHAPS; 10 mM EDTA
MAB (maleic acid buffer):	0.5 M maleic acid; 0,8 M NaCl
1x MBSH:	880 mM NaCl; 24 mM $NaHCO_3$; 100 mM KCl; 4 mM $CaCl_2$; 3,3 mM $Ca(NO_3)_2$; 100 mM HEPES; 8 mM $MgSO_4$; pH 7.4
MEMFA:	MEM; 0.1% (v/v) Tween20; 3.7% (v/v) Formaldehyd; DEPC-H_2O
1x MEM:	100 mM MOPS pH 7.4; 2 mM EGTA; 1 mM $MgSO_4$; DEPC-H_2O
NBT/BCIP-solution:	1% (v/v) NBT/BCIP stock solution (Roche) in AP buffer
Proteinase K solution:	1% (w/v) Proteinkinase K in TBST
20x SSC:	3 M NaCl; 300 mM Na-Citrate pH 7,0
2x SSC-T:	2x SSC; 0.05% (v/v) Tween 20
0,2x SSC-T:	0.2x SSC; 0.05% (v/v) Tween 20
10x TBS:	25 mM Tris-HCl pH 7.4; 137 mM NaCl; 5 mM KCl; 0.7 mM $CaCl_2$; 0.5 mM $MgCl_2$
TBS-T:	1xTBS; 0.1% (v/v) Tween 20
Torula RNA-solution:	50 mg/ml in DEPC-H_2O diluted at 80°C and stored at – 20°C
Triethanolamine solution:	1 M triethanolamine, pH 7.5.

2.2.6.2 Microinjections

Morpholinos (MO) or cDNAs of the particular protein of interest were inserted into frog embryos by microinjection, using a glass needle fixed onto a micromanipulator connected to an air pump. Glass needles were filled with the respective MO/cDNA dilution and for injection the volume was determined by measuring the diameter of expelled droplets using a standardized platinum gauze.

Xenopus laevis embryos were transferred onto an agarose-coated dish containing 0.1x MBSH and fixed in the wells by gently eliminating the buffer. MOs/cDNAs are injected in a volume of 4 nl into one blastomere of a 2-cell stage *X. laevis* embryos. Dextrane-FITC was co-injected as lineage tracer to identify the injected site at neurula stages and to sort left side injected embryos from right side injected embryos.
After injection, the embryos were kept in 1x MBSH for about 1-1.5 hours at room temperature and then cultured in 0.1x MBSH at 14-18°C.

1x MBSH: 880 mM NaCl; 24 mM $NaHCO_3$; 100 mM KCl; 4 mM $CaCl_2$; 3.3 mM $Ca(NO_3)_2$; 100 mM HEPES; 8 mM $MgSO_4$; pH 7.4

2.2.6.3 Care of injected embryos and fixation

Injected *X.laevis* embryos were cultured in 0.1x MBSH at 14-18°C for 24-48 h. Culture medium was changed daily and dead embryos were removed 1 h and 1 day after injection as well as directly before fixation. Embryos were fixed in MEMFA for 1 h at room temperature and stored in methanol at 4°C.

1x MBSH: 880 mM NaCl; 24 mM $NaHCO_3$; 100 mM KCl; 4 mM $CaCl_2$; 3.3 mM $Ca(NO_3)_2$; 100 mM HEPES; 8 mM $MgSO_4$; pH 7.4

MEMFA: MEM; 0.1% (v/v) Tween 20; 3.7% (v/v) Formaldehyd; DEPC-H_2O

2.2.7 In vitro assays

2.2.7.1 TOPFlash reporter gene assay

The TOPFlash reporter gene assay is a method to assess transcriptional activity of TCF/LEF regulated genes, which makes use of the M50 Super 8x TOPFlash reporter construct, an expression vector containing a *Firefly*-luciferase reporter gene under control of TCF/LEF binding sites. Luciferase is an enzyme which oxidates its substrate luciferin in a chemoluminescent reaction. The light emission induced by this reaction can be quantitatively measured as relative light units (RLU) and reflects the relative luciferase activity. The level of activity depends on the amount of expressed luciferase that in turn reflects the amount of expressed TCF/LEF target genes. Treatment of cells with canonical Wnts like Wnt3a, subsequently to transfection of TOPFlash reporter constructs, leads to an increase of luciferase activity compared to untreated cells. This is indicated as fold induction. Any regulatory effect on Wnt signaling can therefore be detected by the ability to reduce or elevate this fold induction. In order to normalise the activation of induced reporter activity, cells are co-transfected with TKrenilla, an expression vector containing a *Renilla*-luciferase gene under control of the constitutive active TK-promotor.

All reporter gene assays were performed in 96 well plates. Briefly, HEK293 cells (1.5x 10^4/well) were transfected with SuperTOPFlash-reporter (20 ng) and TK-Renilla (5 ng) and the respective plasmid DNA as indicated using PromoFectin transfection reagent as described in 2.2.1.5.1. Each transfection was adjusted to 125 ng DNA/transfection with PCDNA3.1 empty vector. Where indicated cells were transfected at 50 – 70% confluency with siRNA using the HiPerFect transfection reagent in 6 cm petri-dishes 24 hrs before seeding the cells for the reporter assays (described in 2.2.1.5.2). 24 hrs after DNA transfection Control-CM or Wnt3a-CM was added to HEK293 cells for another 24 hrs. Where indicated, the MEK-Inhibitor (U0126, 15 µM), the PI3K-Inhibitor (Ly294002, 50 µM), Hyaluronan (HA, 400 µg/ml), Hyaluronidase (HAse, 50 U/ml) or antibodies (BU52; IgG, 3 µg/ml) 1 h prior to Wnt3a induction. 48 hrs after DNA transfection cells were lysed for 30 minutes on ice in 60 µl/well 1X Passive Lysis Buffer (Promega) and subjected to luminescence measurement.

For each measurement (Firefly-luciferase as reporter and *Renilla*-luciferase for normalization) 10 µl of the lysate were transferred into a non-transparent 96 well plate and subjected to luciferase measurement in an automated luminometer. Reaction buffer and substrate were injected automatically into each well (Firefly-luciferase: 70 µl/well luciferin reaction buffer, 20 µl/well luciferin reaction mix; *Renilla*-luciferase: 100 µl/well renilla reaction buffer containing the substrate). Luciferase values of reporter transfected cells were blanked by subtracting the values measured for non-transfected cells. For normalization, *Firefly*-

luciferase activity was divided by *Renilla*-luciferase activity. Statistical analysis of the TOPFlash reporter gene assays was done by using the double sided student´s *t*-test. A p-value less than 0.05 was considered as statistically significant. In parallel to the reporter assay transfected HEK293 cells were subjected to Western-blot analysis in order to confirm overexpression or downregulated expression of the gene of interest.

Gly-Gly-buffer:	25 mM Gly-Gly; 15 mM $MgSO_4$; 4 mM EGTA; pH 7.8
Luciferin reaction buffer:	Gly-Gly-buffer containing 1 mM DTT and 2 mM ATP
Luciferin reaction mix:	Luciferin stock solution diluted 1:5 in Gly-Gly-buffer
Luciferin stock solution:	1 mM Luciferin in Gly-Gly-buffer
Lysis buffer:	5x passive lysis buffer (Promega, Mannheim, Germany) diluted 1: 5 in ddH_2O
Renilla reaction buffer:	25 nM coelenterazin in coelenterazin-buffer
Coelenterazin buffer:	0.1 M KPi; 0.5 M NaCl; 1 mM EDTA; pH 7.6

2.2.7.2 β-catenin- and LRP6-activation assays

In this assays cells were treated with Wnt3a in the context of CD44 loss- or gain of function and activation of β-catenin and LRP6 was analysed by Western-blot analysis.

For loss of function studies, HeLa cells were seeded in 6 well plates (1.5 x 10^5/well) and transfected with CD44pan- or Control-siRNA (5nM) using HiPerFect transfection reagent. For competition experiments using the cytoplasmic CD44 domain, HeLa cells were seeded in 6 well plates (2 x 10^5/well) and transfected as indicated with DNA (1 µg PCDNA3.1 + 500 ng CD44-Cyt or CD44-CytΔEz-BD) using the PromoFectin transfection reagent 5 hrs after seeding. Transfection procedures are described in 2.2.1.5. 72 hrs after siRNA-transfection respectively 48 hrs after DNA-transfection, cells were incubated for 2 hrs (LRP6-assay) or 3 hrs (β-catenin assay) with Control-CM or Wnt3a-CM. Subsequently, cells were washed once with ice cold PBS and solubilized in boiling SDS-sample buffer containing 100 mM DTT. Lysates were subjected to Western-blot analysis in order to detect activation of β-catenin and LRP6.

2.2.7.3 Hyaluronan detection and hyaluronidase treatment

This assay is used to visualize production of Hyaluronan (HA) by HEK293 cells and to confirm degradation of HA by Hyaluronidase.

To this aim, HEK293 cells were seeded on 20 mm glass cover slips in 12-well plates (0.6 x 10^5). 20 hrs later cells were treated with 50 U/ml Bovine Testis Hyaluronidase (HAse) for 24 hrs, washed in PBS and fixed with 4% paraformaldehyde (PFA). Fixed cells were incubated with biotinylated HA-binding protein (HABP, 2.5 mg/ml) overnight. Afterwards, cells were stained with Alexa488-streptavidin conjugates (0.4 µg/ml) for 2 hrs and counterstained with DAPI. The slides were mounted with Fluorescence Mounting Medium and analyzed with a SP5 laser scanning confocal microscope with a 63x objective.

2.2.7.4 Co-Immunoprecipitation (Co-IP)

The co-immunoprecipitation assay was used to analyse the interaction between CD44 and LRP6. To this aim endogenous or overexpressed CD44-proteins were immunoprecipitated using CD44 antibodies and precipitates were then tested by Western-blot analysis for the presence of endogenous or overexpressed LRP6.

For co-immunoprecipitation of endogenous proteins, HeLa cells were seeded in 10 cm plates (1 x 10^6) and incubated for the indicated time points with Wnt3a-CM 24 hrs after seeding. Subsequently, cells were washed with ice cold PBS and lysed in 450 µl HEPES-lysis buffer for 30 minutes. Lysates were transferred to 1.5 ml cooled Eppendorf cups, centrifuged (12 000 rpm for 15 minutes at 4°C) and the supernatant was transferred to new cooled 1.5 ml Eppendorf cups. For immunoprecipitation, CD44 antibodies (Hermes-3, 3µg) were added to the supernatant and incubated over night at 4°C under constant rotation. In between, Protein G agarose beads (30 µl/450 µl) or mouse-IgG sepharose bead conjugates (IgG-control, 3 µg/450 µl) were washed three times and diluted in HEPES-lysis buffer and added to the supernantant for another 3 hrs at 4°C. The precipitates were centrifuged (12 000 rpm for 5 minutes at 4°C) and 30 µl of the supernantant was mixed and boiled with SDS-sample buffer and taken as input control. Precipitates were washed 3x in HEPES-lysis buffer and solubilized in boiling SDS-sample buffer containing 100 mM DTT. Samples were subjected to Western-blot analysis using LRP6-antibodies (Cell signaling C5C7) to detect co-immunoprecipitated LRP6 and CD44-antibodies (Hermes-3) to confirm CD44 immunoprecipitation.

For co-immunoprecipitation of overexpressed LRP6-flag and overexpressed CD44 and CD44ΔCyt, HeLa cells were transfected in 10 cm plates (1x 10^6) with 2 µg LRP6-Flag together with 2 µg rCD44v4-v7 or 2 µg rCD44v4-v7ΔCyt using the PromoFectin transfection reagent. 24 hrs after transfection cells were treated for 30 minutes with Wnt3a-CM and

subjected to co-immunoprecipitation as described above using an rCD44 antibody (1.1ASML) for the immunoprecipitation.

HEPES-lysis buffer: 25 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 1% NP-40, 0.5 mM MgCl2, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na orthovanadate, and 1 mM aprotinine and 1 mM leupeptin

2.2.7.5 Confocal fluorescence microscopy

For loss of function studies, HeLa cells were seeded in 10 cm petri dishes and transfected at 50-70% confluency with control- or CD44pan-siRNA using the HiPerFect transfection reagent as described in 2.2.1.5.2. 24 hrs after siRNA transfection cells were seeded on 20 mm glass cover slips in 12-well plates (0.6x 10^5/well). For β-catenin localization studies cells were treated for 24 hrs either with Control-CM or Wnt3a-CM, washed on time with PBS and fixed with Methanol for 10 min at -20°C. Afterwards cells were washed 3x with PBS, blocked with 5% FCS in PBS for 1 h and incubated with a β-catenin antibody (D10A8,1:100) for 24 hrs at 4°C. Subsequently, cells were washed 3x with PBS, incubated with an donkey anti-rabbit-Alexa 546 antibody (0.4 µg/ml) for 2 hrs at room temperature washed another 3 x, mounted with Fluorescence Mounting Medium and analyzed with a SP5 laser scanning confocal microscope with a 63x objective. In parallel, transfected cells were subjected to Western-blot analysis to confirm hCD44 knockdown. The percentage of cells with nuclear β-catenin was quantified and statistically analyzed using the double-sided student's *t*-test. A value of $p < 0.05$ is considered statistically significant.

For LRP6 localization studies cells were transfected as indicated with PCDNA3.1 empty vector (250 ng) or rCD44s (250 ng) or Mesd (50 ng) together with LRP6-GFP (250 ng). For Ror2 localization studies cells were transfected with PCDNA3.1 empty vector (750 ng) and xROR2-mCherry (250 ng). DNA transfections were performed using PromoFectin transfection reagent as described in 2.2.1.5.1. The total amount of DNA was adjusted with PCDNA3.1 to 1 µg per transfection. For competition experiments using the cytoplasmic CD44 domain (CD44-Cyt); CD44-Cyt mutated in the Ezrin binding site (CD44-CytΔEzBD), and dominant-negative Ezrin (DN-Ezrin),HeLa cells seeded on 20 mm glass cover slips in 12-well plates (0.6x10^5/well) were transfected either with empty vector alone or CD44-Cyt (250 ng), CD44-CytΔEzBD (250 ng) or DN-Ezrin (100 ng) together with LRP6-GFP (250 ng) with PromoFectin. The total amount of DNA was adjusted with PCDNA3.1 to 1 µg per transfection. Where indicated cells were incubated with an ER-marker (ER-TrackerTM Red) for 30 minutes before fixation. 24 hrs after DNA transfection cells were washed 1x with PBS

and fixed with 4% paraformaldehyde for 10 minutes at room temperature. Cells were incubated with DAPI for 30 minutes at room temperature and washed again 2x with PBS. Afterwards, samples were mounted with Fluorescence Mounting Medium and analyzed with a SP5 laser scanning confocal microscope with a 63x objective. In parallel, transfected cells were subjected to Western-blot analysis to confirm hCD44 knockdown and rCD44s overexpression. The ratio between cells with membrane localized LRP6-GFP or Ror2-mCherry to the total amount of LRP6-GFP or Ror2-mCherry transfected cells was quantified and statistically analyzed using the double-sided student's *t*-test. A value of $p < 0.05$ is considered statistically significant.

2.2.8 *Xenopus laevis* studies

X. llaevis embryos were cultured, and injected as described in 2.2.7. 16 ng xCD44-Morpholinos (xCD44-MO) or Control-Morpholinos (Co-MO) were co-injected into one blastomere of xenopus 2-cell stage embryos with 4 pg dextrane-FITC as lineage tracer to identify the injected site at neurula stages via fluorescence microscopy and to sort left side injected from right side injected embryos. Where indicated, xCD44-MO were co-injected with hCD44s (50 pg) or constitutive active β-catenin (30 pg) expression vectors. Embryos were staged according to Niewkoop et al. (1967) and fixed at stages 28-32 in MEMFA (0.1 M MOPS pH 7.2, 2 mM EGTA, 1 mM $MgSO_4$, and 3.7% formaldehyde). *In situ* hybridization for TCF-4 and En-2 is described in 2.2.7.1. In addition to *in situ* hybridization, embryos were analyzed for ventral musculature phenotypes. Images were captured on a Leica MZFLIII microscope using a digital camera (Qimaging) and Improvision software (Openlab). Statistical analysis was performed using the student´s *t*-test. A value of $p < 0.05$ was considered statistically significant.

2.2.9 Statistical analysis

The double-sided student´s *t*-test was used for comparison of two samples. Calculation of the mean averages and standard deviation was performed using at least three biological replicas. P values < 0.05 were considered significant. Error bars indicate standard deviation (SD).

3. Results

Wnt/β-catenin signaling is induced upon binding of canonical Wnt ligands like Wnt3a to their corresponding receptors Fz and LRP. This binding results in an inhibition of the so-called β-catenin destruction complex that is responsible for the proteasomal degradation of cytoplasmic β-catenin. The inhibition of the destruction complex allows accumulation of β-catenin in the cytoplasm and its subsequent translocation to the nucleus. In the nucleus β-catenin binds to DNA bound TCF/LEF-transcription factors, turning them into transcriptional activators, a step that leads to the transcription of TCF/LEF regulated genes (reviewed in Logan and Nusse, 2004).

Remarkably, many of these Wnt-target genes are themselves components of the pathway, indicating that the Wnt-pathway is regulated through feedback-control mechanisms. This feedback can either be negative, resulting in a decrease of Wnt-signaling activity but also positive, leading to an enhanced activity (reviewed in Logan and Nusse, 2004). One prominent Wnt target gene is the transmembrane glycoprotein CD44 (Wielenga et al., 1999), a well-known regulator of growth factor (GF) signaling (reviewed in Orian-Rousseau and Ponta, 2008). The functions of CD44 as a signaling regulator are diverse and are often dependent on specific isoforms that are generated by alternative splicing of ten variant exons in the extracellular domain. The finding that the signaling modulator CD44 is a target gene of the canonical Wnt-pathway, raised the question of its potential participation in the feedback-regulation of Wnt/β-catenin signaling. Indeed, CD44 can be chondroitin- and heparansulphated. These posttranslational modifications are also found on well-known Wnt-regulators. Overlapping functions in limb development, hair regrowth, tissue regeneration and colorectal cancer give further hints for a collaboration of CD44 and Wnt-signaling. The finding that binding of the ECM component hyaluronan to CD44 can induce TCF/LEF regulated transcription and furthermore that silencing of CD44 can prevent β-catenin stabilization, indicate that CD44 might be part of a regulatory Wnt-network. Preliminary work performed during my Diploma thesis provided further evidence for a regulatory function of CD44 in the Wnt-pathway. As this work was the basis of my PhD thesis two results of my Diploma thesis are included in the results part of this PhD thesis. These results (Fig. 4 A and B) are indicated in the figure legends with a hash key (#).

3.1 CD44 is a positive co-regulator of Wnt3a induced TCF/LEF regulated transcription

In order to analyze whether CD44 plays a role in the canonical Wnt-pathway, I tested whether a knockdown of CD44 had any effect on the activation of canonical Wnt-signaling, using TCF/LEF regulated transcription as read-out. To this aim, I made use of a Wnt-sensitive TCF/LEF reporter gene assay, a well-established and highly sensitive method to study Wnt/β-catenin signaling (Molenaar et al., 1996). This assay is based on a so-called TOPFlash (TCF/LEF optimal promoter) reporter construct, which harbors a *firefly*-luciferase reporter gene under the control of a minimal CMV promoter and multiple TCF/LEF binding sites. As the Wnt/β-catenin signaling pathway activates TCF/LEF regulated transcription, induction of canonical Wnt-signaling activates the expression of the luciferase reporter gene. Luciferase is an enzyme that oxidates its substrate luciferin in a measurable chemiluminescent reaction. The light intensity measured in this reaction correlates with the expressed luciferase and reflects thereby the level of Wnt activation. Thus, any effects on the activation of Wnt-signaling are reflected by an increase or decrease of luciferase activity. In order to activate the canonical Wnt-pathway at the level of the Wnt-ligands, cells were treated with Wnt3a conditioned medium (Wnt3a-CM) that was obtained by harvesting the supernatant of stably Wnt3a-transfected L-cells, stably transfected with Wnt3a and that secret Wnt3a ligands in the cell culture medium (Shibamoto et al., 1998). As control, cells were treated with medium that was obtained by harvesting the supernatant of non-transfected L-cells (control-CM).

To address whether CD44 loss of function has an effect on canonical Wnt-signaling, HEK293 cells were transfected either with unspecific control-siRNA or siRNA against all CD44 isoforms and co-transfected with the TOPFlash reporter construct. Subsequently, these cells were treated either with Wnt3a-CM or with control-CM medium. Afterwards the HEK293 cells were analyzed for luciferase activity. In parallel cell lysates were subjected to Western-blot analysis to confirm the knockdown of CD44. In the control-siRNA transfected cells treatment with Wnt3a-CM led to increase of almost 50-fold of the luciferase activity compared to the control-CM treated cells. In CD44-siRNA transfected cells, instead, Wnt3a-treatment led only to a 30-fold induction (Fig. 4 A), indicating a requirement of CD44 for a proper activation of the canonical Wnt-pathway.

Conversely, co-transfection of HEK293 cells with the TOPFlash reporter together with different amounts of CD44 standard (CD44s), the smallest isoform of CD44 that does not contain any variant exon, showed that ectopic expression of CD44 increases Wnt3a-CM induced Wnt-reporter activation significantly and in a dose dependent manner (Fig. 4 B). Indeed, in empty vector transfected cells treatment with Wnt3a led to a 25-fold increase in

the luciferase activity compared to cells treated with control-CM. Remarkably, ectopic expression of hCD44s was able to enhance this Wnt3a-mediated activation of the Wnt-reporter up to almost 100-fold (Fig. 4 B). Both, efficient knockdown by the CD44-siRNA and increased expression levels of CD44 transfectants was confirmed by Western-blot analysis (Fig. 4 A and B).

Taken together these CD44 loss- and gain of function experiments reveal a role of CD44 as a positive regulator of Wnt/β-catenin signaling. Given that CD44 is a Wnt target gene, these findings indicate that CD44 is involved in a positive feedback loop that controls Wnt activity.

In addition to the standard isoform of CD44, the expression of specific CD44 variant isoforms containing variant exons v4 and v6 is highly upregulated in the context of Wnt-hyperactivation (Wielenga et al., 1999). As we assume feedback-regulation of Wnt-signaling by CD44 and since the inclusion or exclusion of specific variant exons can influence the properties of CD44, it is possible that different CD44 isoforms act differently in the Wnt-pathway. Thus, experiments were performed to address whether CD44 isoforms that contain v4 or v6 differ in their ability to enhance Wnt-signaling compared to CD44s that lacks the v4 and v6 exons. In these experiments, HEK293 cells were co-transfected with the TOPFlash reporter construct and either CD44s, CD44v6 or CD44v4-v7 (including both v4 and v6), treated with Wnt3a-CM and analyzed for luciferase activity. Remarkably, all tested isoforms augmented Wnt3a-induced activation of the TOPFlash reporter to the same extent, indicating that the function of CD44 as a positive Wnt-regulator is not influenced by inclusion of additional variant exons and that the standard CD44 isoform is sufficient (Fig. 4 C).

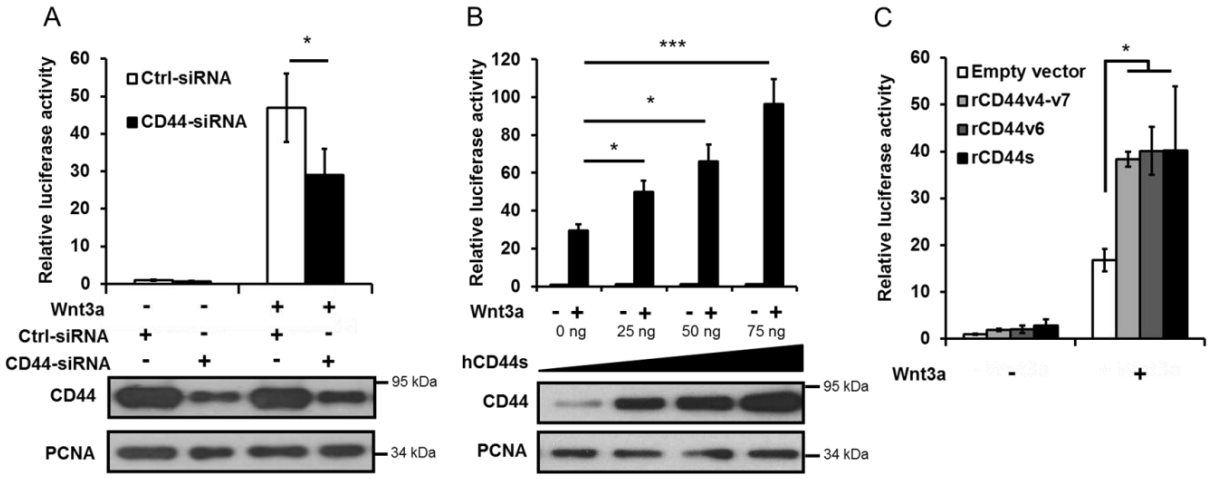

Fig. 4 CD44 is a required positive regulator of Wnt3a induced TCF/LEF regulated transcription.
A) HEK293 cells transfected with control-siRNA or siRNA against all CD44 isoforms were transfected with the TOPFlash-reporter and control TK-renilla vectors. Cells were treated either with control- or Wnt3a-CM for 20 hrs and analyzed for luciferase activity 48 hrs after DNA transfection. B) HEK293 cells transfected with TOPFlash-reporter and TK-renilla together with increasing amounts of hCD44s (0, 25, 50, 75 ng) were treated with control- or Wnt3a-CM for 20 hrs and analyzed for luciferase activity 48 hrs after DNA transfection. C) HEK293 cells transfected with TOPFlash-reporter and TK-renilla together with equal amounts of either rCD44v4-v7, rCD44v6 or rCD44s, were treated either with control- or Wnt3a-CM for 20 hrs and analyzed for luciferase activity 48 hrs after transfection. Data represent an average from at least 5 independent experiments performed in triplicates, with the indicated standard deviations (*p< 0.05,***p<0.005, statistical significance was analyzed using the student´s *t*-test). Lysates were in parallel subjected to Western-blot analysis with antibodies against CD44 in order to confirm the knockdown respectively the overexpression of CD44. # Experiment A and B were obtained during my Diploma thesis.

3.2 CD44 is required for Wnt3a induced activation of β-catenin

The results from the TOPFlash assay raised the hypothesis that CD44 is indeed required for activation of the Wnt-pathway. If this hypothesis holds true a knockdown of CD44 should also affect the activation of endogenous Wnt-pathway components.

β-catenin is a pivotal molecule in the Wnt-pathway and changes in its cytoplasmic concentration are decisive for transduction of Wnt-signaling. In resting cells, cytoplasmic β-catenin is bound to an intracellular degradation complex that constantly phosphorylates free cytosolic β-catenin on serine 37 (Ser37) and threonine 41 (Thr41) (van Noort et al., 2002). The phosphorylation of these specific sites on β-catenin triggers the ubiquitination and subsequent proteasomal degradation of β-catenin and by this the concentration of cytosolic β-catenin is kept low. Activation of the Wnt-pathway leads to an inhibition of the degradation complex and β-catenin is no longer phosphorylated on Ser37 and Thr41 and thereby not ubiquitinated and not degraded by the proteasome. Hence, upon Wnt induction the hypophosphorylated β-catenin can accumulate in the cytoplasm and translocate to the nucleus where it binds to TCF/LEF transcription factors thereby activating the expression of Wnt-target genes. The following experiment addressed whether the downregulation of CD44

has an effect on the activation and accumulation of hypophosphorylated β-catenin. To this end, HeLa cells were transfected either with unspecific control-siRNA or siRNA against all CD44 isoforms. 48 hrs later, these cells were then treated either with Control-CM or Wnt3a-CM for 3 hours and subjected to Western-blot analysis using an antibody that recognizes the active hypophosphorylated β-catenin (Activated Beta-Catenin antibody, ABC) (van Noort et al., 2002). In concordance with the results obtained from the TOPFlash assay, HeLa cells transfected with CD44-siRNA failed to accumulate activated β-catenin upon Wnt3a induction, whereas control-siRNA treated cells showed increased levels of hypophosphorylated β-catenin after stimulation with Wnt3a (Fig. 5). In contrast to the hypophosphorylated β-catenin, the silencing of CD44 expression had no effect on the overall protein level of β-catenin within the cell, as demonstrated by an antibody against total β-catenin. This is due to high amounts of membrane bound β-catenin that is not recognized by the degradation complex. The knockdown of CD44 was proven by an antibody against CD44 and equal loading was confirmed using an antibody against actin.

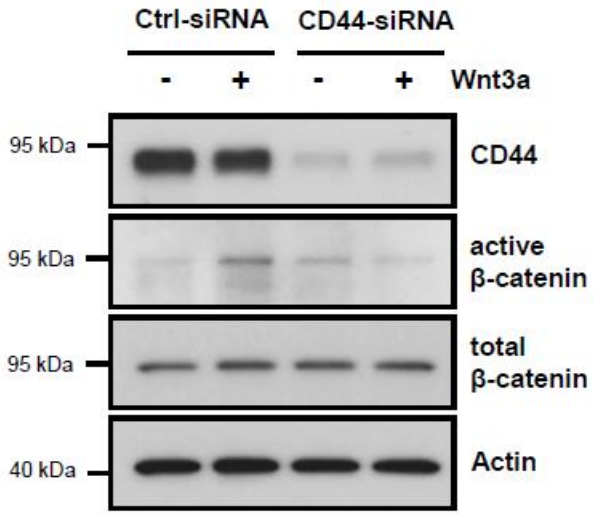

Fig. 5 CD44 is required for Wnt3a induced β-catenin activation.
HeLa cells were transfected with control-siRNA or siRNA against all CD44 isoforms. 48 hrs after siRNA-transfection, cells were treated with control- or Wnt3a-CM for 3 hrs. Subsequently, cells were subjected to Western-blot analysis. Antibodies against dephosphorylated β-catenin were used to assess β-catenin activation and antibodies against CD44s to confirm the knockdown of CD44. Antibodies against total β-catenin and Actin were used as loading controls. Data show one representative out of 4 independent experiments.

To test whether the knockdown of CD44 also has an effect on the Wnt-induced nuclear translocation of β-catenin, HeLa cells were treated with unspecific control-siRNA or siRNA against all CD44 isoforms and induced with Wnt3a for 20 hrs. Subsequently, the cells were fixed and subjected to immunofluorescence staining using an antibody against β-catenin and counterstained with DAPI to visualize the nucleus. The cells were analyzed by confocal fluorescence microscopy and the percentage of cells with nuclear β-catenin was

evaluated. In control-siRNA transfected cells, treatment with Wnt3a lead to an increase of β-catenin in the cytoplasm and furthermore β-catenin could be detected in the nucleus of 76% of the cells (Fig. 6), whereas almost no cells (4% of analyzed cells) with nuclear β-catenin were detected in cells treated with control-CM. In cells treated with CD44-siRNA nuclear β-catenin was only found in 22% of cells induced with Wnt3a, showing that a knockdown of CD44 strongly suppresses Wnt3a-induced β-catenin nuclear translocation. Again, the knockdown of CD44 expression was confirmed by Western-blot analysis (Fig. 6). Altogether, these results show that silencing CD44 expression interferes with Wnt3a-induced activation and nuclear translocation of β-catenin, the critical step in the Wnt/β-catenin pathway. This strongly supports the hypothesis that CD44 plays an essential role and important function in the Wnt/β-catenin signaling pathway.

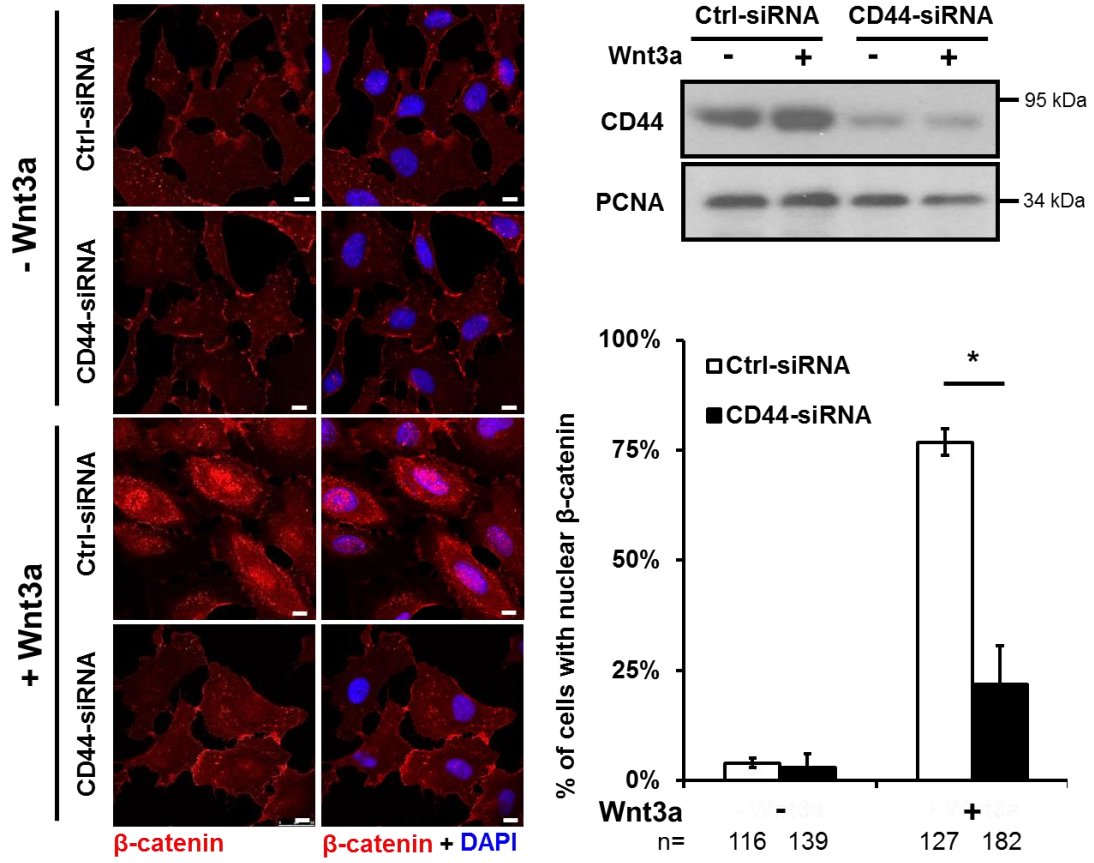

Fig. 6 CD44 is required for Wnt3a induced nuclear translocation of β-catenin.
HeLa cells were transfected with control-siRNA or siRNA against all CD44 isoforms. 48 hrs after siRNA-transfection, cells were treated with control- or Wnt3a-CM for 24 hrs. Subsequently, cells were subjected to immunofluorescence staining using antibodies against β-catenin (red). The nucleus was stained with DAPI (blue). Afterwards, cells were analyzed by confocal microscopy (left panel: β-catenin only; right panel: overlay; scale bar = 10 µm) and the percentage of cells with nuclear β-catenin was evaluated (diagram). Data represent mean +/- SD of all confocal microscopy experiments (4 independent experiments; >25 cells per experiment were evaluated), n indicates the total number of analyzed cells. Statistical significance was analyzed using the student´s *t*-test (*p< 0.05). The knockdown of CD44 was confirmed by Western-blot analysis.

The data obtained so far describe CD44 as an essential positive co-regulator of canonical Wnt-signaling. The mechanism of action of CD44 in the Wnt-cascade still needs to be investigated. As shown in Fig. 4 B, several CD44 isoforms, among them the smallest isoform CD44s, augmented Wnt-signaling to the same extent, implying that a function common to all CD44 isoforms is involved in the role of CD44 in the Wnt-pathway. The following chapters address which common functions of CD44 might be involved and describe the molecular mechanism of CD44 mediated Wnt/β-catenin signaling regulation.

3.3 Analysis of the molecular mechanism of CD44 mediated Wnt/β-catenin signaling regulation

3.3.1 CD44-mediated amplification of Wnt/β-catenin signaling is independent of HA

One function that is conserved amongst all CD44 family members is the ability to bind to hyaluronan (HA) through their extracellular domains (reviewed in Orian-Rousseau, 2010). The glycosaminoglycan HA is a component of the extracellular matrix and widely distributed throughout epithelial, neural and connective tissues. It is worth noting that the binding of HA to CD44 has already been shown to activate TCF/LEF regulated transcription in several cancer cell lines, most probably indirectly by two different mechanisms. On the one hand, HA binding to CD44 activates PI3K/Akt-signaling via activation of ErbB2 receptors, a step that induces in turn cytosolic β-catenin accumulation and subsequent nuclear translocation (Bourguignon et al., 2007c; Misra et al., 2008). On the other hand, the interaction between CD44 and HA was shown to upregulate the expression of the histone acetyltransferase p300 that acts as a transcriptional co-activator for TCF/LEF regulated gene expression (Bourguignon et al., 2009). Thus, further experiments addressed whether the regulation of Wnt-signaling by CD44 might be mediated or influenced by its binding to HA.

To address whether HA binding to CD44 is necessary for the regulation of Wnt-signaling, I first tested whether addition of HA to cells overexpressing CD44 or not had any effect on canonical Wnt-signaling. To this aim, HEK293 cells, transfected with the TOPFlash reporter construct together with an empty vector or CD44s-cDNA, were treated with control-CM or Wnt3a-CM without additional HA; or control-CM respectively Wnt3a-CM in combination with HA.

The addition of HA had no significant effect on Wnt induced TCF activation, neither in combination with the control-CM nor in cells induced with Wnt3a-CM (Fig. 7 A). HA treatment did also not influence the augmentation of Wnt-signaling by CD44. However, HEK293 cells

produce HA endogenously as shown by the staining of HEK293 with a biotinylated HA binding protein (Fig. 7 B, left panel). Thus, it is possible that CD44 is already bound to HA and therefore no effect of additional HA can be detected. To circumvent this problem, HEK293 cells were treated with hyaluronidase (HAse), an enzyme that degrades HA and tested whether the removal of HA affected the Wnt3a induced activation of the TOPFlash reporter in cells transfected with an empty vector or in cells transfected with hCD44s-cDNA. Remarkably, although HA was removed from the cell surface upon HAse treatment (Fig. 7 B, left panel), this removal had neither an effect on Wnt-induced activation of Wnt/β-catenin signaling nor on the amplification of Wnt-signaling mediated by CD44 gain of function (Fig. 7 B, right panel). Thus, HA seems not to play any role for the regulation of Wnt/β-catenin signaling by CD44.

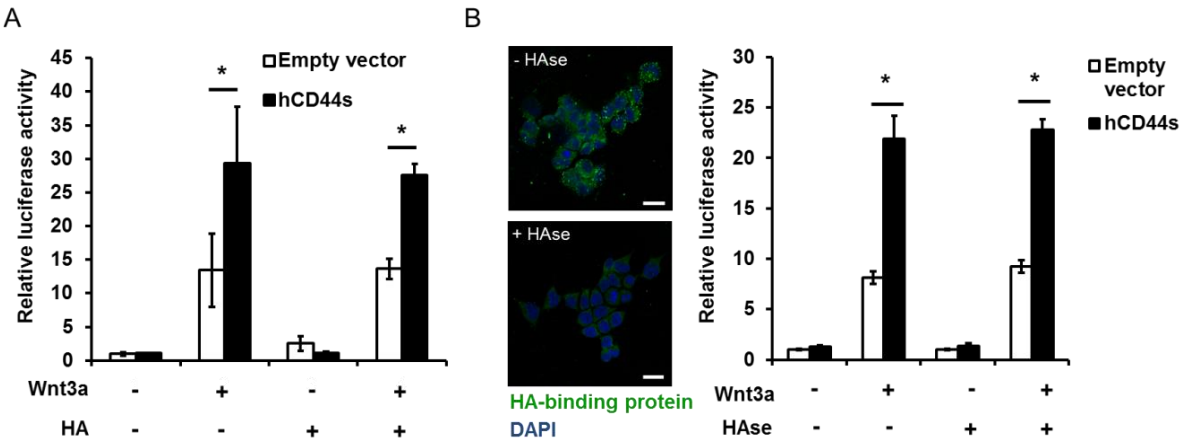

Fig. 7 HA levels have no influence on CD44 mediated regulation of Wnt/β-catenin signaling.
A) HEK293 cells were transfected with TOPFlash and TK-renilla vectors together with empty vector or hCD44s-cDNA. Cells were treated either with control- or Wnt3a-CM for 20 hrs in absence or presence of HA (400 µg /ml) and analyzed for luciferase activity. B) HEK293 cells were transfected with TOPFlash-reporter and TK-renilla vectors and co-transfected with an empty vector or hCD44s-cDNA. Cells were treated either with control- or Wnt3a-CM for 20 hrs in the absence or presence of Hyaluronidase (50U/ml) and analyzed for luciferase activity. In parallel HEK293 cells, untreated or treated with Hyaluronidase (50U/ml) for 20 hrs, were stained with a biotinylated HA binding protein and Alexa488 coupled streptavidin to confirm that HA was removed from cells. Data represent an average from 4 independent experiments performed in triplicates with the indicated standard deviations (*p< 0.05, statistical significance was analyzed using the student´s *t*-test).

To confirm this in an alternative assay, HA binding to CD44 was inhibited by means of a CD44 antibody (BU52) that prevents the interaction between CD44 and HA (Avigdor et al., 2004). Indeed, treatment of HEK293 cells with this antibody did not suppress Wnt3a activation or the CD44-mediated augmentation of Wnt-signaling as shown by the TOPFlash reporter assay (Fig. 8 A). In agreement with these results, overexpression of CD44s mutated in the HA binding site (hCD44s-HAmut) (Peach et al., 1993) was as efficient in amplifying Wnt3a induced transcription of TCF/LEF regulated genes as was the overexpression of wild-type CD44 (Fig. 8 B). In parallel to the TOPFlash assay, the transfected cells were subjected

to Western-blot analysis in order to prove that both, the wildtype CD44 and the CD44 mutated in the HA binding domain were expressed equally.

Altogether, these results show that neither addition nor removal of HA as well as interference with CD44/HA interaction had any effect on the enhancement of Wnt3a induced β-catenin signaling by CD44. This demonstrates that CD44 regulates Wnt-signaling independent of its binding to HA.

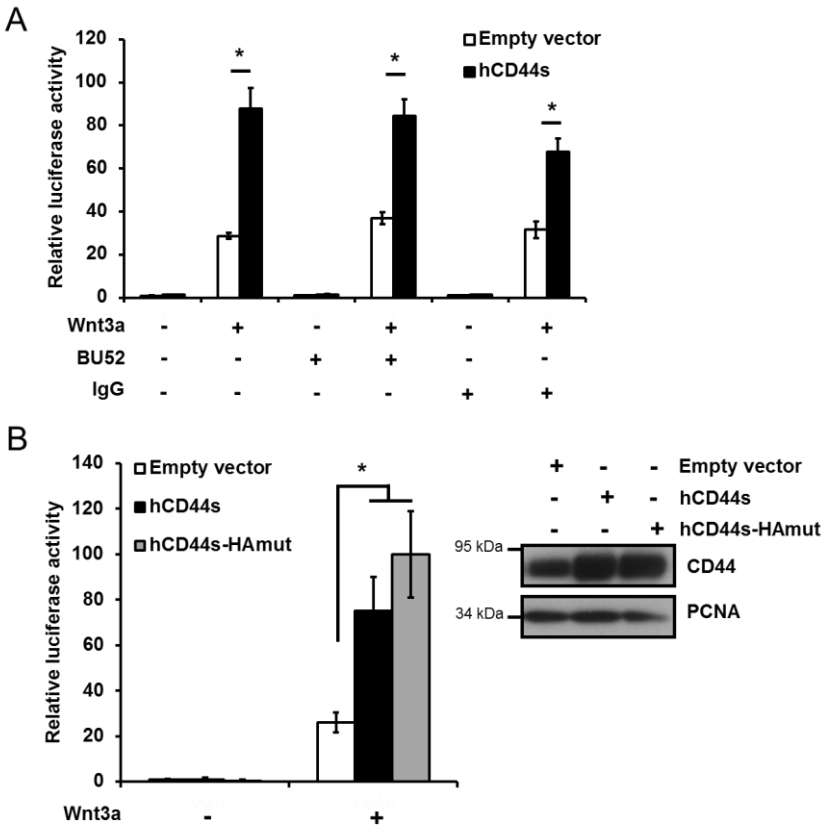

Fig. 8 CD44 does not require binding to HA to regulate Wnt/β-catenin signaling.
A) HEK293 cells were transfected with TOPFlash and TK-Renilla vectors together with empty vector or hCD44s. Cells were treated either with control- or Wnt3a-CM for 20 hrs in the absence or in the presence of antibodies that block the interaction between HA and CD44 (BU52, 3 µg/ml). IgG antibodies were used as negative control. Subsequently, cells were analyzed for luciferase activity. B) HEK293 cells were transfected with TOPFlash and TK-Renilla vectors together with empty vector or hCD44s or hCD44s mutated in the HA binding domain (hCD44s-HAmut). Cells were treated either with control- or Wnt3a-CM for 20 hrs and analyzed for luciferase activity. Lysates were in parallel subjected to Western-blot analysis with antibodies against hCD44 in order to confirm equal expression of hCD44s and hCD44s-HAmut. Data represent an average from 5 independent experiments performed in triplicates with the indicated standard deviations (*p< 0.05, statistical significance was analyzed using the student´s *t*-test).

As several CD44 isoforms augment Wnt/β-catenin signaling and since binding of HA to CD44 is not involved in the function of CD44 in the canonical Wnt-cascade, another common feature of the CD44 family members was addressed, namely their interaction with intracellular binding partners via the cytoplasmic CD44 domains. Indeed, besides their ability to bind to HA, CD44 proteins bind to several intracellular proteins including ERMs (Legg and Isacke, 1998), ankyrin (Bourguignon et al., 1991), and IQGAP1 (Bourguignon et al., 2005b),

all involved in the modulation of several signaling pathways. Moreover, the linkage of CD44 to the actin-cytoskeleton via ERM proteins is required for signaling from several cell surface receptors such as RTKs (Orian-Rousseau et al., 2007; Tremmel et al., 2009). Thus, the following experiments adressed whether the intracellular domain of CD44 and the linkage to intracellular binding partners is also important for the function of CD44 in the Wnt-pathway.

3.3.2 CD44 requires the binding of Ezrin to its intracellular domain in order to modulate Wnt/β-catenin signaling

In order to analyze whether the cytoplasmic domain of CD44 is required for the regulation of canonical Wnt-signaling, HEK293 cells were transfected with the TOPFlash reporter together with either a full-length rat CD44v4-v7 construct (rCD44v4-v7) or a rCD44v4-v7 deletion mutant that lacks the cytoplasmic CD44 domain (rCD44v4-v7ΔCyt), to examine whether CD44 requires its intracellular domain to enhance TCF/LEF regulated transcription induced by Wnt3a. In contrast to wildtype (wt) rCD44v4-v7, rCD44v4-v7ΔCyt was not able to increase Wnt3a-induced TOPFLASH reporter activity (Fig. 9 A). The expression of both, rCD44v4-v7 and rCD44v4-v7ΔCyt was confirmed by Western-blot analysis.

This result demonstrates that the presence of its intracellular domain is a prerequisite for CD44 to operate as Wnt-regulator. As several membrane-anchored or intracellular proteins can interact with CD44 via its intracellular domain, further experiments were performed to test whether interference with these interactions affects the activation of Wnt/β-catenin signaling by Wnt3a-ligands. To this aim, HEK293 cells were transfected with the TOPFlash reporter and additionally with an expression vector containing the cytoplasmic domain of CD44 (CD44-Cyt). It has already been demonstrated that this CD44-Cyt can act as a dominant-negative (DN) competitor for the binding of intracellular proteins to endogenous CD44 (Orian-Rousseau et al., 2002). Consistently, ectopic expression of CD44-Cyt significantly inhibited Wnt-induced activation of the TOPFlash reporter (Fig. 9 B). This result further proves the requirement of the intracellular domain for CD44 to modulate the Wnt-pathway.

Among the various proteins that bind to the cytoplasmic domain of CD44, the CD44-ERM (Ezrin-Radixin-Moesin proteins) interplay is the best characterized and the binding of ERM-proteins to CD44 is most often involved in CD44 induced signaling (Orian-Rousseau et al., 2007; Tremmel et al., 2009; Tsukita et al., 1994; Yonemura et al., 1998). ERM proteins function as linker that connect membrane bound or transmembrane proteins to the actin-cytoskeleton (reviewed in Fehon et al., 2010). In my PhD work, experiments were performed to investigate whether ERM proteins and their binding to CD44 is involved in the Wnt-

pathway. In order to analyze whether the binding of ERM proteins to CD44 plays a role in Wnt-signaling, the effect of an ectopically expressed CD44 cytoplasmic domain mutated in the ERM binding domain (CD44-CytΔEz-BD) on Wnt-signaling was tested in the TOPFlash assay. This mutant of the dominant-negative CD44 cytoplasmic domain allows the binding of ERM proteins to the intracellular domain of endogenous CD44, however it still competes for the binding of all other intracellular CD44 binding partners (Legg and Isacke, 1998). HEK293 cells were transfected with the TOPFlash reporter and co-transfected either with an empty vector construct, the DN CD44-Cyt or the CD44-CytΔEz-BD. Afterwards, these cells were treated with Wnt3a and the activation of the TOPFlash reporter was evaluated. In cells transfected with the DN CD44-Cyt the activation of the TOPFlash reporter was significantly reduced compared to cells transfected with an empty vector construct (Fig. 9 B). In contrast to the DN CD44-Cyt, the ectopic expression of the CD44-CytΔEz-BD construct showed almost no effect on Wnt3a induced TOPFlash reporter activation (Fig. 9 B). This indicates that CD44 indeed requires the association with ERM proteins via its cytoplasmic domain for its function in Wnt signaling.

In addition to the TOPFlash reporter assay in HEK293 cells, the crucial role of the CD44 ICD for canonical Wnt-signaling was confirmed on Wnt-induced activation of endogenous β-catenin in HeLa cells. Indeed, transfection of the DN CD44-Cyt prevented β-catenin activation as shown by Western-blot analysis using the antibody against hypophosphorylated active β-catenin (Fig. 9 C). Furthermore and in concordance with the results of the TOPFlash assay, ectopic expression of the cytoplasmic CD44 domain mutated in the ERM binding site showed no inhibitory effect on Wnt3a induced activation of β-catenin, demonstrated by an equivalent level of active β-catenin in empty vector and CD44-CytΔEz-BD transfected cells (Fig. 9 C).

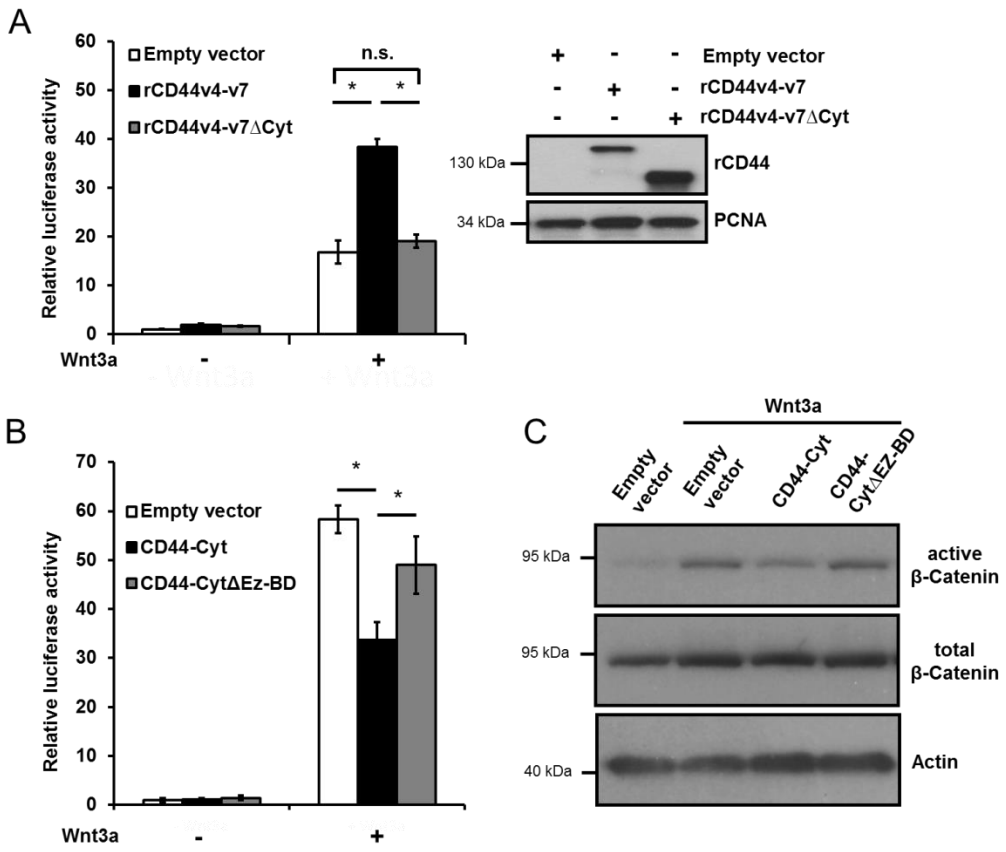

Fig. 9 CD44 requires the cytoplasmic domain and its linkage to intracellular binding partners to modulate Wnt/β-catenin signaling.
A) HEK293 cells were transfected with TOPFlash and TK-Renilla vectors together with empty vector, rCD44v4-v7 or rCD44v4-v7ΔCyt, treated either with control- or Wnt3a-CM for 20 hrs and analyzed for luciferase activity. In parallel lysates were subjected to Western-blot analysis to prove expression of the CD44 constructs. B) HEK293 cells were transfected with TOPFlash and TK-Renilla vectors together with empty vector, CD44-Cyt (CD44 cytoplasmic domain), or CD44-CytΔEzBD (CD44-Cyt mutated in the Ezrin binding domain), treated either with control- or Wnt3a-CM for 20 hrs and analyzed for luciferase activity. Data represent an average from 5 independent experiments performed in triplicates with the indicated standard deviations (*p< 0.05; n.s.=not significant; statistical significance was analyzed using the student´s *t*-test). C) HeLa cells were transfected either with empty vector, CD44-Cyt or CD44-CytΔEzBD, treated with control- or Wnt3a-CM for 3h and subjected to Western-blot analysis. Activation of Wnt-signaling was addressed by antibodies against dephosphorylated β-catenin. Antibodies against total β-catenin and Actin were used as loading controls.

The data obtained till now strongly suggest that the regulatory role of CD44 in the Wnt/β-catenin pathway is highly dependent on its intracellular domain and most probably mediated by its binding to ERM-proteins. Thus, further experiments were performed to address a specific role of ERM proteins in the Wnt-pathway.

3.3.3 ERM-linkage to F-actin and Ezrin are crucial for Wnt/β-catenin signaling

In order to analyze which ERM proteins are expressed in HEK293 cells whole cell lysates of HEK293 cells where subjected to Western-blot analysis using antibodies against Ezrin, Radixin or Moesin. The results of this experiment revealed that Ezrin is the predominant ERM member in HEK293 cells, as shown by a strong band in the Western-blot, compared to Radixin and Moesin that are only expressed in low amounts (Fig. 10 A). Thus, in the next experiments the specific requirement of Ezrin for canonical Wnt-signaling was analyzed. For this, TOPFlash assays were performed on HEK293 cells, transfected either with control-siRNA or with siRNA against Ezrin. The knockdown of Ezrin strongly suppressed the activation of the TOPFlash reporter by Wnt3a (Fig. 10 B), supporting an important function of Ezrin for Wnt-pathway activation. The knockdown of Ezrin was confirmed by Western-blot analysis.

As Ezrin links transmembrane or membrane associated proteins to the actin-cytoskeleton I tested whether interference with this function affects activation of Wnt/β-catenin signaling. To this aim, HEK293 cells were transfected with the TOPFlash reporter construct and a dominant-negative Ezrin mutant where the F-actin binding site was deleted (DN-Ezrin) (Algrain et al., 1993). This mutant binds to the transmembrane partners of ERMs but is no longer able to link them to the actin-cytoskeleton, thus acting as competitive inhibitor against endogenous Ezrin proteins. Ectopic expression of this mutant strongly suppressed TOPFlash activation in HEK293 upon treatment with Wnt3a-CM (Fig. 10 C). This indicates that like the binding of CD44 to ERM proteins, also the binding of ERM proteins to the actin-cytoskeleton is required for a proper activation of the Wnt-pathway.

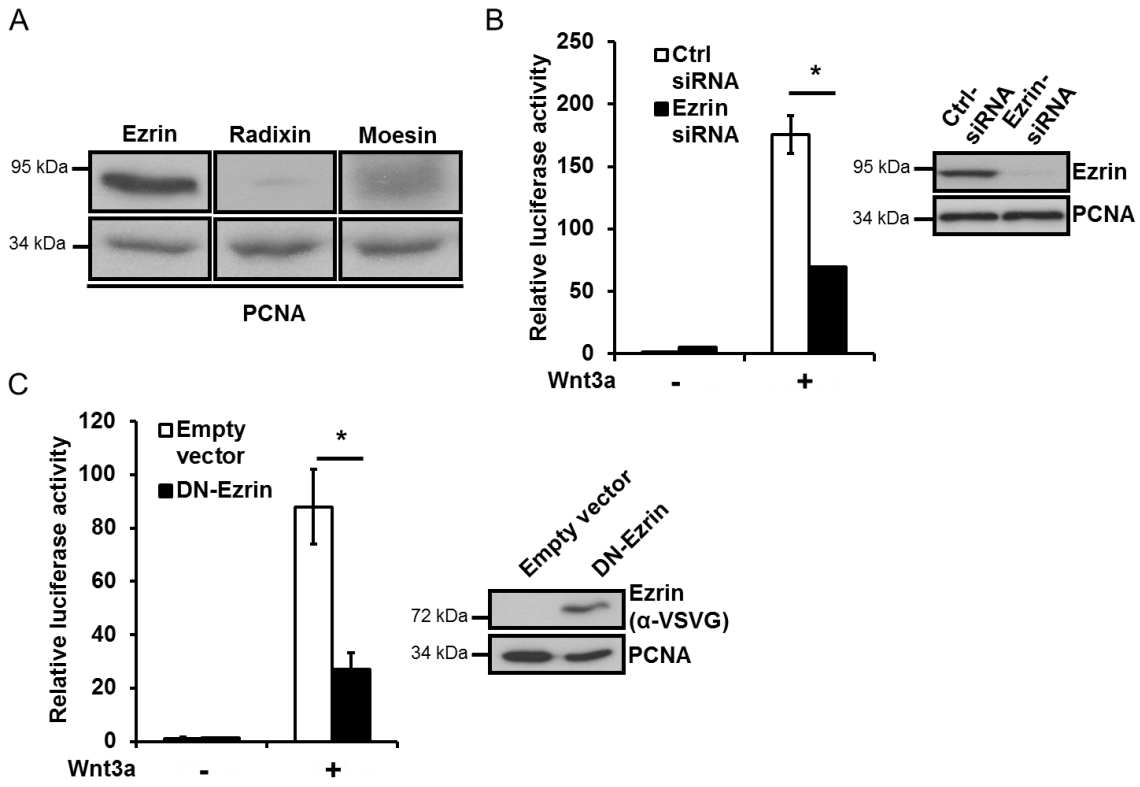

Fig. 10 Ezrin and the linkage of ERM-proteins to F-actin are crucial for Wnt/β-catenin signaling.
A) HEK293 whole cell lysates were subjected to Western-blot analysis in order to detect expression levels of Ezrin, Radixin and Moesin. B) HEK293 cells transfected with control-siRNA or siRNA against Ezrin were transfected with TOPFlash and TK-Renilla vectors. Cells were treated either with control- or Wnt3a-CM for 20 hrs and analyzed for luciferase activity 40 hrs after transfection. C) HEK293 cells were transfected with TOPFlash and TK-Renilla vectors together with empty vector or a dominant-negative VSVG-tagged mutant of Ezrin (DN-Ezrin) that is unable to bind to F-actin. Cells were treated either with control- or Wnt3a-CM for 20 hrs and analyzed for luciferase activity. Data represent an average from 3 independent experiments performed in triplicates with the indicated standard deviations (*p< 0.05, statistical significance was analyzed using the student´s *t*-test). Lysates were in parallel subjected to Western-blot analysis with antibodies against the VSVG-tag to confirm expression of DN-Ezrin and antibodies against Ezrin, to confirm reduced Ezrin protein levels upon Ezrin-siRNA transfection.

Taken together, both, downregulation of Ezrin expression as well as inhibition of the binding of ERM proteins to the actin-cytoskeleton significantly suppressed the ability of Wnt3a to activate TCF/LEF regulated transcription. In combination with the finding that inhibition of CD44 binding to ERM proteins has the same negative effect on Wnt-activation, these data suggest that the linkage of CD44 to the actin-cytoskeleton via Ezrin is decisive for the mechanism of CD44 mediated modulation of the Wnt/β-catenin pathway.

3.3.4 The effect of CD44 on the Wnt signaling pathway is RTK independent

CD44 proteins are well known co-regulators of RTK-signaling (reviewed in Orian-Rousseau and Ponta, 2008). Two prominent pathways activated via RTKs are the mitogen-activated protein kinase pathway (MAPK-pathway), also known as MEK/Erk-pathway and the PI3K/Akt-signaling cascade (reviewed in Grant et al., 2002; McKay and Morrison, 2007). Both pathways were shown to be able to activate β-catenin signaling in the absence of Wnts, on the one hand, by stimulating phosphorylation of LRP6 (MEK/Erk) and on the other hand, by triggering stabilization and nuclear translocation of β-catenin (PI3K/Akt) (Krejci et al., 2012; Misra et al., 2008). Thus, it is possible that the regulatory function of CD44 in the Wnt-pathway is indirect and mediated by its function as a co-regulator of RTK signaling. In order to rule this out, the MEK/Erk- and PI3K/Akt pathways were blocked with specific chemical inhibitors and the consequences on the augmentation of Wnt-signaling by CD44 were analyzed.

To address the MEK/Erk-pathway, HEK293 cells were transfected with the TOPFlash reporter together with a control empty vector or hCD44s-cDNA and treated either with Control-CM or Wnt3a-CM in the absence or presence of the specific MEK-inhibitor U0126 (Favata et al., 1998).

Although, the inhibition of MEK/Erk-signaling decreased the activation of TCF/LEF regulated transcription by Wnt3a, the amplification of Wnt-signaling by ectopic CD44 expression remained unchanged (Fig. 11 A). The inhibition of MEK/Erk-signaling by U0126 was confirmed by Western-blot analysis using Erk-phosphorylation as read-out (Data not shown). These results indicate that CD44 regulates Wnt-signaling independent of RTK signaling via the MEK/Erk-pathway.

Also a treatment with the PI3K-inhibitor Ly294002 (Vlahos et al., 1994) failed to counteract the augmentation of Wnt-signaling by CD44. Like for MEK/Erk-inhibition, the blocking of PI3K/Akt-signaling affected Wnt3a induced activation of the TOPFlash reporter, however the fold elevation of Wnt-signaling by CD44 remained the same (Fig. 11 B). As the antibodies against activated phosphorylated Akt, did not work in our hands the inhibition of PI3K/Akt-signaling by LY294002 treatment could not be confirmed by Western-blot analysis. However, LY294002 lead to a reduction in Wnt-pathway activation as already described (Tomita et al., 2006), indicating that the inhibitor is indeed functional.

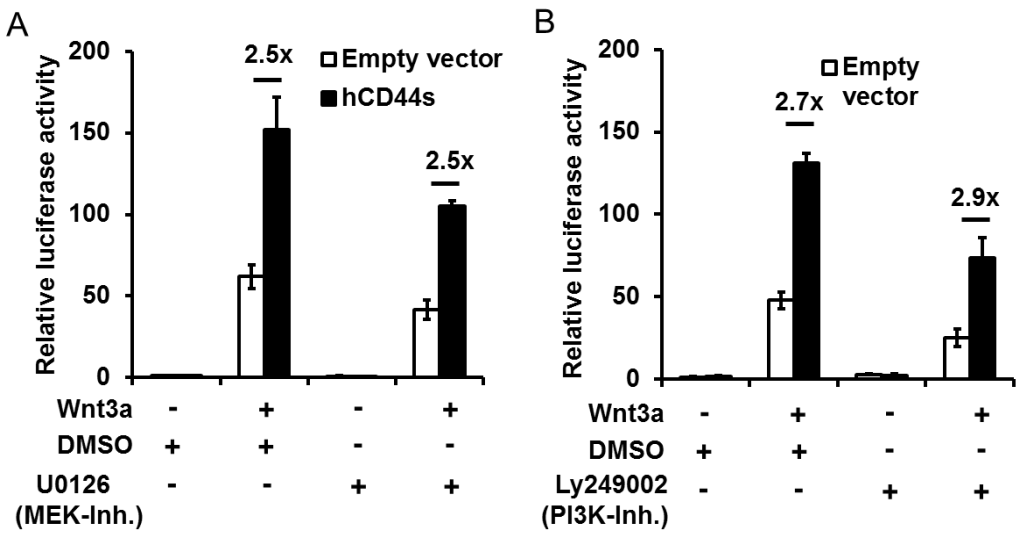

Fig. 11 CD44 regulates Wnt/β-catenin signaling independent of MEK/Erk or PI3K/Akt-signaling.
A) HEK293 cells were transfected with TOPFlash or TK-Renilla vectors together with empty vector or hCD44s. Cells were treated either with DMSO or the MEK-Inhibitor U0126 (15 µm) 1h prior to the addition of control- or Wnt3a-CM. 20 hrs after Wnt-induction cells were analyzed for luciferase activity. B) HEK293 cells were transfected similar to A), treated either with DMSO or the PI3K-Inhibitor Ly294002 (50 µm) 1h prior to the addition of control- or Wnt3a-CM. 20 hrs after Wnt-induction cells were analyzed for luciferase activity. Numbers indicate fold amplification of reporter activity by CD44. Data represent an average from 4 independent experiments performed in triplicates with the indicated standard deviations (*p< 0.05, statistical significance was analyzed using the student´s *t*-test).

As neither inhibition of MEK/Erk-signaling nor the blocking of PI3K/Akt-signaling showed any effect on the enhancement of Wnt-signaling by CD44, it is unlikely that CD44-mediated regulation of the Wnt-pathway is dependent on its function in RTK signaling.

So far, it was shown that CD44 indeed is required for Wnt-signaling and even able to enhance the activation of Wnt3a induced TCF/LEF target gene transcription. Furthermore, this is most probably depending on the linkage of CD44 to the actin-cytoskeleton via Ezrin. There is strong evidence that the interaction between CD44 and its major ligand HA has no implication in the regulation of Wnt-signaling by CD44 and in addition, the function of CD44 in the Wnt-pathway seems also to be completely independent of its role as co-factor for RTK-signaling. However, the exact step controlled by CD44 was to this point still not identified.

3.3.5 CD44 acts at the level of the Wnt receptors

The canonical Wnt-pathway is a cascade of several molecular events, starting from the binding of Wnt-ligands to their receptors Fz and LRP5/6. Subsequently, LRP5/6 gets activated by phosphorylation through kinases like GSK3-β or CK1-γ. These phosphorylated sequences are docking sites for Axin, a scaffold protein of the cytoplasmic β-catenin degradation complex. Upon LRP6 phosphorylation, Axin is recruited to LRP6 at the membrane, leading to an inhibition of the β-catenin degradation complex. In addition to this, the reception of Wnts by Fz and LRP5/6 activates Dsh, a small phosphoprotein that acts downstream of Fz. The activation of Dsh inhibits another protein of the β-catenin degradation complex, the kinase GSK3. The inhibition of GSK3 prevents the phosphorylation of β-catenin within the degradation complex. The inactivation of the β-catenin destruction complex inhibits the proteasomal degradation of β-catenin, which results in an increase of free cytosolic β-catenin. Subsequently, β-catenin enters the nucleus, binds to TCF/LEF transcription factors and activates Wnt-target gene transcription (reviewed in Logan and Nusse, 2004). Experimentally, the Wnt/β-catenin signaling pathway can be activated at consecutive steps of the cascade. At the level of the ligands, the Wnt-cascade can be induced by treatment of cells with medium containing Wnt3a. Overexpression of LRP6 activates the Wnt-pathway at the receptor level (Tamai et al., 2000). Intracellularly and biochemically downstream of LRP6, Wnt-signaling can be activated by overexpression of Dsh (Wehrli et al., 2000) and even further downstream by ectopic expression of β-catenin (Aberle et al., 1997). In order to pinpoint the step at which CD44 plays a role in the Wnt-cascade, the Wnt-pathway was epistatically activated as described above, in the context of CD44 loss- or gain of function. The activation of the TOPFlash reporter was used as a read-out. These so-called “epistasis” experiments should unravel which steps of the Wnt-pathway are modulated by CD44 and conversely at which steps an overexpression or a knockdown of CD44 shows no effect on the Wnt-cascade.

In order to analyze the effects of CD44 loss of function, HEK293 cells were transfected either with control-siRNA or siRNA targeting all CD44 isoforms. These cells were additionally transfected with the TOPFlash reporter construct. The Wnt-pathway was activated either by treatment with Wnt3a-CM or by co-transfection of LRP6, Dsh or β-catenin. Subsequently, the cells were analyzed for TOPFlash activation. As expected and already shown before, the downregulation of CD44 expression reduced activation of the TOPFlash reporter when induced by Wnt3a-CM (Fig. 12 A). Like for Wnt3a, the activation by LRP6 was decreased upon downregulation of CD44. However, whenever the Wnt-pathway was activated downstream of LRP6 for example by Dsh or β-catenin, silencing of CD44

expression had no effect. In parallel to the TOPFlash assay, the cell lysates were analyzed by Western-blot analysis in order to prove the reduced expression of CD44.

In concordance with the loss of function experiments, ectopic expression of CD44 in the same experimental set up enhanced the TOPFlash reporter activation when activated by treatment with Wnt3a-CM or by overexpression of LRP6, whereas no CD44-mediated increase of TOPFlash activity was seen upon activation by overexpression of Dsh or β-catenin (Fig. 12 B). The expression of the transfected rCD44 was confirmed by Western-blot analysis.

Altogether, these results suggest that CD44 regulates Wnt-signaling at the membrane level as both, loss and gain of function of CD44, had strong influence on Wnt-signaling when activated by the extracellular Wnt-ligand Wnt3a or by ectopic expression of the Wnt-receptor LRP6. However, silencing or overexpression of CD44 had no effect on TCF/LEF activation when activated by intracellular Wnt-transducers.

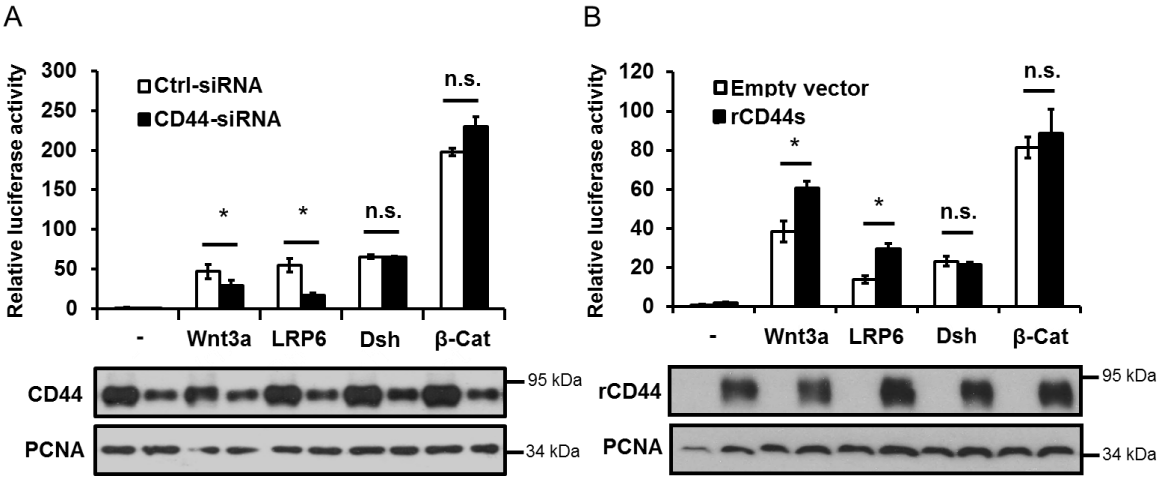

Fig. 12 CD44 regulates Wnt/β-catenin signaling at the membrane level
A) HEK293 cells were transfected with control-siRNA or siRNA against all CD44 isoforms. 20 hrs later, cells were transfected with TOPFlash and control TK-Renilla vectors. Wnt-signaling was activated either by treatment with Wnt3a-CM or by co-transfection of LRP6 (20ng), Dsh (20ng) or constitutive active β-catenin (3ng). 40 h after DNA transfection, cells were analyzed for luciferase activity. B) HEK293 cells were transfected with TOPFlash and TK-Renilla vectors together with empty vector or hCD44s. Wnt-signaling was induced as described in A) 40 hrs after transfection, cells were analyzed for luciferase activity. Data represent an average from 5 independent experiments performed in triplicates with the indicated standard deviations (*p< 0.05; n.s.= not significant, statistical significance was analyzed using the student´s *t*-test). In parallel to the luciferase assays cells were lysed and subjected to Western-blot analysis in order to confirm knockdown or overexpression of CD44.

Since in the previous experiments the binding of CD44 to Ezrin and Ezrin itself have been shown to be crucial for Wnt-signaling, epistasis experiments were performed in the context of Ezrin loss of function to analyze whether downregulation of Ezrin affects Wnt-signaling at the same levels of the Wnt-cascade as CD44. To this aim, HEK293 cells were treated either with control-siRNA or siRNA targeting Ezrin and additionally transfected with the TOPFlash reporter construct. Like in the previous experiments, the Wnt-pathway was activated either by treatment with Wnt3a-CM or by co-transfection of LRP6, Dsh or β-catenin.

Subsequently, the cells were analyzed for TOPFlash activation. Surprisingly, downregulation of Ezrin expression did not only decrease the TOPFlash reporter activation when induced by Wnt3a-CM and ectopic expression of LRP6, but also the activation of the pathway by ectopic expression of Dsh or β-catenin was hampered significantly (Fig. 13). Although, the inhibitory effect of the Ezrin knockdown was more severe at the level of Wnt3a and LRP6 when compared to pathway stimulation by Dsh or β-catenin, these results indicate that Ezrin might have additional functions in the Wnt/β-catenin signaling pathway besides its function in association to CD44.

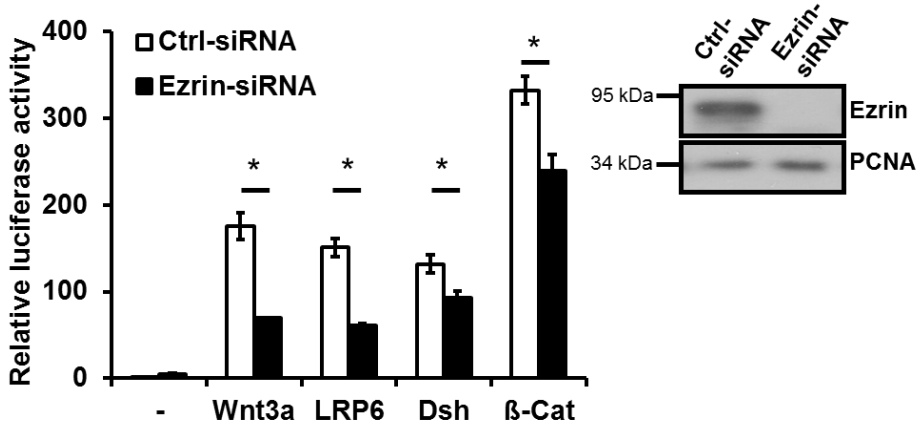

Fig. 13 Ezrin knockdown affects Wnt/β-catenin signaling downstream of LRP6.
HEK293 cells were transfected with control-siRNA or siRNA against Ezrin. 20 hrs later, cells were transfected with TOPFlash and control TK-Renilla vectors. Wnt-signaling was activated either by treatment with Wnt3a-CM or by co-transfection of LRP6 (20ng), Dsh (20ng) or constitutive active β-catenin (3ng). 40 h after DNA transfection, cells were analyzed for luciferase activity. Data represent an average from 5 independent experiments performed in triplicates with the indicated standard deviations (*p< 0.05, statistical significance was analyzed using the student´s *t*-test). In parallel to the luciferase assay cells were lysed and subjected to Western-blot analysis in order to confirm the knockdown of Ezrin.

The results of the epistasis experiments indicate that CD44 acts at the level of the Wnt-membrane reception. Remarkably, the effects of CD44 loss and gain of function on the Wnt-pathway seem to be more drastic on the level of LRP6 when compared to the effects on Wnt3a-induction (Fig. 13). This finding prompted us to specifically study the role of CD44 on LRP6.

3.3.6 CD44 interacts with LRP6 and is required for LRP6 activation

CD44 was has been shown to interact with another member of the LRP family at the cell surface, LRP1, with implications in tumor cell adhesion (Perrot et al., 2012). Thus, it is possible that CD44 might also form a complex with LRP6. The following experiments addressed whether CD44 and LRP6 could co-precipitate in the presence or absence of Wnt3a. To this end, HeLa cells were treated for different time points with Wnt3a-CM, lysed and subjected to immunoprecipitation using protein G beads coupled to a panCD44 antibody (Hermes-3), recognizing all isoforms of CD44. As a control, IgG coupled protein G beads were used for the immunoprecipitation. The immunoprecipitates were then subjected to Western-blot analysis using antibodies specific for LRP6 in order to test whether LRP6 might have been pulled down together with CD44. The LRP6 and CD44 bands in the Western-blot were quantified using the program ImageJ. In order to estimate the amount of co-immunoprecipitated LRP6, the intensity values of the LRP6 bands in the Western-blot were normalized to the intensity values of the bands of the immunoprecipitated CD44. As shown in lane 1 in Fig. 14, no LRP6 was co-immunoprecipitated with CD44 in cells that were not induced with Wnt3a, demonstrated by the absence of a LRP6 band in the Western-blot (lane 1). However, after five minutes of Wnt3a induction LRP6 was found in complex with CD44 (Fig. 14 lane 2). The highest intensity of the LRP6 band was detected after 30 min of Wnt3a-treatment (Fig. 14 lane 4) and even after 60 min of Wnt-induction LRP6 was still co-immunoprecipitated with CD44 (Fig. 14 lane 5). In the control sample were IgG coupled beads were used for the pulldown, no LRP6 could be detected in the Western-blot (Fig. 14 lane 6), confirming a specificity of the co-immunoprecipitated LRP6 to CD44. A comparison between the immunoprecipitates and the whole cell lysates of HeLa cells confirmed the correct molecular weight of the pulled down CD44 and LRP6 proteins (Fig. 14 lane 7).

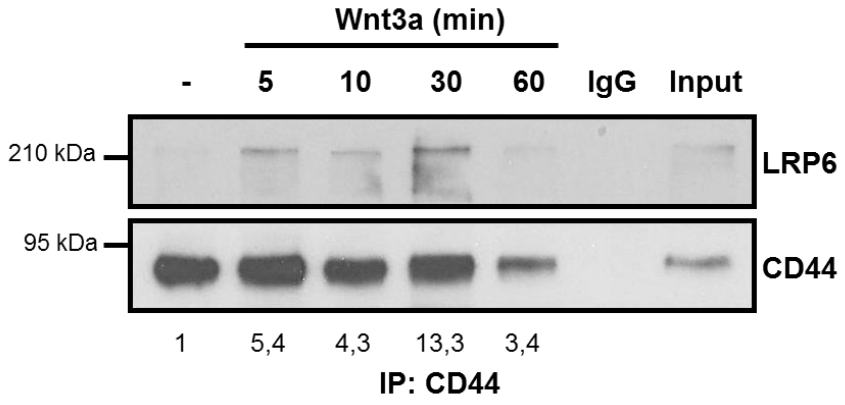

Fig. 14 LRP6 complexes with CD44 upon Wnt3a-induction.
HeLa cells were treated for the indicated timepoints with Wnt3a-CM, lysed and lysates were immunoprecipitated with antibodies against CD44. In order to address interaction between CD44 and LRP6, immunoprecipitates were subjected to Western-blot analyzis and probed for LRP6. Antibodies against CD44s were used to analyze the quantity of CD44 proteins in the immunoprecipitate. Numbers indicates the fold increase of co-immunoprecipitated LRP6 normalized to the immunoprecipitated CD44. Data show one representative out of 4 independent experiments.

The previous finding indicating that CD44 requires its intracellular domain in order to control the Wnt-pathway suggests that the CD44 cytoplasmic domain might be necessary for the interaction between CD44 and LRP6. In order to analyze the dependency of CD44/LRP6 complex formation on the cytoplasmic CD44 domain I tested whether a CD44 mutant that lacks the intracellular domain is still able to interact with LRP6 upon Wnt-induction, as it was the case for the full length CD44. To this aim, HeLa cells were transfected either with the full-length version of rCD44v4-v7 or with a mutant of rCD44v4-v7 lacking the intracellular domain (rCD44v4-v7ΔCyt) together with a flag-tagged version of LRP6. These cells were treated either with control-CM or Wnt3a-CM for 30 minutes, lysed and subjected to immunoprecipitation. In order to immunoprecipitate specifically the ectopically expressed rCD44s, protein G beads were coupled to an antibody against rCD44 that is not recognizing human CD44 proteins. IgG coupled protein G beads were used as a control. Subsequently, the immunoprecipitates were analyzed by Western-blot analysis using an antibody against rCD44 and an antibody recognizing the Flag-tag of the transfected Flag-LRP6. Whole cell lysates of the analyzed HeLa cells were probed in order to confirm the correct molecular weight of the immunoprecipitated proteins.

As shown in Fig. 15 (lane 1 and 2) in uninduced cells no co-immunoprecipated LRP6 was found, neither in cells transfected with full length CD44 nor in cells transfected with the truncated CD44. However, in cells induced with Wnt3a, LRP6 was found in complex with the transfected full-length rCD44 (Fig. 15 lane 3). On the contrary, the LRP6 band was missing in the immunoprecipitates of cells transfected with the mutant rCD44 that lacks the intracellular domain (Fig. 15 lane 4). When IgG coupled beads were used for the pulldown, no LRP6 band was detected, demonstrating the specificity of the co-immunoprecipitated LRP6 to

CD44 (Fig. 15 lane 5). A comparison between the immunoprecipitates and the whole cell lysates of the analyzed HeLa cells confirmed the correct molecular weight of the pulled down rCD44v4-v7, rCD44v4-v7ΔCyt and LRP6 proteins (Fig. 15 lane 6 and 7). Altogether, the result of this co-immunoprecipitation demonstrates that Wnt3a fails to induce a complex between CD44 and LRP6, if the intracellular domain of CD44 is missing. Consequently, CD44 requires its cytoplasmic domain to interact with LRP6.

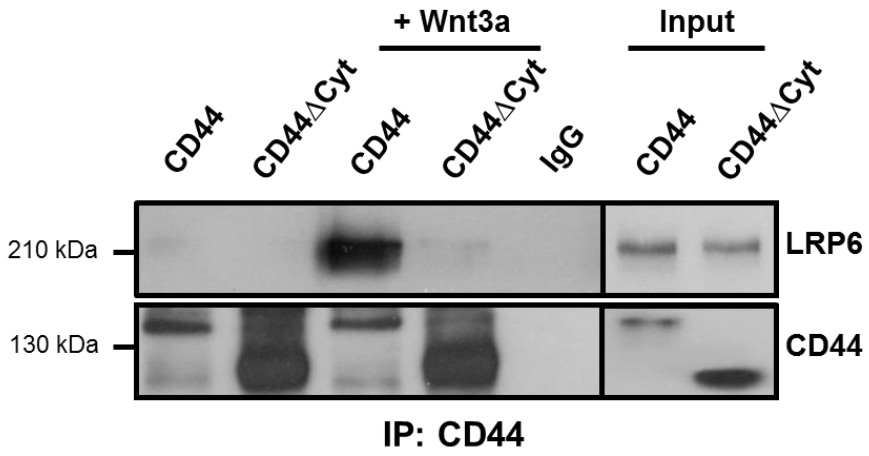

Fig. 15 CD44 requires its cytoplasmic domain to complex with LRP6.
HeLa cells were transfected with rCD44v4-v7 or rCD44v4-v7Δcyt together with flag-tagged LRP6. 24 hrs after transfection cells were treated either with control- or Wnt3a-CM for 30 minutes, lysed and lysates were immunoprecipitated with an antibody against rCD44. In order to address interaction between CD44 and LRP6, immunoprecipitates were subjected to Western-blot analysis and probed for flagged-LRP6. Antibodies against rCD44s were used to analyze the quantity of rCD44 proteins in the immunoprecipitates. Data show one representative out of 4 independent experiments.

The previous results, showing that a loss of CD44 inhibits Wnt-signaling at the receptor level and that CD44 can be found in complex with LRP6 upon Wnt-stimulation, suggest a role of CD44 in the activation of the Wnt-receptor LRP6. One early event in the Wnt-signaling cascade is the phosphorylation of LRP6 by GSK3-β at serine 1490 (S1490), a step that is induced by canonical Wnts (Zeng et al., 2005). In order to test whether CD44 is required for the Wnt induced phosphorylation of LRP6, HeLa cells were transfected with control-siRNA or siRNA against all CD44 isoforms and treated with Wnt3a for 2 hours. Subsequently, the cells were lysed and subjected to Western-blot analysis using an antibody against LRP6 phosphorylated at S1490. In control cells, treatment with Wnt3a-CM increased the levels of phosphorylated LRP6 (Fig. 16). Downregulation of CD44 expression strongly reduced the Wnt-induced phosphorylation of LRP6, showing that CD44 is indeed already required for the phosphorylation of this Wnt-receptor (Fig. 16).

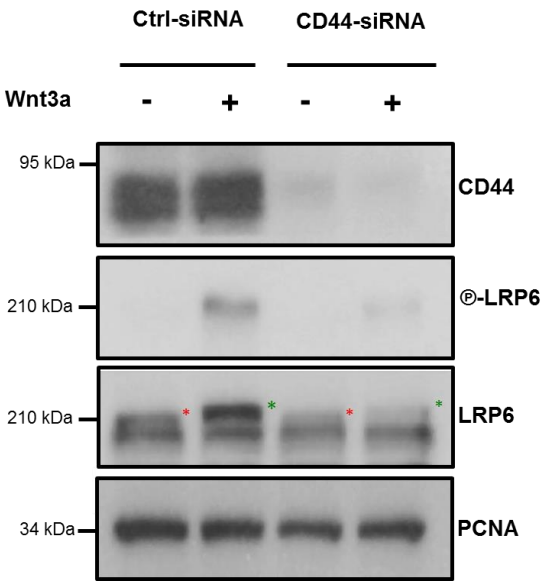

Fig. 16 CD44 is required for Wnt3a-induced phosphorylation of LRP6.
HeLa cells were transfected with control-siRNA or siRNA against all CD44 isoforms and treated either with control- or Wnt3a-CM for 2 hrs. Cells were subjected to Western-blot analysis, 48 h after siRNA transfection and antibodies against phosphorylated LRP6 (ser1490) were used to assess LRP6-activation. Antibodies against CD44 were used to confirm the knockdown of CD44. Antibodies against total LRP6 (T1479) and PCNA were used as loading controls. Data show 1 representative experiment out of 4 independent experiments.

LRP6 proteins are usually detected as two bands with different molecular weights in SDS-PAGE/Western-blot. The lower and faster migrating LRP6 band is the immature and ER located form of LRP6. The upper and slower migrating band represents the mature form of LRP6 at the plasma membrane. The slower migration of the mature LRP6 in the SDS-PAGE is due to multiple glycosylations of LRP6 that take place during the maturation process and that highly increase its molecular weight (Hsieh et al., 2003). Upon induction with Wnt3a, the upper band is normally shifted up due to a higher phosphorylation status of the membrane LRP6 (Davidson et al., 2005). However, this Wnt3a-induced up-shift of the LRP6 protein band was not seen upon knockdown of CD44 (compare green asterisks in tot. LRP6 panel of Fig. 16). Most strikingly, the silencing of CD44 expression led to a reduction of the upper band of endogenous LRP6 in stimulated as well as in unstimulated cells, indicating that CD44 loss of function might reduce the amount of membrane located, mature LRP6 and therefore be necessary for the membrane localization of LRP6.

3.3.7 CD44 is required for the membrane localization of LRP6

In order to visualize a potential effect of CD44 on LRP6 membrane localization, CD44 expression was downregulated in HeLa cells by means of siRNA and the localization of transfected fluorescence labelled LRP6-GFP was analyzed by fluorescence microscopy. Control-siRNA transfected cells were used as controls. The percentage of cells with membrane located LRP6 was quantified by dividing the number of cells with membrane located LRP6 by the total number of analyzed cells. The experiment was performed four times and the mean percentage of cells with membrane located LRP6 as well as the standard deviation was calculated. The knockdown of CD44 was confirmed by Western-blot analysis. As shown in Fig. 17, in 80 % of the cells transfected with control-siRNA, LRP6-GFP was mainly located at the membrane and only a low amount of LRP6 could be found surrounding the nucleus. However, in cells transfected with CD44-siRNA most of the transfected LRP6-GFP remained in close proximity to the nucleus and less LRP6 was located at the membrane. In that case membrane located LRP6 was found only in 25% of all analyzed cells. Importantly, ectopic expression of rCD44s in cells treated with human CD44-siRNA could restore the membrane localization of LRP6, which was again found mainly at the membrane in 75 % of the cells (Fig. 17).

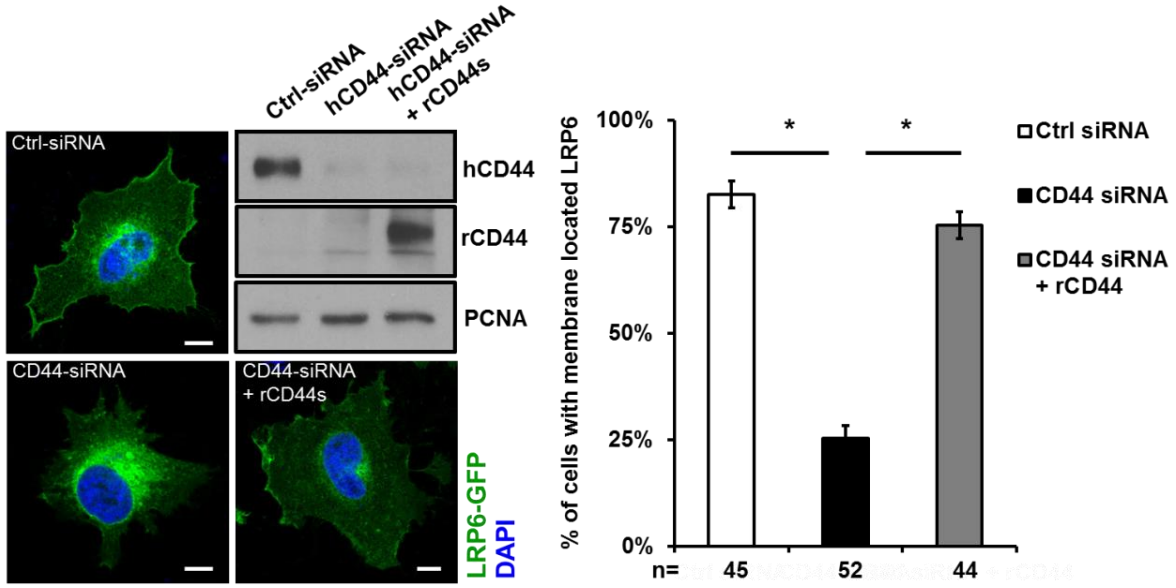

Fig. 17 CD44 is required for cell surface expression of LRP6.
HeLa cells transfected either with control-siRNA or siRNA against human CD44 were transfected with LRP6-GFP, where indicated cells were co-transfected with rCD44s-cDNA. The nucleus was stained with DAPI (blue). Localization of LRP6-GFP (green) was addressed by confocal fluorescence microscopy (scale bar = 10 µm). The percentage of analyzed cells with membrane located LRP6 was quantified (diagram). Data represent mean +/- SD of all confocal microscopy experiments (4 independent experiments), n indicates the total number of analyzed cells. Statistical significance was analyzed using the student´s *t*-test (*p< 0.05). The knockdown of hCD44 and overexpression of rCD44s was confirmed by Western-blot analysis.

These results demonstrate that LRP6 indeed needs CD44 for its cell surface localization. In order to prove whether this phenotype is specific for LRP6, the dependency of the membrane localization of another single pass transmembrane Wnt-receptor, Ror2, on CD44 was analyzed. HeLa cells were transfected in a first step with unspecific control-siRNA or with siRNA against CD44 and in a second step with Ror2-mCherry. The knockdown of CD44 expression was confirmed by Western-blot analysis. Like for LRP6, four independent experiments were performed and the mean percentage of cells with membrane located Ror2 was quantified. In contrast to LRP6, in the context of CD44 loss of function, Ror2 was still localized at the cell membrane (Fig. 18), demonstrating that a requirement of CD44 for the cell surface expression of membrane proteins is specific to LRP6.

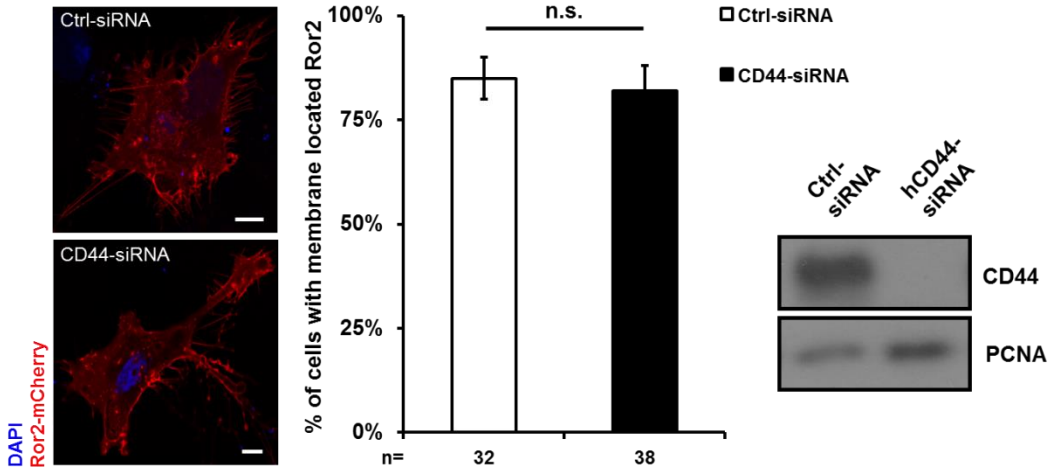

Fig. 18 Membrane expression of Ror2 is independent of CD44.
HeLa cells transfected either with control-siRNA or siRNA against all CD44 isoforms were transfected with Ror2-mCherry, the nucleus was stained with DAPI (blue). Localization of Ror2-mCherry (red) was addressed by confocal fluorescence microscopy (scale bar = 10 µm). Percentage of analyzed cells with membrane located Ror2-mCherry was quantified. Data represent mean +/- SD of all confocal fluorescence microscopy experiments (at least 4 independent experiments), n indicates the total number of analyzed cells. Statistical significance was analyzed using the student´s *t*-test (n.s.= not significant). The knockdown of CD44 was confirmed by Western-blot analysis.

Without CD44, LRP6 fails to reach the plasma membrane. As CD44 requires its intracellular domain for its interaction with LRP6, the cytoplasmic domain of CD44 might also be required for an appropriate membrane localization of LRP6. In order to analyze this, HeLa cells were transfected with LRP6-GFP together with the dominant-negative cytoplasmic CD44 domain (CD44-Cyt) that presumably competes with endogenous CD44 and blocks any interaction to intracellular proteins. 24 hrs after transfection, the cells were analyzed by confocal fluorescence microscopy and the percentage of cells with membrane located LRP6 was evaluated. In line with the results obtained with the knockdown of CD44, interference with the binding of proteins to the cytoplasmic domain of CD44 significantly decreased the

amount of cells with membrane located LRP6. In 75% of the empty vector transfected cells, LRP6 was found mainly at the membrane. In contrast to this, LRP6 appeared at the membrane in only 30% of cells transfected with the dominant-negative cytoplasmic CD44 domain (Fig.19).

This result indicates that the membrane localization of LRP6 is mediated via the intracellular domain of CD44. As previously shown, mutation of the Ezrin binding domain in the ectopically expressed cytoplasmic CD44 domain significantly reduced its ability to block activation of Wnt-signaling at the level of TCF/LEF regulated transcription and activation of β-catenin (Fig. 9 A and B), indicating that the interaction between CD44 and Ezrin is pivotal for the function of CD44 in the Wnt-pathway. Hence, I analyzed whether the mutation of the Ezrin binding domain also affects the ability of the dominant-negative CD44 cytoplasmic domain to suppress cell surface expression of LRP6. Fluorescence microscopy analysis of cells co-transfected with the cytoplasmic CD44 domain containing mutated Ezrin binding sites (CD44-CytΔEzBD) together with LRP6-GFP revealed that LRP6-GFP was found at the membrane of more than 60% of analyzed cells (Fig. 19). Thus, the ability to block LRP6 membrane expression was drastically reduced compared to the transfection of the dominant-negative CD44-Cyt that reduced the amount of cells with membrane located LRP6 to 30% (Fig. 19).

This result indicates that LRP6 membrane expression requires Ezrin binding to CD44 and most probably the linkage of CD44 to the actin-cytoskeleton via Ezrin. In order to prove whether indeed functional Ezrin is required for cell surface expression of LRP6, I competed with the binding of endogenous Ezrin to F-actin by ectopic expression of DN-Ezrin. As previously described this DN-Ezrin is able to bind to membrane proteins but not to the actin-cytoskeleton. Confocal fluorescence microspcopy analysis was performed to whether this ectopically expressed DN-Ezrin represses LRP6 membrane localization. As shown in Fig. 19, LRP6-GFP was found at the membrane of only 25% of cells that were co-transfected with DN-Ezrin and LRP6-GFP, showing that linkage of membrane proteins to F-actin via Ezrin is required for the cell surface expression of LRP6. Together with the result showing that upon interference with the binding of CD44 to Ezrin LRP6-GFP was also not found at the membrane, these findings indicate that the linkage of CD44 to the actin-cytoskeleton is essential for the proper membrane localization of the Wnt co-receptor LRP6.

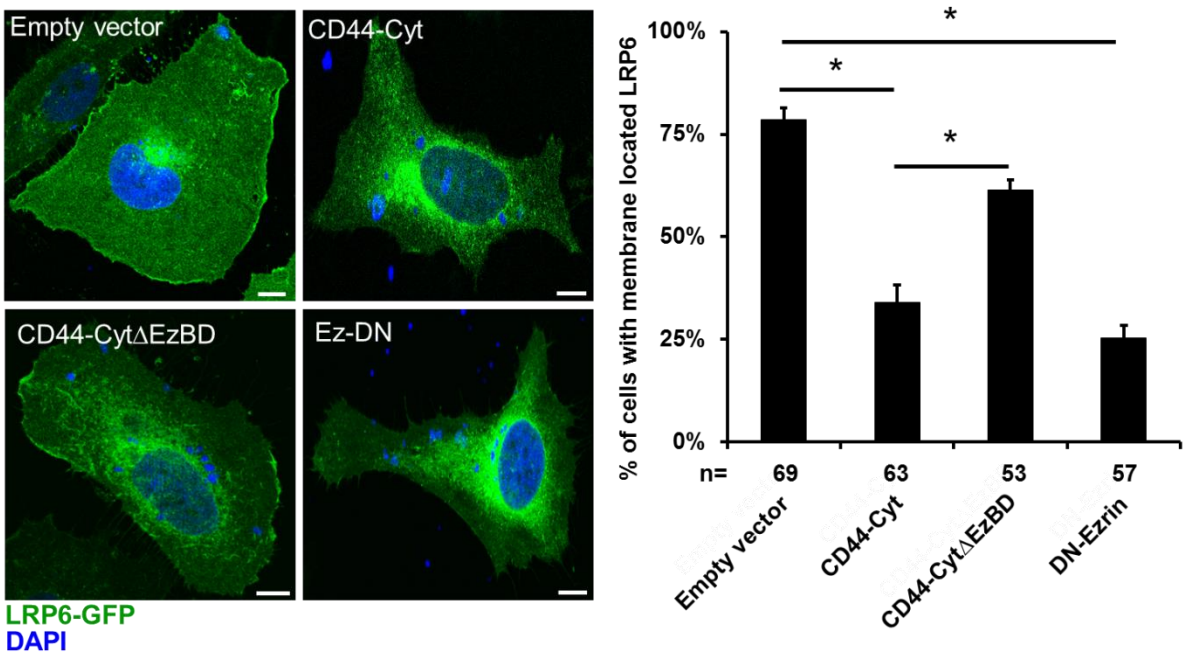

Fig. 19 The linkage of CD44 to F-actin via Ezrin is important for LRP6 cell surface localization.
HeLa cells were transfected with LRP6-GFP together with an empty vector or plasmids encoding the cytoplasmic domain of CD44 (CD44-Cyt), CD44-Cyt mutated in the Ezrin binding sites (CD44-CytΔEzBD), or Ezrin with a deleted actin binding site (DN-Ezrin). Localization of LRP6-GFP (green) was addressed by confocal fluorescence microscopy (scale bar = 10 µm). The nucleus was stained with DAPI (blue). Percentage of analyzed cells with membrane located LRP6 was quantified. Data represent mean +/- SD of all experiments (4 independent experiments), n indicates the total number of analyzed cells. Statistical significance was analyzed using the student´s *t*-test (*p< 0.05).

So far my experiments show that the downregulation of CD44 expression induces a mislocalization of LRP6 in the perinuclear compartment where the endoplasmatic reticulum (ER) is located. The next question is whether LRP6 is really retained in the ER or in other cellular compartments. To test this, control- or CD44-siRNA treated cells, transfected with LRP6-GFP were counterstained with an ER marker (ER-TrackerTM Red). Subsequently, the cells were analyzed by fluorescence microscopy, in order to address whether the LRP6-GFP colocalizes with this ER marker. A colocalization of LRP6 with the ER marker is indicated as yellow staining in the fluorescence microscopy pictures. The percentage of cells with membrane located LRP6 was quantified by dividing the number of cells with membrane located LRP6 by the total number of analyzed cells. In addition the number of cells with ER located LRP6 was quantified and divided by the total number of cells in order to obtain the percentage of cells with ER located LRP6.

Indeed, in cells treated with CD44-siRNA the transfected LRP6-GFP mainly colocalized with the ER marker (65% of analyzed cells) and only in a small amount of transfected cells LRP6 was found at the membrane (26% of analyzed cells) (Fig. 20, upper panel). In contrast to this, in cells transfected with control-siRNA the LRP6-GFP was

expressed at the cell membrane of 73% of transfected cells and only in 15% of cells LRP6 was located presumably in the ER (Fig. 20 upper panel).

Like all type I transmembrane proteins, LRP6 harbors a signal sequence at its N-terminus that targets it to the ER. The ER located chaperone Mesd (mesoderm development candidate 2) takes part in the folding and maturation of LRP6. Consequently, in the absence of Mesd, LRP6 is retained in the ER and fails to reach the cell membrane (Hsieh et al., 2003; Koduri and Blacklow, 2007). Moreover, co-expression of Mesd enhances surface expression of epitope-tagged LRP6 (Li et al., 2006). The following experiment addressed whether it is the processing or maturation of LRP6 that is affected by CD44 loss of function, or rather the transport of the mature LRP6 to the membrane.

To this aim, HEK293 cells were transfected with control- or CD44-siRNA and additionally transfected with LRP6-GFP and Mesd, in order to find whether Mesd can restore the cell surface expression of LRP6 in a CD44 loss of function background. The cells were analyzed by confocal fluorescence microscopy and the percentage of cells with membrane located LRP6 was quantified. In addition the percentage of cells with ER located LRP6 was evaluated. The amounts of Mesd transfected cells with membrane or ER located LRP6-GFP where then compared to the values of cells that were not additionally transfected with Mesd.

The percentage of control-siRNA transfected cells with membrane located LRP6-GFP was not increased by ectopic expression of Mesd (Fig. 20 left panel). However, the intensity of the LRP6-GFP staining at the membrane of Mesd transfected cells was higher when compared to control-siRNA treated cells that were transfected with LRP6 alone. This result shows that Mesd increases the amount of LRP6 at the membrane. In CD44-siRNA transfected cells, ectopic expression of Mesd drastically and significantly reduced the percentage of cells with ER located LRP6 (24% of analyzed cells) compared to cells that were transfected with CD44-siRNA alone (65% of analyzed cells). Remarkably, the ectopic expression of Mesd in CD44-siRNA transfected cells did not increase the percentage of cells with membrane located LRP6 although LRP6 was not retained anymore in the ER. In these cells LRP6 was rather accumulating in the cytoplasm (Fig. 20 right panel).

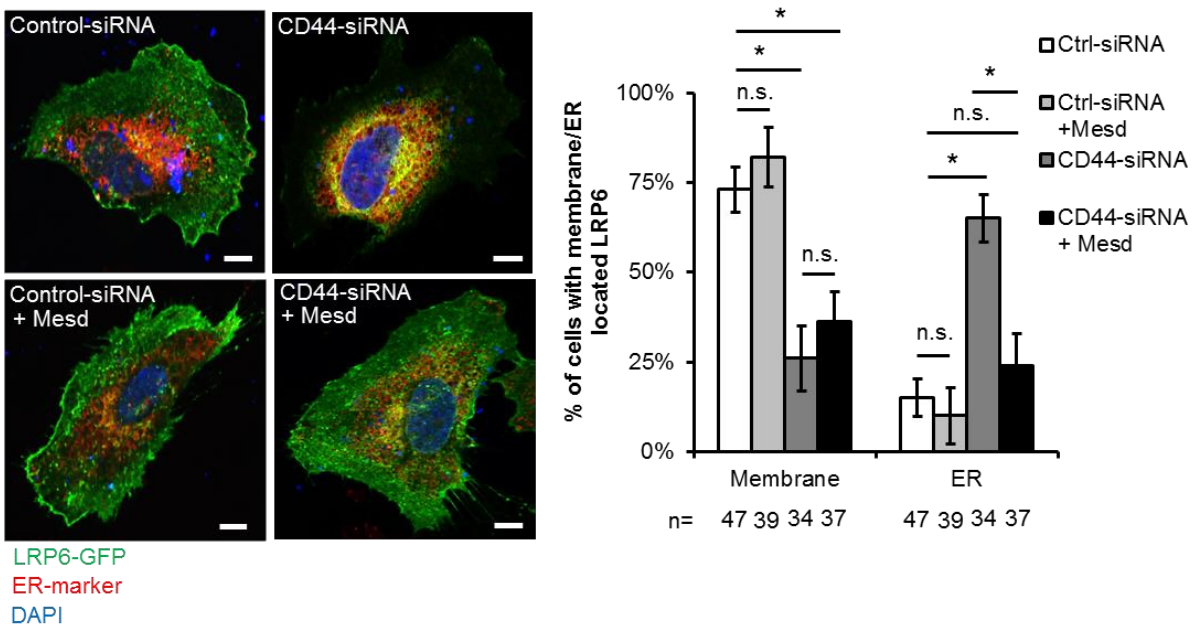

Fig. 20 Mesd can counteract CD44 knockdown mediated maintenance of LRP6 in the ER but not restore LRP6 cell surface expression.
HeLa cells transfected either with control-siRNA or siRNA against all CD44 isoforms were additionally transfected with LRP6-GFP (green) and counterstained with an ER-marker (red). The nucleus was stained with DAPI (blue).Where indicated cells were co-transfected with Mesd-cDNA. Localization of LRP6-GFP was addressed by confocal fluorescence microscopy (scale bar = 10 µm). Data represent mean +/- SD of all experiments (4 independent experiments), n indicates the total number of analyzed cells. Statistical significance was analyzed using the student´s *t*-test (*p< 0.05; n.s.=not significant).

Altogether, these results demonstrate that in the absence of CD44, LRP6 is not expressed at the cell surface and is restricted to the ER. Most strikingly, even though ectopic expression of Mesd triggers the release of LRP6 from the ER, LRP6 is still not able to reach the cell membrane in the absence of CD44. These findings suggest that CD44 might play a crucial role for the targeting of the matured LRP6 to the membrane. Wnt-induced LRP6 phosphorylation is a prerequisite for the activation of the Wnt/β-catenin signaling cascade. As this phosphorylation takes place at the plasma membrane (reviewed in Niehrs and Shen, 2010), the requirement of CD44 for LRP6 cell surface expression might explain the negative effect of the CD44 knockdown on LRP6-phosphorylation, β-catenin activation and in turn the inhibition of TCF/LEF regulated transcription.

The physiological relevance of the collaboration between CD44 and the Wnt siganlosome was tested in vivo as described in the last part of my PhD.

3.4 CD44 is required for Wnt-target gene expression and CNS patterning in *Xenopus laevis* embryos

In order to confirm the requirement of CD44 for Wnt/β-catenin signaling *in vivo*, the *X. laevis*) model, a well-established animal model to study the Wnt-signaling pathway (Moon, 1993) was used. Indeed, CD44 is expressed in *X. laevis* (Ori et al., 2006). Sequence analysis revealed that the xenopus sequence contains all important CD44 domains, including HA-binding sites, the FERM domain, a site for ankyrin binding and the PDZ-peptide (Fig. 21 A).

The sequence homology between human and xenopus CD44, indicates that the function of CD44 for the Wnt/β-catenin pathway might be conserved. To prove this, the human, rat and *X. laevis* CD44s (xCD44s) were transfected together with the TOPFlash reporter in HEK293 cells and their ability to enhance Wnt3a induced reporter activation was assessed. Indeed, overexpression of the xCD44s as well as human or rat CD44s increased TCF/LEF regulated transcription significantly, indicating a conserved function of CD44 as Wnt regulator (Fig. 21 B).

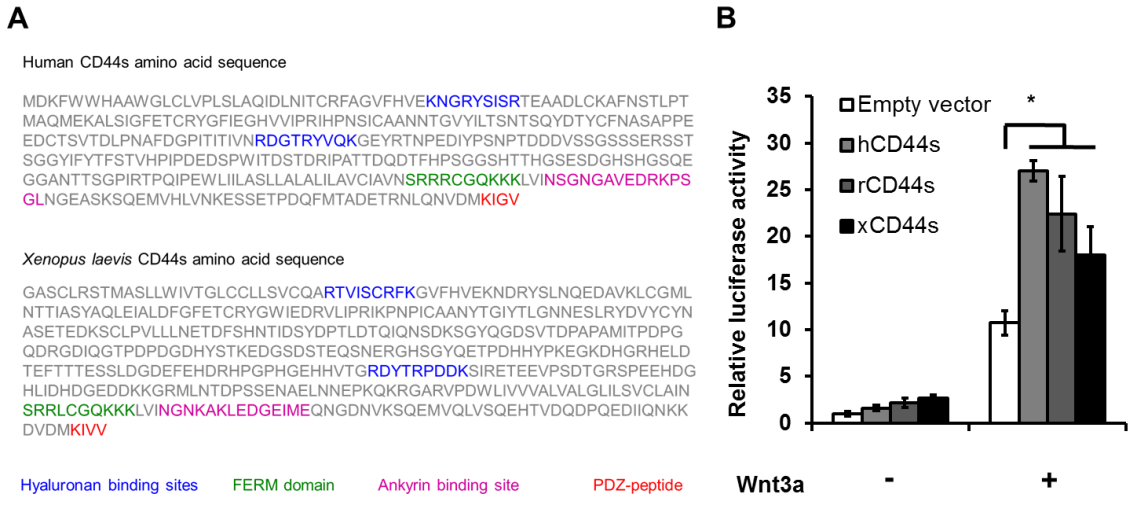

Fig. 21 CD44s sequence and the function in Wnt/β-catenin signaling is conserved across species.
A) Comparison between the protein sequences of human and *Xenopus laevis* CD44s. Conserved functional domains are indicated in colors. B) HEK293 cells were transfected with TOPFlash and control TK-Renilla vectors together with 75 ng of cDNAs for hCD44s, rCD44s or xCD44s. Upon stimulation with Wnt3a-CM or control-CM (20 hrs), cells were lysed and subjected to luciferase measurements 48 hrs after DNA transfection. Data represent mean +/- SD from representative experiments (at least 4 independent experiments) performed in triplicates. Statistical significance was analyzed using the student´s t-test (*p< 0.05).

During *X. laevis* embryogenesis, Wnt-signaling is essentially required for midbrain and isthmus development in the central nervous system (CNS) (reviewed in Wodarz and Nusse, 1998). Here, the activation of Wnt-signaling induces the transcription of the Wnt-target genes *tcf-4* and *engrailed-2* (*en-2*), that can be detected using whole-mount *in situ* hybridization analysis of tailbud stage embryos (stages 28-40) (Konig et al., 2000). As CD44 is also expressed in the developing *X. laevis* brain at this stage (Ori et al., 2006), it was possible to address the physiological relevance of CD44 function in Wnt-signaling by analyzing the effect of CD44 loss of function on the expression of the Wnt-target genes in the developing brain. To this aim, CD44 morpholino antisense oligonucleotides (CD44-MO) were injected into one blastomere of a two-cell stage *X. laevis* embryo, causing a downregulation of CD44 expression in either the left or the right body-half of the embryo. The uninjected half served as control side. Injection of unspecific antisense oligonucleotides where used as control. FITC-Dextran, which can be visualized by fluorescence microscopy, was co-injected as lineage tracer in order to separate right side from left side injected embryos.

In *X. laevis*, CD44 has been shown to be crucial for the development of the ventral musculature (Ori et al., 2006). Indeed, xCD44-MO injected *X. laevis* embryos fail to develop a properly organized ventral musculature, resulting in a phenotype that is easily detectable by light microscopy (see Fig. 22). As antibodies against xCD44 are not available, it was not possible to confirm the reduction of endogenous CD44 protein levels in the morphants. Thus, in order to prove that the morpholino against CD44 really interfered with CD44 function, the MO-injected embryos were fixed at tailbud stages (stages 28-40 (according to Nieuwkoop, 1967)) and analyzed for a disorganized musculature phenotype. Whereas all of the control-MO injected embryos displayed wild type muscle structures, more than 40% of xCD44-MO injected embryos failed to develop a ventral musculature, demonstrated by curvature of the embryos and the lack of muscle tissue (Fig. 22, arrows). However, co-injection of hCD44s-cDNA together with xCD44-MO reduced the number of embryos with musculature phenotypes to only 10% of injected embryos, demonstrating that these defects are indeed specific to the loss of CD44 (Fig. 22).

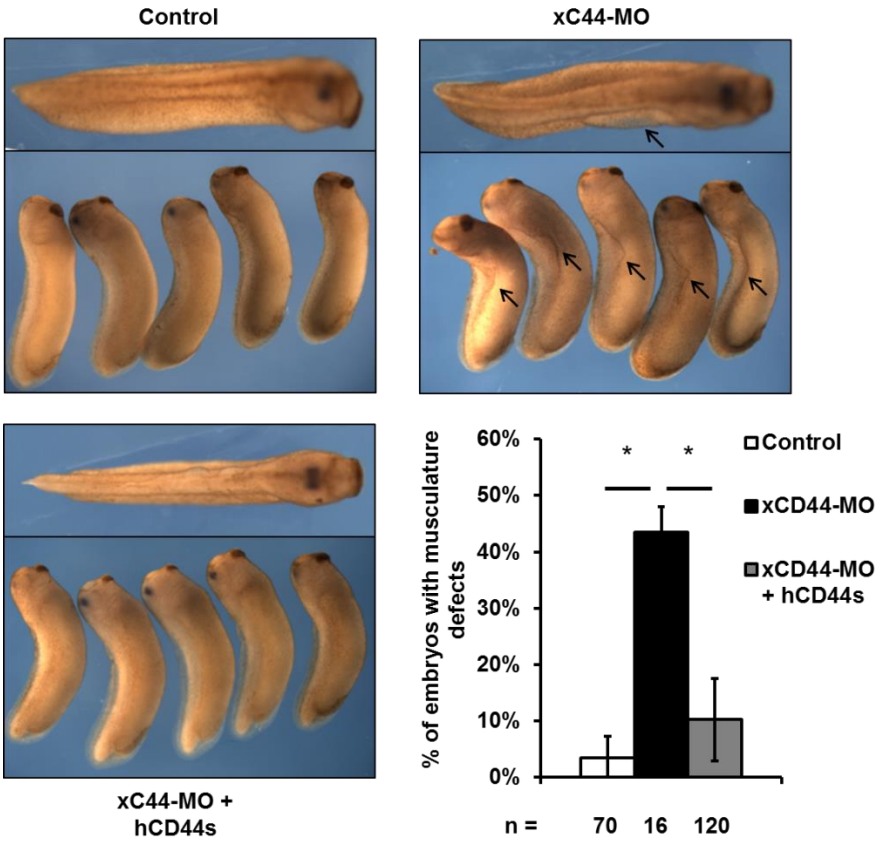

Fig. 22 CD44 knockdown causes musculature defects in Xenopus laevis.
Embryos were injected in the right blastomere at 2-cell stage either with Co-MO, xCD44-MO alone, or xCD44-MO together with hCD44s-cDNA. FITC labeled Dextran was co-injected to assess the side of injection. Embryos were fixed at stage 32 and the percentage of embryos with musculature defects (arrows) was quantified. Data represent mean +/- SD from at least 4 independent experiments. Numbers indicate the amount of analyzed embryos. Statistical significance was analyzed using the student´s *t*-test (*p< 0.05).

Since the CD44 morpholinos indeed work, the consequence of a CD44 knockdown on the expression of the Wnt-target genes *tcf-4* and *en-2* in the developing brain was further analyzed. To this aim, unspecifc control-MO or xCD44-MO mixed with FITC-Dextran were injected in one blastomere of *X. laevis* 2-cell stage embryos. At neurula stage (stages 14-20) the expression of the lineage tracer FITC-Dextran was analyzed by fluorescence microscopy in order to evaluate the side of injection. At tailbud stage (stages 28-40), the embryos were fixed and analyzed by *in situ* hybridization for the expression of *tcf-4* and *en-2*. This analysis revealed that the expression of both target genes was significantly reduced on the injected side in at least 30% of injected embryos (Fig. 23 A and B). Importantly, co-injection of hCD44s partially rescued this phenotype, confirming that the reduced expression of the Wnt-target genes is a result of the knockdown of CD44. In addition, also injection of β-catenin-cDNA rescued this phenotype, showing that downregulated expression of *tcf-4* and *en-2* by CD44-MO is indeed specific to Wnt/β-catenin signaling (Fig. 23 A and B).

In summary, these data provide compelling evidence for the *in vivo* relevance of CD44 function in Wnt-signaling during CNS development. As Wnt-signaling is required for proper patterning of the CNS, CD44 might be a novel regulator of this Wnt-driven CNS patterning. However, a more detailed analysis of the role of CD44 in CNS patterning and development remains to be investigated.

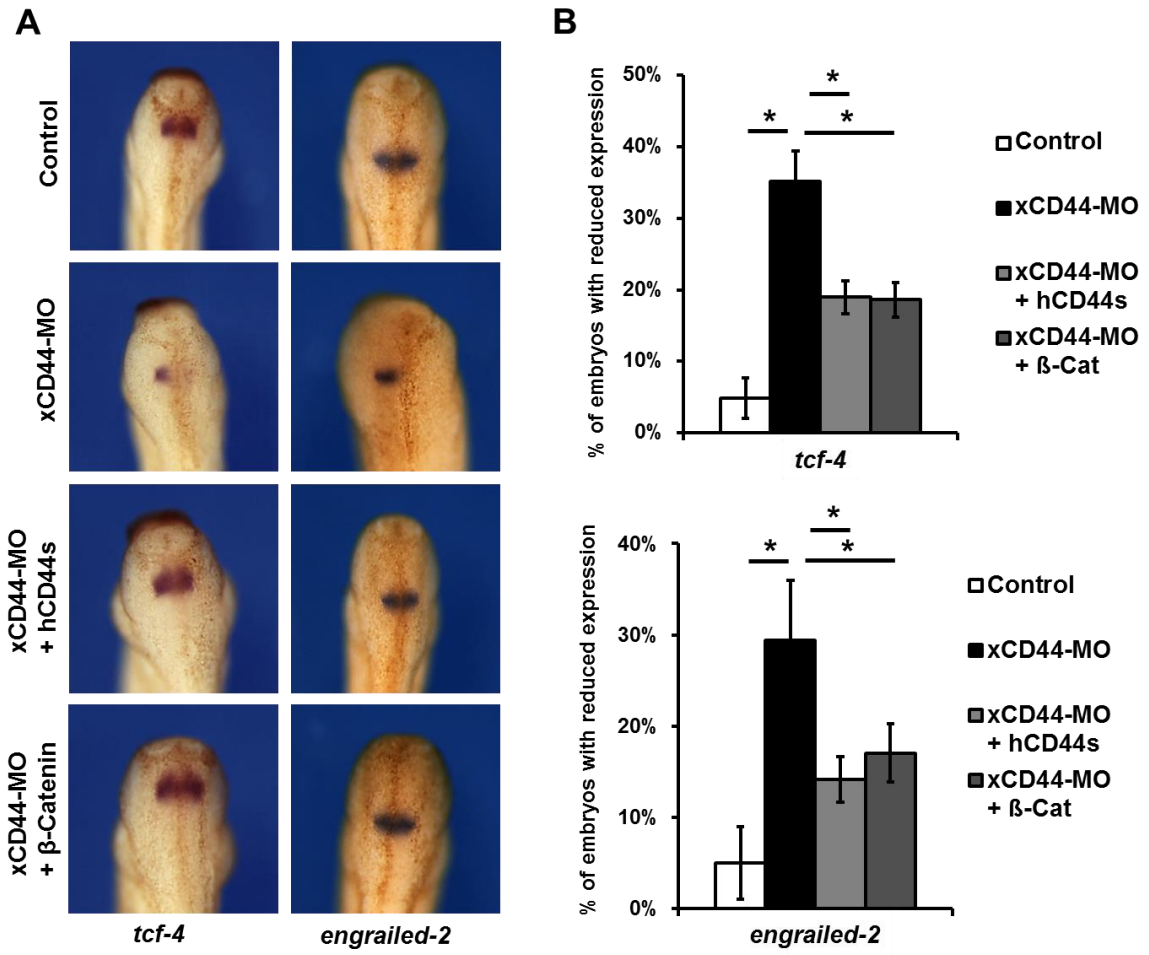

Fig. 23 CD44 is required for Wnt-target gene expression and CNS patterning in Xenopus laevis.
A) *In situ* hybridization for the canonical Wnt-target genes *tcf-4* and *engrailed-2* in 32 stage *X. laevis* embryos. Embryos were injected in the right blastomere at 2-cell stage either with Co-MO, xCD44-MO alone, or xCD44-MO together with hCD44s or β-catenin cDNAs. FITC labeled Dextran was co-injected to assess the side of injection. B) Quantification of experiments described in A) Data represent mean +/- SD from at least 4 independent experiments. Numbers indicate the amount of analyzed embryos. Statistical significance was analyzed using the student´s *t*-test (*p< 0.05).

Altogether, the results of this PhD thesis demonstrate a crucial function of CD44 as an essential positive co-regulator of Wnt/β-catenin signaling. This function was shown to be independent of the binding of CD44 to hyaluronan. Furthermore, the results of this work exclude that CD44 enhances Wnt-signaling indirectly via regulation of RTK signaling via the MEK/Erk- and the PI3K/Akt-pathways. However, CD44 requires its intracellular domain and

the interaction with ERM-proteins like Ezrin in order to modulate the Wnt-pathway. In addition it was shown that ERM proteins, that are able to link membrane proteins to the actin-cytoskeleton, and especially Ezrin are required for proper activation of the Wnt-cascade. Moreover, this study revealed that CD44 acts at the level of the Wnt-receptor LRP6. Indeed, CD44 was found to be in complex with LRP6 and to be necessary for LRP6 activation upon Wnt-induction. Most strikingly, CD44 is indispensable for cell surface expression of LRP6, the location where Wnt-induced LRP6 activation occurs. Remarkably, complex formation of CD44 and LRP6 and also the membrane localization of LRP6 seem to be highly dependent on the cytoplasmic domain of CD44 and most probably on its linkage to the actin-cytoskeleton via Ezrin. Importantly, the pivotal role of CD44 for canonical Wnt-signaling was confirmed *in vivo*, where the knockdown of CD44 expression inhibited the expression of the Wnt-target genes *tcf-4* and *en-2*.

4. Discussion

The Wnt/β-catenin signaling pathway controls cell proliferation, -growth, -differentiation and -migration and plays therefore key roles in numerous important developmental processes, like gastrulation, body axis formation, somitogenesis as well as CNS- and limb development (reviewed in Logan and Nusse, 2004). Remarkably, Wnt-β-catenin signaling is not only important during development but is also essential throughout life. Indeed, canonical Wnt-signaling was shown to regulate the homeostasis of bones, the hematopoietic system and tissues like the intestinal epithelium or the skin (Enshell-Seijffers et al., 2010; van Genderen et al., 1994). Consequently, a misregulation of Wnt/β-catenin signaling is connected to developmental defects, malformations, severe degenerative and metabolic diseases and cancer (reviewed in Clevers and Nusse, 2012).

CD44 is a positive feedback-regulator of Wnt/β-catenin signaling

This PhD work identifies the Wnt target gene CD44 as an essential positive feedback-regulator of Wnt/β-catenin signaling. Indeed, in HEK293 and HeLa cell lines, in which CD44 expression has been shown to be regulated by β-catenin activation (Li and Zhou, 2011; Sarkar et al., 2011), a knockdown of CD44 strongly suppressed Wnt3a induced activation and nuclear translocation of β-catenin and TCF/LEF regulated transcription (Fig. 4 A, 5 and 6). Most importantly, a knockdown of CD44 resulted in a complete loss of Wnt-target gene expression in the developing *X. laevis* CNS, demonstrating an essential function of CD44 in vivo (Fig. 23).

As in the case of CD44, the expression of many other components of the Wnt/β-catenin pathway is regulated by canonical Wnt-signaling and over the last decades feedback-regulation has emerged as a key feature of Wnt/β-catenin signaling.

Wnt/β-catenin signaling is tightly regulated by numerous proteins and the complexity of Wnt-signaling regulation is already illustrated by the number of Wnt-ligands and -receptors. Up till now, 19 Wnt-ligands, 10 Wnt-receptors of the Fz-family, 2 LRP coreceptors as well as RTKs like Ror2 and Ryk have been identified to control Wnt-reception at the cell surface (reviewed in Kikuchi et al., 2007). The feedback-regulation is also highly complex and the proteins involved in this feedback-regulation range from Wnt-ligands and -receptors, over intracellular pathway regulators, to downstream transcription factors that mediate Wnt-target gene transcription.

Fz receptors, for example, can be up or downregulated upon Wnt induction and therefore influence Wnt-signalling in positive and negative ways (Cadigan et al., 1998; Muller et al., 1999; Sato et al., 1999; Willert et al., 2002). Also the levels of other Wnt-binding

proteins, such as LRP or HSPG are controlled by Wnt-signaling, providing further fine-tuning of Wnt activity at the cell surface (Baeg et al., 2001; Wehrli et al., 2000). In addition, it was shown that Dkk1, a potent inhibitor of canonical Wnt-signaling, is a target of β-catenin/TCF regulated transcription (Gonzalez-Sancho et al., 2005; Niida et al., 2004). Also cytoplasmic regulators like Axin2 or β-TRCP are targets of the Wnt-pathway (Jho et al., 2002; Lustig et al., 2002; Spiegelman et al., 2000). Axin2, for example, was shown to be upregulated in colorectal adenomas and carcinomas, caused by aberrant Wnt-signaling in human tumors and also in adenomas of $Apc^{MIN/+}$ mice (Lustig et al., 2002). Moreover, LEF1 has been shown to be expressed in colon cancer as a β-catenin target, in order to favor a positive feedback loop for canonical Wnt-signaling (Hovanes et al., 2001).

The discovery that the cell adhesion molecule CD44 participates in this regulation provides further insight in the regulatory network that controls the activity of Wnt-signaling. As the hyperactivation of Wnt-signaling results in uncontrolled cell growth and proliferation, the initial step of cancer formation, a negative feedback-regulation is crucial to inactivate Wnt-signaling once its activation is no longer required. Furthermore, negative feedback might be an important factor to inhibit hyperactivation of Wnt-signaling that is caused by mutation of pathway components. This might for example be the case in colon cancer, where it is thought that the expression of negative feedback-regulators have the function to inhibit Wnt-signaling and reduce the severity of the perturbations that result from a loss of APC (reviewed in Logan and Nusse, 2004). Positive feedback even enhances the activity of the Wnt-signal and stabilizes the activation of the pathway. As mentioned above, this might result in uncontrolled cell proliferation and endanger the whole organism. The question is now: What should be the benefit of a positive feedback-regulation of Wnt/β-catenin signaling by CD44?

CD44 is not the only Wnt-target gene that is able to provide positive feedback to Wnt/β-catenin signaling. Also the expression of other positive regulators like Wnt3a (Zhang et al., 2009), LEF1 (Hovanes et al., 2001), or receptors of the Fz-family have been identified to be upregulated upon activation of canonical Wnt-signaling (Sato et al., 1999; Willert et al., 2002). The fact that several major components of the Wnt-pathway give positive feedback to the Wnt/β-catenin pathway provides evidence that positive feedback-regulation plays a key role in Wnt/β-catenin signaling.

As in the case of Wnt-hyperactivation, also loss of function of the Wnt-pathway is linked to severe human disorders like tetra-amelia (absence of limbs) (Niemann et al., 2004), neural tube defects (Kibar et al., 2007), Alzheimer (De Ferrari et al., 2007), osteoporosis (Mani et al., 2007) and eye vasculature defects (Little et al., 2002; Toomes et al., 2004) that in the worst case can lead to complete blindness (Robitaille et al., 2002). Loss of canonical

Wnts results also in defects in somitogenesis and early gastrulation (reviewed in Logan and Nusse, 2004).

This demonstrates that it is also crucial to prevent Wnt-signaling from hypoactivation or from a complete switch-off. Furthermore, a positive feedback might be needed to accelerate the response to extracellular cues and to increase or stabilize the levels of Wnt-activity if necessary. The question is: where would such a positive-feedback-regulation, especially between CD44 and Wnt/β-catenin signaling make sense?

CD44 and Wnt/β-catenin signaling in development

Positive-feedback loops between CD44 and Wnt-signaling might be required during development. Indeed, there are many events during embryonic development that require high Wnt-activity, mostly as an inducer of cell proliferation. Thus, Wnt-knockout phenotypes can most often be explained by a loss of cell proliferation (reviewed in Logan and Nusse, 2004).

Limb development, for example, is highly dependent on canonical Wnt-signaling as shown by the human disease tetra-amelia. Here, a loss of function mutation in the *wnt3*-gene results in an absence of all for limbs (Niemann et al., 2004). Especially here, a positive feedback via CD44 could play an important role as it was shown that CD44 is necessary for the induction of proliferative signals that are required for the correct outgrowth of the limbs (Sherman et al., 1998). Although the function of CD44 in limb development was connected to its regulation of FGF-signaling, the similar phenotypes between a loss of CD44 and a loss of Wnt-signaling suggest that the CD44/Wnt-interplay might also be implicated in limb development.

Moreover, a positive feedback loop between CD44 and Wnt-signaling might be implicated in somitogenesis. For example, in *X.laevis,* Wnt-signaling is essential for the formation of somites (Aulehla et al., 2003). CD44 is highly expressed in the presomitic mesoderm and later on in the somites. Here, CD44 is also involved in somitogenesis, as shown by musculature defects upon CD44 knockdown (Ori et al., 2006).

Also during CNS development the interplay between CD44 and Wnt-signaling might be important. Wnt-signaling is essential for isthmus development in the central nervous system (CNS) of *X. laevis* (reviewed in Wodarz and Nusse, 1998). A knockdown of xTCF-4 translation results in isthmus malformations, as shown in brain explants, by a reduction of the mesencephalic part of the isthmus (Kunz et al., 2004). In my PhD thesis, I could show that a knockdown of CD44 suppresses the expression of TCF4 in the CNS of *X. laevi*s embryos (Fig. 23), suggesting that CD44 is also involved in isthmus development. However, whether a knockdown of CD44 has the same phenotypes as TCF4-morphants requires further investigation. Interestingly, in the isthmus, *Tcf4* is a target gene and a positive co-regulator of

Wnt-signaling (Kunz et al., 2004), suggesting an involvement of positive feedback-regulation of Wnt-signaling in isthmus development. The fact that the Wnt-target gene CD44 is required for the expression of TCF4, highly indicates a participation in this positive feedback-loop.

CD44 and Wnt/β-catenin signaling in tissue homeostasis and wound repair

Positive feedback-regulation of Wnt/β-catenin signaling might also be crucial for tissue homeostasis. Indeed, high Wnt-activity is not only important during development. There are also many physiological events in adults that require constant moderate or high levels of Wnt-activity. Here, a positive feedback might be required to keep Wnt-signaling in an *ON*-state. In addition to this, positive feedback-loops might be necessary to raise the levels of Wnt-activity during tissue repair, for example upon injury.

In the skin, Wnt/β-catenin signaling is a critical regulator of tissue homeostasis, wound repair and hair regrowth and constant Wnt-signaling activity is detected in the hairfollicle, a region where stem and proliferative progenitor cells reside (reviewed in Reya and Clevers, 2005). Here, a hypoactivation of Wnt/β-catenin signaling causes an inhibition of hair regrowth, as shown by ablation of β-catenin in mice (Enshell-Seijffers et al., 2010). Moreover, loss of Wnt-signaling results in impaired wound repair as demonstrated in *Tcf3/4*-knockout mice (Nguyen et al., 2009). In addition to this important steady-state level of Wnt-signaling that is required for tissue homeostasis, Whyte and colleagues showed that augmentation of Wnt-signaling can even improve wound healing (Whyte et al., 2013). Thus, in the skin a positive feedback for Wnt-signaling might be an important factor to provide constant levels of Wnt-signaling to maintain the tissue under physiological conditions. In the case of injury positive feedback might be necessary to provide high Wnt-signals that are required for efficient wound repair.

CD44 is also expressed in the hair follicles, the regions where the stem and progenitor cells residue and where high Wnt-activity is detected (Yasaka et al., 1995). Furthermore, in combination with expression of ALDH (aldehyde dehydrogenase), CD44 is a predicted marker of epidermal stem cells (Szabo et al., 2013). Interestingly, selective suppression of CD44 expression in keratinocytes causes delayed hair regrowth and impaired wound repair (Kaya et al., 1997; our own unpublished data), similar to the phenotypes that are seen upon loss of Wnt/β-catenin signaling. Together with the results obtained during my PhD thesis that define CD44 as positive feedback-regulator, these findings indicate that positive feedback of CD44 to Wnt-signaling might be an important issue in skin homeostasis and wound healing.

Another organ in which a positive feedback loop between Wnt-signaling and CD44 might be important is the intestine. The intestinal epithelium is a highly dynamic tissue that is

constantly renewed every 4-5 days. This renewal requires permanently active Wnt-signals in the intestinal stem- and proliferative progenitor cells. These cells produce the high number of intestinal epithelial cells which are necessary for this rapid tissue turnover (reviewed in Clevers, 2006). The importance of a proper activity of Wnt-signaling is underscored by the consequences of an abrogation of canonical Wnt-signaling by either removal of β-catenin or TCF4 or by overexpression of the Wnt inhibitor Dkk1. The inactivation of canonical Wnt-signaling caused in all cases a complete loss of proliferation of intestinal progenitor cells at the bottom of the intestinal crypts and had severe effects on stem cell maintenance and tissue homeostasis(Korinek et al., 1998; Kuhnert et al., 2004b; Pinto et al., 2003). Especially in this intestinal stem- and proliferative progenitor cells, the Wnt-target gene CD44 is highly expressed (Zeilstra et al., 2013). Like in the skin, a positive feedback of CD44 to Wnt-signaling might be a crucial factor for the maintenance of Wnt-activity, which is required for the constant renewal of the intestinal epithelium.

Ongoing studies in our lab address this issue. We established a Cd44 floxed mouse, using the Cre-lox system for gene inactivation. In these floxed animals, exon 3 of Cd44 is flanked with two lox-P sites. Crossing of these mice with Cre-recombinase expressing mice will lead to excision of exon 3 of Cd44 and subsequently to a disruption of its protein synthesis by a stop codon. We crossed these mice with *Villin-Cre^{ERT2}* mice, in which the expression of the Cre-recombinase is controlled by a tamoxifen inducible Villin-promotor. Treatment of the *Cd44$^{fl/fl}$-Villin-Cre^{ERT2}* mice with tamoxifen resulted in the absence of CD44 in the intestinal crypts. Remarkably, tamoxifen treated *Cd44$^{fl/fl}$-Villin-Cre^{ERT2}* mice suffered much more severe from DSS (dextran sulphate sodium) induced colitis (Metzger et al., unpublished). Wnt-signaling plays an important role during intestinal inflammation and is required for a proper tissue regeneration (Kuhnert et al., 2004a). The results from our study indicate that a positive feedback of CD44 on Wnt-signaling might be required under pathological conditions like inflammation. A positive feedback of CD44 to Wnt-signaling might be crucial to provide the high levels of Wnt-activity which are required for a proper regeneration of the tissue. The fact that CD44 expression is upregulated during colitis and intestinal regeneration further supports this hypothesis (Rosenberg et al., 1995).

CD44 might be indeed an important positive Wnt-feedback-regulator with implications in development, tissue homeostasis or regeneration. However, there are a lot of other proteins that can also give positive feedback to Wnt-signaling. In addition, there are numerous known negative feedback-regulators. Thus, the question arises why Wnt/β-catenin signaling needs such a complex mechanism of regulation and why especially a feedback-regulation by CD44?

Feedback-regulation of Wnt/β-catenin signaling might be adjusted to specific cellular environments

As Wnt-signaling is active during numerous developmental events and in a high number of different tissues, thus Wnt-signaling might require a high diversity of regulators. The cellular context and microenvironment differ between tissues and give multiple inputs in the Wnt-pathway. Thus, it is possible that the high number of Wnt-regulators is necessary to implement the high variety of influencing factors that can act on the Wnt-pathway.

The fact that CD44 is especially expressed in stem cell niches, like the intestinal crypts or the hairfollicle (Szabo et al., 2013; Wielenga et al., 1999; Yasaka et al., 1995; Zeilstra et al., 2013), indicates that CD44 might be a specific Wnt-regulator in these locations, adjusted to the particular environment of these stem cell niches. For example, intestinal crypt cells need in addition to Wnt3 also other growth factors to maintain their stemness, for example EGF (Sato et al., 2011). Remarkably, signaling from EGF-receptors also underlies the regulation of CD44 proteins (Bourguignon et al., 1997; Sherman et al., 2000; Yu et al., 2002). Furthermore, like Wnts, EGFs can drive the expression of CD44 (Zhang et al., 1997). And in addition to this and most recently, a bidirectional feedback-loop between Wnt/β-catenin- and EGFR/ERK-signaling was discovered (Georgopoulos et al., 2014). This feedback-loop was shown to be required for the homeostasis of epithelial tissues. Altogether, these findings lead to the hypothesis that CD44 might be involved in the integration of a positive feedback-loop between the Wnt/β-catenin pathway and EGFR/ERK-signaling in stem cell niches like the intestinal crypts, in order to promote the tissue homeostasis in intestine.

Another reason for the high number of Wnt/β-catenin signaling regulators: Compensation

As CD44 might exert an essential function in Wnt/β-catenin signaling and as this pathway is essential for so many developmental processes, as well as for tissue homeostasis and regeneration, it is rather surprising that the CD44 germline knockout has no severe Wnt-related phenotypes (Protin et al., 1999; Schmits et al., 1997). However, Wnt/β-catenin signaling is of such a fundamental importance that it is vital to guarantee a proper pathway regulation, even in the loss of function of some components. This can be achieved if one protein can take over the function of another protein, what is called compensation.

This might also be one explanation for the existence of ten different Fz-receptors and 19 different Wnt-ligands. Several Fz-receptors can bind to the same Wnt-ligand and vice-versa, a specific Wnt-ligand can address different Fz-receptors. This might be a mechanism to compensate the loss of function of Wnt-receptors or -ligands. Also the Wnt-co-receptors LRP5 and LRP6, as well as the scaffold proteins Axin and Axin-2, show redundant functions concerning the regulation of Wnt-pathway activation (Chia and Costantini, 2005; Zhong et al., 2012), suggesting a mutual compensation. Also, TCF/LEF transcription factors as well as GSK3-α and -β can take over the function of each other to regulate the Wnt-pathway (Brantjes et al., 2001; Doble et al., 2007; Okamura et al., 1998). Thus, the lack of a Wnt-related phenotype in the CD44 germline knockout mice might be due to the fact that another protein takes over the function of CD44 in case of its absence.

Such a substitution has already been shown in the case of the co-regulatory function of CD44v6 in HGF/Met-signaling. In the absence of CD44v6, ICAM-1 still allows c-Met receptor activation *in vivo* and *in vitro* and replaced the CD44 co-receptor function for c-Met in liver regeneration upon partial hepatectomy (Olaku et al., 2011). As ICAM-1 is also expressed in the intestinal epithelium (Kelly et al., 1992), this could be a candidate that might be able to take over the function of CD44 in intestinal development and homeostasis in the case of its loss.

Another candidate that can act as substitute for CD44, is the transmembrane protein Rhamm (Receptor for HA-Mediated Motility), which is, similar to CD44, a receptor for HA (Hardwick et al., 1992). Also Rhamm can be found on intestinal epithelial cells (Li et al., 1999a) and there is some evidence that Rhamm might compensate for CD44. This evidence comes from the following observation: Although treatment with antibodies against CD44 reduced collagen-induced arthritis (CIA) in wildtype mice (Nedvetzki et al., 1999), CIA is enhanced in the CD44 germline knockout mice. This is most probably a result of enhanced expression of Rhamm. This might compensate for the loss of CD44 and trigger the inflammatory response during CIA in the absence of CD44 (Nedvetzki et al., 2004b).

However, whether ICAM1 or Rhamm can substitute the function of CD44 in Wnt-pathway regulation needs to be investigated.

CD44 and Wnt/β-catenin signaling in cancer

A functional feedback-regulation between CD44 and Wnt/β-catenin signaling might be relevant during development and to assure a proper tissue homeostasis and regeneration in adults. Here, especially a possible contribution of CD44 in the Wnt-driven maintenance of stem cells or proliferation of progenitor cells might be an important issue. However, a deregulation of this feedback-loop might have severe effects and highly contributes to the formation and progression of cancer.

Gain of function of Wnt-signaling can be found in almost all colorectal cancer types (reviewed in Lustig and Behrens, 2003). Canonical Wnt-signaling is thought to be the major inducer of this disease and the earliest mutations that are found in the adenoma-to carcinoma cascade concern components of the Wnt-pathway (Vogelstein and Kinzler, 1993). Wnt-signaling normally promotes proliferation of progenitor cells and is silenced when these cells exit the intestinal crypts (Gregorieff and Clevers, 2005). However, due to mutations that result in a hyperactivation of Wnt-signaling, these cells do not stop to proliferate and cause the formation of aberrant crypt foci (ACF), the earliest lesions detectable in colorectal cancer. Due to additional mutations (e.g. in Kras and p53) these ACF progress to adenomas and carcinomas (Vogelstein and Kinzler, 1993). Not surprisingly, due to the hyperactivation of Wnt-signaling, CD44 is highly expressed in ACFs as well as in colorectal adenomas and carcinomas.

My data, showing that CD44 can give positive feedback to Wnt-signaling, suggest that CD44 might be also involved in tumor initiation in the intestine. This is indeed the case, Zeilstra and colleagues showed that a knockout of CD44 in $Apc^{MIN/+}$ mice, a mouse model of colorectal cancer, drastically reduced the number of ACFs, adenomas and carcinomas in the intestine, whereas the growth and the progression of these tumors were not affected (Zeilstra et al., 2008). Remarkably, the knockout of CD44 in $Apc^{MIN/+}$ mice resulted also in a significant survival advantage in these mice (from 22 weeks in $Cd44^{+/+}/Apc^{MIN/+}$ mice, to 35 weeks in $Cd44^{-/-}/Apc^{MIN/+}$ mice) (Zeilstra et al., 2013).

Until now, it was thought that the reduction of the tumor number is a result of the function of CD44 as a Wnt-target and due to an increased apoptosis in the absence of CD44. However, the findings made during my PhD thesis suggest that the decrease in the tumor number could also be a result of a lack of CD44 as an essential positive regulator of the Wnt-pathway. Indeed, the hyperproliferation of intestinal epithelial cells is a result of increased Wnt-signaling, which in turn enhances the expression of CD44. This might then even further

amplify Wnt-hyperactivity. Thus, it is possible that the positive-feedback-regulation of Wnt/β-catenin signaling highly contributes to Wnt-driven tumor initiation.

Furthermore, like Wnt-signaling is required to maintain the stemness of intestinal stem cells in the crypts, the Wnt-pathway also contributes to the maintenance of colorectal cancer stem cells (Pinto and Clevers, 2005). In addition to their expression in the normal intestinal stem cells, CD44 proteins can also be found on colorectal cancer stem cells (Zeilstra et al., 2013). As these cells require Wnt-activation to maintain there stemness, CD44 might also contribute to maintenance and self-renewing capacity of these cells. Thus this might be another, but not mutually exclusive, explanation why the knockout of CD44 in $Apc^{MIN/+}$ mice resulted in a decreased tumor number and an increased lifetime.

Indeed, activating mutations of the Wnt-pathway are not restricted to cancer of the intestine. Wnt-hyperactivity is also detectable in several other cancer types e.g. of the liver, brain, breast, pancreas, or the skin (reviewed in Lustig and Behrens, 2003). Also CD44 expression is connected to human tumors originating from cells of the brain, breast, pancreas and the skin (reviewed in Naor et al., 2002). Thus, it is possible that the CD44/Wnt/β-catenin interplay might also play a role in the formation of these cancer types. However, this remains to be investigated. As Wnt/β-catenin signaling is the driving force of many cancers, it became a great challenge to find effective drugs to inhibit the Wnt-pathway in patients. My findings that CD44 is essentially required for the activation of the pathway, together with the findings of others that a loss of CD44 can counteract Wnt-driven tumorigenesis in colorectal cancer, might provide ideas for new therapeutical approaches against intestinal cancer.

How CD44 might contribute to Wnt/β-catenin signaling: A review of the molecular mechanism of CD44 mediated Wnt-regulation

CD44 proteins are well-known signaling regulators and were shown to be involved in in many different important growth factor induced signaling pathways, like ErbB2-, FGFR-, VEGFR-, c-Met and CXCR4-signaling (Bourguignon et al., 2007c; Fuchs et al., 2013; Orian-Rousseau et al., 2002; Tremmel et al., 2009). The finding that CD44 is also a regulator for Wnt/β-catenin signaling broadens even more the spectrum of signaling pathways regulated by CD44 proteins. The question that arises is how a protein like CD44 can be involved in so many different pathways? Are the functions of CD44 in the regulation of growth factor-induced signaling common to all signaling pathways or are they different for each signaling pathway? The next question is, whether there is a specific function of CD44 in the Wnt-pathway?

The mode of action of CD44 in signaling regulation is indeed not common for all pathways. The functions of CD44 in growth factor induced signaling are very diverse and range from direct binding of growth factors and their presentation to the corresponding receptor as it is the case for FGF (Sherman et al., 1998) or HGF and VEGF (our own unpublished data) to the recruitment of enzymes like MMP7 that are required for the activation of ErbB4 by HB-EGF (Bennett et al., 1995; Yu et al., 2002). Furthermore, CD44 has been shown to be able to trigger dimerization of ErbB2- and ErbB3-receptors upon neuregulin induction (Sherman et al., 2000). CD44 can regulate signaling in combination with its ligand HA, as it is the case for CXCR4-signaling (Fuchs et al., 2013). Thus it seems that CD44 has different modes of action and specific functions for each pathway, although all CD44 proteins are encoded by one single gene. Thus, the question of the diversity of CD44 action in signaling regulation can be raised: How can one single gene provide so many different functions?

Alternative splicing generates numerous CD44 isoforms

Although all CD44 proteins are encoded by one single gene, there is a huge heterogeneity of CD44 proteins. This is due to posttranscriptional alternative splicing events that generate a huge number of CD44 isoforms. This alternative splicing allows the inclusion or exclusion of 10 variant exons in different combinations in the extracellular domain of CD44 (Screaton et al., 1992). It has been shown that the presence of specific variant exons can be crucial for the function of CD44 as a signaling regulator.

The regulation of growth factor-induced signaling by CD44 is often highly dependent on the presence of variant exons. The inclusion of variant exon 6 is essential for CD44 to act as co-regulator for HGF/Met and VEGF-A/VEGFR-signaling (Matzke et al., 2005; Tremmel et al., 2009), most probably by binding to the growth factors and their presentation to the

corresponding receptors (Volz et al., in preparation). Inclusion of variant exon 3, instead, allows CD44 to bind FGFs (Sherman et al., 1998). Moreover, only v3 containing isoforms are able to recruit MMP7 to active HB-EGF/ErbB4-signaling (Bennett et al., 1995; Yu et al., 2002). Ongoing studies in our lab address the specificity of CD44 isoforms in signaling regulation of the ErbB-receptor family, a perfect model for both specificity and diversity of RTK-signaling. The ErbB-receptors 1-4 can dimerize in different combinations to promote intracellular signaling (reviewed in Olayioye et al., 2000). These dimerizations are induced by the binding of different ligands to the ErbB-receptors. Remarkably, different ligands address the same receptor pair as for example EGF, TGFα, Amphiregulin or Epiregulin can all bind to ErbB1 to trigger dimerization of ErbB1/ErbB1 or ErbB1/ErbB2. Furthermore, one growth factor can address different ErbB-receptors. For example, Epiregulin can bind to ErbB1 and ErbB4 or Neuregulin-1 or -2 to ErbB3 and ErbB4 (reviewed in Sweeney and Carraway, 2000). The study of the role of CD44 in this system showed a ligand-specific recruitment of CD44 isoforms. Whereas, for example, receptor dimerization and activation of ErbB-receptors by TGFα occurs independently of CD44, CD44v6 isoforms are pivotal for the activation of ErbB-signaling by EGF (our own unpublished data). The activation of ErbB-signaling by HB-EGF, however, requires the presence of CD44v3 (Yu et al., 2002).

Is the function of CD44 in the Wnt-pathway dependent on the inclusion of variant exons and isoform specific?

In my PhD thesis, I compared different CD44 isoforms (CD44s, CD44v6, CD44v4-v7) for their ability to enhance Wnt3a induced TCF/LEF regulated transcription. Remarkably, all these isoforms augmented Wnt/β-catenin signaling to the same extent (Fig. 4 C). The result that even the smallest CD44 isoform that lacks all variant exons was able to increase Wnt/β-catenin signaling, demonstrates that CD44 does not require the inclusion of any variant exon to function in the Wnt-pathway. This result further implicates that it is a common feature of all CD44 isoforms that is required for CD44 to act as a regulator of the Wnt-pathway.

Posttranslational modifications increase the heterogeneity of CD44 proteins

CD44 proteins do not only exist in numerous isoforms, their heterogeneity can be further increased by posttranslational modifications like glycosylations e.g. heparansulphation, chondroitin-sulphation or keratansulphation. Also these modifications can define whether CD44 proteins can regulate a specific signaling pathway or not. For example, variant exon v3 contains a motif for heparansulphation and only heparansulphated CD44v3 isoforms can act as HSPGs and bind to heparin binding proteins like FGF (Bennett et al., 1995; Jones et al., 2000; Lokeshwar et al., 1994; Sherman et al., 1998). These posttranslational modifications might be needed in the regulation of Wnt-signaling by CD44. However, the results of my PhD work clearly show that it is not the role of an HSPG that

CD44 plays in the Wnt-pathway. The fact that also CD44 isoforms lacking v3 can enhance Wnt-signaling (Fig. 4 C), excludes that CD44 requires heparansulphation for the augmentation of Wnt/β-catenin signaling. This finding is rather surprising, as the heparansulphation of other HSPGs, like glypicans or syndecans, has been shown to enable them to bind Wnt-ligands and modulate their signaling (Goutebroze et al., 2003; Granes et al., 2000). For example, glypican-3 binds directly to Wnt-proteins via heparan-sulfated glycosaminoglycan side chains and acts upstream of Dsh in the signaling cascade. These data would be consistent with a role of HSPGs in controlling the availability of Wnts for interactions with their receptors Fz and LRP (De Cat et al., 2003). It has been proposed that HSPGs are able to stabilize Wnts from being degraded or to reduce the Wnt diffusion, thereby maintaining the local concentration of Wnt ligands, rendering them available for their receptors (Hacker et al., 1997; Pfeiffer et al., 2002). Another hypothesis is that HSPG directly facilitate the formation of Wnt/Fz-signaling complexes by acting as co-receptors (Lin and Perrimon, 1999).

Yet, there is another posttranslational modification that could be important for the function of CD44 in the Wnt-pathway and that might specify whether CD44 proteins can participate in Wnt-regulation or not: Chondroitinsulphation. This posttranslational modification allows CD44 to act as a CSPG and might enable CD44 to control Wnt-signaling similar to HSPGs. Indeed, CSPGs are, like HSPGs, able to bind Wnts and to act as positive co-factor for Wnt3a-signaling (Nadanaka et al., 2008). Furthermore, also a specific function of CSPG-CD44 proteins in signaling regulation has already been identified. Chondroitinsulphated CD44-proteins are regulators of IFN-γ signaling and here it is thought that CD44 binds to IFN-γ in order to increase the local concentration of IFN-γ at the cell surface, thereby fascilitating its binding to high affinity IFN-γ-receptors (Hurt-Camejo et al., 1999).

In contrast to the heparansulphation site that is exclusively found in variant exon v3, the chondroitinsulphation is located on a serine-glycine motif in the constant exon 5. Hence, all CD44 isoforms can be chondroitin-sulphated and act as CSPG (Greenfield et al., 1999). Therefore, the hypothesis that CD44 might require chondroitin-sulphation in order to modulate Wnt-signaling would fit to my observations that an inclusion of variant exons within the stem region is not required for CD44 to augment Wnt-signaling. However, if chondroitinsulphation is a prerequisite for CD44 to regulate Wnt-signaling still needs to be addressed. Treatment of cells with chondroitinase could reveal whether lack of chondroitin-sulphation interferes with the augmentation of Wnt-signaling by CD44. Furthermore, it could be analyzed whether a CD44, mutated in the CS-binding site, is still able to enhance Wnt-signaling. This might shed light on the question whether chondroitinsulphation is required for CD44 to regulate Wnt-signaling or not.

In addition to heparansulphation and chondroitinsulphation, CD44 can also be keratansulphated (Sleeman et al., 1998). However, any report about a regulation of Wnt-signaling by keratansulphated proteins or the binding of Wnt ligands to keratansulphate-residues are missing.

Specificity of CD44 proteins can be defined by the microenvironment

In addition to the presence of variant exons and posttranslational modifications, a function of CD44 as signaling regulator might depend on its ability to bind to extracellular matrix components. Indeed, CD44 can interact with ECM components like hyaluronan (HA), osteopontin (Lin and Yang-Yen, 2001), collagen, laminin and fibronectin (Borland et al., 1998; Naor et al., 1997). Moreover, CD44 has been identified as major receptor for HA and HA binding to CD44 is required in different signaling pathways. Thus, it is possible that the function of CD44 in Wnt-signaling regulation is dependent on the presence of HA. As the binding sites for hyaluronan (HA) are located in the constant part of the extracellular domain, binding to HA is a common feature of all CD44 proteins (Peach et al., 1993; Thorne et al., 2004). A contribution of HA to the regulation of Wnt-signaling by CD44 would therefore also match with my results that several CD44 isoforms can augment Wnt-induced TCF/LEF activation to the same extent.

The binding of HA to CD44 can trigger complex formation between CD44 and growth factor receptors. This was shown for ErbB2-receptors that could be co-immunoprecipitated with CD44 in the presence of HA (Bourguignon et al., 2007c). Accordingly, a complex formation of CD44 and the Wnt-receptors induced by HA would be one possibility why CD44 might need HA to contribute to Wnt-signaling.

On the other hand, ECM components like HA can store, mask, present or sequester growth factors and thereby modulate their signaling (reviewed in Eble, 2009). Indeed, HA has been shown to directly interact with growth factors like CXCL12, TGF-1β, IL-8 or bFGF (Hintze et al., 2012; Pichert et al., 2012; Purcell et al., 2012; Walicke, 1988). Hence, there is the possibility that CD44 might indirectly bind to Wnt-ligands via HA molecules. Recently, our group revealed an important function of HA binding to CD44 in modulating CXCR4-signaling. Here, we could demonstrate that the interaction between HA and CD44 modulates the activation of CXCR4 by its ligand CXCL12, depending on the size of the HA molecules (Fuchs et al., 2013). Whereas small HA fragments block CXCL12 mediated signaling transduction, the presence of high molecular weight HA molecules is even able to enhance CXCL12 activated CXCR4-signaling in the presence of CD44. One hypothesis is that the glycosaminoglycan HA binds to the growth factors and accumulates them in close proximity to their receptors. Thus, the same could be true for the function of CD44 in the Wnt-pathway, although there is up till now no report about a binding between Wnt proteins and HA.

However, the results obtained during my PhD thesis excluded any involvement of HA in the CD44 mediated regulation of Wnt-activated β-catenin signaling. Neither treatment with exogenous HA nor removal of HA by the means of hyaluronidase had any effects on Wnt-induced activation of TCF/LEF regulated transcription. Furthermore, both did not alter the augmentation of Wnt-signaling by CD44 (Fig. 7). Furthermore, blocking the interaction between CD44 and HA via CD44 specific antibodies did not interfere with the function of CD44 in Wnt-signaling (Fig. 9 A). Finally, a CD44 mutant that is not able to bind HA amplified the Wnt-pathway to the same level as wildtype-CD44 (Fig. 9 B).

Remarkably, HA binding to CD44 has already been shown to activate β-catenin-mediated TCF/LEF regulated transcription, in human ovarian- and colorectal cancer cells. Here HA/CD44 interaction has been shown to activate the ErbB2/PI3K-pathway that in turn can activate β-catenin mediated TCF/LEF regulated transcription (Bourguignon et al., 2007c; Misra et al., 2008). Furthermore, interaction of HA with CD44 can increase the expression of the histone acetyltransferase p300, a transcriptional co-activator for TCF/LEF regulated transcription in breast cancer cells (Bourguignon et al., 2009). Also here the interaction of HA and CD44 results in an activation of Wnt-target gene expression.

As it has been shown that the binding of HA to CD44 can activate β-catenin signaling and Wnt-target gene transcription, the results of my work, showing that HA and the interaction between HA and CD44 play no role in Wnt/β-catenin signaling are rather surprising. However, the experiments performed by others, showing that HA/CD44 interaction can induce Wnt-target gene transcription were performed in other cell lines than the cells used in my work. Thus, it is possible that an involvement of HA/CD44 interaction in the activation of the Wnt-cascade is highly dependent on the cellular context. Indeed, it was shown that the function of several Wnt regulators is often cell type and context specific. The receptor tyrosine kinase Ror2, for example, has been shown to inhibit the activity of the Wnt3 or Wnt3a but also to potentiate Wnt1 and Wnt3a activity depending on the cell line, the type of the available Wnt-receptors or the presence or absence of Wnt5a (Green et al., 2008). Also Dkk-proteins, normally known as potent inhibitors of canonical Wnt-signaling can potentiate Wnt-signalling depending on the cellular context (Li et al., 2002; Mao and Niehrs, 2003; Wu et al., 2000). Although Dkk2 inhibits Wnt3a-signaling in NIH3T3 and 293T fibroblasts, it was shown that a transfection of these cells with Dkk2 together with LRP6 is able to stimulate canonical Wnt-signalling (Li et al., 2002; Mao and Niehrs, 2003). Thus, it is possible that the contribution of HA to the regulation of Wnt-signaling by CD44 is also context dependent.

As mentioned above, HA is not the only ECM component that can interact with CD44. In addition, CD44 has been shown to bind to osteopontin (Weber et al., 1996), collagen,

laminin and fibronectin (Borland et al., 1998; Naor et al., 1997). Thus, it would be worth testing, whether these ECM molecules might contribute to the positive function of CD44 in the Wnt-pathway. Fibronectin was recently shown to collaborate with Wnt-signaling in the regulation of satellite stem cell homeostasis (Bentzinger et al., 2013) and might therefore be a good candidate for further investigation. Also a role of osteopontin in CD44/Wnt-regulation should be addressed in follow up work. Like HA, osteopontin has been shown to activate β-catenin signaling via the PI3K/Akt pathway (Robertson and Chellaiah, 2010) and CD44/osteopontin interaction can activate PI3K/Akt signaling (Lin and Yang-Yen, 2001).

Positive feedback-regulation of Wnt/β-catenin signaling by CD44 can be direct or indirect

There are numerous possibilities how CD44 might regulate the Wnt-pathway. On the one hand, there is the possibility that CD44 regulates Wnt-signaling by direct interaction with Wnt-components. For example, CD44 might interact with Wnt-ligands in order to bring them in close proximity to their receptors, as it is the proposed function of CD44 in FGF, HGF or VEGFA signaling. Furthermore, CD44 might interact with Wnt-receptors, for example in order to stabilize a ternary complex with Wnt, Fz and LRP6. A similar function of CD44 is known for ErbB-signaling, where CD44 has been shown to trigger dimerization and activation of ErbB2 and ErbB3 upon neuregulin induction (Bourguignon et al., 1997; Sherman et al., 2000). Furthermore, CD44 might bind to secreted Wnt-antagonists like sFRPs or Dkks and counteract their inhibitory effect on the Wnt-pathway. CD44 might also be necessary for an activation of the Wnt-receptors Fz or LRP6. A requirement of CD44 for receptor phosphorylation has already been shown for c-Met and VEGFR (Orian-Rousseau et al., 2007; Tremmel et al., 2009).

However, as CD44 is a well-known and potent regulator of RTK- or CXCR4-signaling it is possible that CD44 controls Wnt-signaling activity more indirectly via the regulation of RTK- or CXCR4-signaling. Remarkably, both have been shown to activate the Wnt-pathway intracellularly at several steps of the cascade. Binding of CXCL12 to CXCR4, for example has been shown to activate β-catenin via the MEK/Erk-pathway (Luo et al., 2006). Downstream signaling from RTKs via the MEK/Erk-pathway can trigger phosphorylation of LRP6 and thereby activate canonical Wnt-signaling (Krejci et al., 2012). RTK-signaling via the PI3K/Akt-pathway can furthermore activate the pathway at the level of β-catenin, by inactivating GSK3-β and even further downstream at the level of TCF/LEF-regulated transcription, by inducing the expression of p300, a transcriptional coactivator of TCF/LEF transcription factors (Bourguignon et al., 2009).

However, a blocking of both, MEK/Erk- and the PI3K/Akt-signaling, by specific chemical inhibitors had no effect on the augmentation of Wnt-signaling by CD44 (Fig. 11).

These results exclude that the positive effect of CD44 gain of function is indirectly mediated via its function as RTK- or CXCR4-regulator, at least via this two intracellular pathways.

Nevertheless, it is still possible that the regulation of RTK- or CXCR4-signaling by CD44 might contribute to a regulation of the Wnt-pathway. Indeed, activation of MEK/Erk-signaling by FGFs, EGFs or NGFs can trigger phosphorylation and thereby activation of the Wnt-receptor LRP6 and furthermore amplify Wnt3a induced TCF/LEF regulated transcription (Krejci et al., 2012). Most recently, also HGF was shown to induce LRP6 phosphorylation, most probably via the PI3K/Akt-pathway (Koraishy et al., 2014). Interestingly, in addition to CD44, RTKs like EGFR or Met, as well as RTK activating growth factors like VEGF or FGFs, are upregulated upon activation of canonical Wnt-signaling (reviewed in Logan and Nusse, 2004). Thus, Wnt-signaling might induce a positive feedback through MEK/Erk-signaling by activating RTK and GF expression. The fact that the Wnt-target gene CD44 also provides positive feedback and regulates both, RTK/MEK/Erk- and Wnt-signaling, indicates that CD44 might be involved in or even be crucial for a positive feedback-loop between RTK-signaling and the canonical Wnt-pathway. However, this theory needs to be further proven in follow up work.

In addition to MEK/Erk- or PI3K/Akt-signaling, there are also other pathways that can be regulated by CD44 and that have impact on canonical Wnt-signaling. Crosslinking of CD44 with immobilized CD44 antibodies, for example, has been shown to activate PKC regulated migration of T-cells (Fanning et al., 2005). Furthermore, PKC-signaling can be activated by the interaction of CD44 with EGFR (Kim et al., 2008b). PKC signaling has also impact on canonical Wnt-signaling, shown by a study of Chen and colleagues (Chen et al., 2010). In this study, interference with PKC-signaling by a chemical inhibitor or dominant-negative PKC could inhibit cytoplasmic accumulation of β-catenin and Wnt-mediated transcriptional activation. Moreover, activation of Src-signaling by ErbB2 can trigger activation of β-catenin/TCF-regulated transcription (Haraguchi et al., 2004) and CD44 has been shown to be involved in the activation of Src-signaling, with implications in breast tumor cell invasion (Bourguignon et al., 2010). In addition, it has been found that CD44 can complex with Signal Transducers and Activators of Transcription-3 (STAT-3) and Janus kinase-2 (JAK-2), in order to promote JAK/STAT-signaling (Bourguignon et al., 2008). This pathway is also able to induce β-catenin nuclear accumulation and TCF/LEF regulated transcription (Kawada et al., 2006). Inhibition of these pathways by the use of specific inhibitors like the PKC inhibitor Calphostin C (Kobayashi et al., 1989), the Src-kinase inhibitor Saracatinib (Hennequin et al., 2006) or the Janus kinase inhibitor AG490 (Nielsen et al., 1997) and subsequent analysis of the effect on CD44 mediated amplification of Wnt-

signaling, might unravel whether these pathways contribute to the regulation of Wnt-signaling by CD44.

Which steps of the canonical Wnt-cascade are regulated by CD44?

The regulation of Wnt-signaling is highly complex and the activation of the pathway can be controlled at each step of the signaling cascade. Secreted proteins like sFRPs, for example, can antagonize Wnt-signaling by binding to Wnts, thereby preventing their interaction with Fz-receptors. Transmembrane HSPGs like glypican-3 can bind to Wnt ligands and accumulate them in close proximity to their receptors, thereby enhancing Wnt-activity. Inhibitors of the Dkk-family can bind to LRP6 and trigger its internalization. This inhibits Wnt-signaling as LRP6 is no-longer present at the membrane in order to bind Wnt-ligands. Also the phosphorylation of the Wnt-receptor LRP6 is tightly regulated by several kinases. Furthermore, the levels of free cytosolic β-catenin are regulated by numerous proteins that build a β-catenin degradation complex. Also within the nucleus TCF/LEF regulated transcription is controlled by several transcriptional co-activators or –repressors (reviewed in Logan and Nusse, 2004).

Hypothetically, CD44 can regulate Wnt-signaling at any of these levels. To rule out precisely, which Wnt-components are regulated by CD44, might help to understand how CD44 functions in Wnt/β-catenin signaling.

In order to address this issue, I performed so called “epistasis-experiments” in which I activated the TCF/LEF regulated transcription artificially at several consecutive steps and analyzed, which steps of the cascade are affected by CD44 loss- and gain of function. These experiments placed CD44 at the level of the Wnt-receptors. Indeed, ectopic expression of CD44 as well as the knockdown CD44 affected TCF/LEF regulated transcription when activated at the level of Wnt3a or LRP6, however no effect was seen when the pathway was activated at the level of Dsh or β-catenin (Fig.12).

These findings indicate a role of CD44 in Wnt-membrane reception, whereas intracellular downstream events are not affected by CD44. Moreover, these results exclude that CD44 regulates Wnt-signaling exclusively indirect via activation of β-catenin, for example via its regulatory function for RTK-signaling. If this would be the case, the effects of a CD44 loss and gain of function on TCF/LEF regulated transcription would also be visible, when the pathway is activated at the level of Dsh or β-catenin.

A possible contribution of CD44 to LRP6-signalosome formation

One very interesting aspect of the results of the epistasis experiment is that CD44 was able to influence the activation of Wnt-signaling at the level of LRP6, whereas the pathway activation by Dsh was not affected by CD44. This is interesting since it is not clearly defined whether Dsh acts upstream or downstream of LRP6. On the one hand, overexpression of Dsh in a LRP6 negative background can activate β-catenin signaling (Wehrli et al., 2000) and it has been shown that activated Dsh can interact with the β-catenin destruction complex through the GSK3-β inhibitor GBP/FRAT. This prevents the phosphorylation of β-catenin and thus its degradation (Jonkers et al., 1997; Li et al., 1999b; Salic et al., 2000; Yost et al., 1998). These findings indicate a role of Dsh downstream of LRP6, which would be consistent with the result of my epistasis experiments.

However, more recently and in contradiction with an exclusive role of Dsh downstream of LRP6, Bilic and colleagues showed that Dsh is required for LRP6 activation. This indicates a role of Dsh upstream of LRP6 (Bilic et al., 2007). In their model they propose that Wnt induces the formation of Dsh-aggregates at the membrane, where they co-cluster LRP6 and other Wnt-components into so-called “LRP6-signalosomes”. This clustering provides high local receptor concentration that triggers LRP6 phosphorylation and activation by its kinase CK1γ. Taken into account that Dsh acts upstream of LRP6, the finding that CD44 regulates Wnt-signaling at the level of LRP6 but not at the level of Dsh is rather surprising (Fig. 12).

However, it was shown that oligomerization of LRP6, for example by a synthetic multimerizer, can bypass the need for Dsh (Cong et al., 2004). Hence, one explanation why CD44 affects Wnt-signaling at the level of LRP6 but not at the level of Dsh might be that CD44 complexes with LRP6, in order to form LRP6 oligomers that can activate the Wnt-pathway independent of Dsh. The fact that CD44 has been found to physically interact with LRP1, another member of the LDL-receptor family (Perrot et al., 2012), makes it even more likely that CD44 might form a complex with LRP6.

Indeed, by co-immunoprecipitation studies, I could demonstrate that CD44 forms a Wnt-inducible complex with LRP6 (Fig. 14). The binding of CD44 to LRP1 occurs in the fourth cluster of a basic LRP domain within the extracellular part of LRP1. Such basic domains are also present in the extracellular domain of LRP6 (see 1.3.2 LRP5/6) (reviewed in Schneider and Nimpf, 2003). Thus, it is most likely that the binding of CD44 to LRP6 also takes place in one of its basic LRP domains. A mutation of this domain of LRP6 and a subsequent analysis whether these mutants can still interact with CD44, might unravel if these basic LRP domains are common domain within LRPs that enable them to interact with CD44.

The fact that CD44 interacts with LRP6 only upon induction with Wnt-ligands, furthermore indicates that CD44 might indeed be part of the LRP6-signalosome.

Indeed, I could show that CD44 complexes with LRP6 already 5 minutes after Wnt-induction (Fig 14). Interestingly, Bilic and colleagues showed by real-time confocal microscopy that the coalescence of LRP6 proteins at the membrane and the subsequent recruitment of Axin to LRP6 occur later, after 15 minutes of Wnt-treatment (Bilic et al., 2007). The finding that the interaction between CD44 and LRP6 occurs previously to the described aggregation of LRP6 and recruitment of Axin, indicates that CD44 might already be necessary to establish the LRP6-signalosome. Remarkably, in my co-immunopreciptiation experiments, I could show that the strongest LRP6 band in the Western-blot of CD44-immunoprecipitates appears after 30 minutes of Wnt-induction, whereas less amounts of LRP6 could be found in the CD44-immunoprecipitates of HeLa cells that were treated with Wnt3a for 60 minutes. These results indicate that the interaction between CD44 and LRP6 reaches its peak 30 minutes after stimulation with Wnt. Also LRP6/Axin-aggregates, hence LRP6-signalosomes, have been shown to remain stable over a time course of 30 minutes (Bilic et al., 2007). Thus, my findings that CD44 interacts with LRP6 previously to the formation of LRP6-signalosomes and that this interaction occurs over the same period of time as the LRP6-signalosomes remain stable, suggest that CD44 might not only be required for the establishment of the LRP6-signalosome but might also contribute to its stability. An involvement of CD44 in signalosome formation is also the predicted model of CD44 function in HGF/MET-signaling. Here, the binding of HGF to c-Met and CD44v6 recruits ERM-proteins to the plasma membrane that connect the HGF/Met/CD44v6-complex to the actin-cytoskeleton. This recruits the guanine nucleotide exchange factor (GEF) Son of sevenless (Sos) that in turn activate Ras and its downstream signaling via the MEK/Erk-pathway (reviewed in Orian-Rousseau and Ponta, 2008).

The potential function of CD44 in Wnt-induced LRP6-internalization

In addition, there is also the possibility that CD44 does not only interact with LRP6 in order to establish and/or stabilize LRP6-signalosomes, but also internalizes with LRP6 upon Wnt-induction. Canonical Wnts have been shown to trigger the internalization of LRP6 via caveolin or clathrin dependent internalization routes. Whereas caveolin dependent internalization of LRP6 rather promotes cytoplasmic accumulation of β-catenin and TCF/LEF target gene transcription (Yamamoto et al., 2006), the results of clathrin-mediated endocytosis of LRP6 are contradictory. On the one hand, it has been shown that clathrin mediated endocytosis of LRP6 is required for Wnt-signaling (Blitzer and Nusse, 2006), on the other hand, Jiang and colleagues showed that clathrin mediated endocytosis downregulates Wnt-signaling (Jiang et al., 2012).

As the internalization of LRP6 has been shown to start already 10 minutes (caveolin) (Yamamoto et al., 2006), respectively 30 minutes (clathrin) (Blitzer and Nusse, 2006) after stimulation with Wnt, it is likely that CD44 that is found in a complex with LRP6 for at least 30 minutes (Fig. 14), is internalized together with LRP6. This is even more likely as it was shown that the interaction of LRP1 and CD44 triggers their joint internalization via the clathrin-mediated pathway (Perrot et al., 2012). However, here it is LRP1 that promotes CD44 internalization in order to regulate tumor cell adhesion. A CD44-regulated endocytosis of transmembrane receptors has recently been demonstrated by our group. Immunofluorescence studies showed that CD44v6 internalizes together with c-Met upon HGF treatment via the clathrin mediated pathway (Hasenauer et al., 2013). In addition, c-Met was not able to internalize upon HGF induction in the absence of CD44v6. Altogether, these findings suggest that CD44 might internalize together with LRP6 upon Wnt-induction, most probably via the clathrin mediated pathway. As it has been shown that this internalization can be required for a proper activation of Wnt/β-catenin signaling, a possible internalization of LRP6 and CD44 would not be in contradiction with my findings that CD44 is needed to activate the Wnt-pathway.

Real-time confocal microscopy analysis and colocalization studies of fluorescently labelled CD44-, LRP6- and Axin proteins are necessary to provide further evidence for the theory that CD44 takes part of LRP6-signalosome formation and internalization. These studies are currently ongoing in our lab and might shed light on the hierarchy of the CD44/LRP6 interaction in the formation of the LRP6-signalosomes and furthermore on the dynamics and localization of the CD44/LRP6 complexes.

A possible regulation of Wnt-induced LRP6-activation by CD44

Most notably, the aggregation of LRP6 into LRP6-signalosomes initiates a key step of Wnt-signaling transduction, the phosphorylation and thereby activation of LRP6 by GSK3-β. GSK3-β phosphorylates LRP6 at serine 1490 (Zeng et al., 2005). In addition to a Wnt-inducible complex formation between CD44 and LRP6, I could demonstrate that CD44 is required for this Wnt-induced phosphorylation of LRP6 (Fig. 16). As CD44 has been shown to be able to recruit kinases, for example PKC or members of the Src-family (reviewed in Ponta et al., 2003), it might be that CD44 interacts with LRP6, in order to bring LRP6 together with its kinase GSK3-β. However, there is up till now no report about any interaction between CD44 and GSK3-β and co-immunoprecipitation experiments performed in our lab could not detect any complex formation between CD44 and GSK3-β (data not shown).
Yet, there is another explanation why Wnt fails to trigger the phosphorylation of LRP6 in the absence of CD44. It is described that the Wnt-induced phosphorylation of LRP6 is rather a consequence than the cause of the aggregation of LRP6 in signalosomes (reviewed in Niehrs and Shen, 2010). Thus, LRP6 needs to form aggregates in order to get phosphorylated by GSK3-β. As discussed above, CD44 might be required for the initiation and/or stabilization of LRP6-signalosomes. Consequently, the knockdown of CD44 might inhibit phosphorylation of LRP6 by GSK3-β because LRP6 is not able to form signalosomes in the absence of CD44.

CD44 regulates LRP6-cell surface localization

Yet, there is another possibility why Wnts require CD44 to activate LRP6-receptors. LRP6 normally appears as two bands of different molecular weight in the Western-blot. The lower molecular weight band represents the immature LRP6 that is located in the endoplasmatic reticulum. The higher molecular weight band represents the mature LRP6 at the cell surface (Hsieh et al., 2003). The higher molecular weight of the mature, membrane located LRP6-proteins come from multiple glycosylations of LRP6 during the maturation process (Hsieh et al., 2003). Remarkably, the upper LRP6 band did not appear in the Western-blot of cells treated with CD44-siRNA (Fig.16). This strongly suggests that CD44 is required for a proper membrane localization of LRP6.

In addition, ectopically expressed LRP6-GFP was not present at the cell surface upon knockdown of CD44 (Fig. 17). Remarkably, this phenotype was specific for LRP6 as another Wnt-receptor, Ror2, appeared normally at the cell surface in the absence of CD44 (Fig. 18). As my studies only addressed the localization of ectopically expressed LRP6-GFP, additional experiments should be performed, in order to confirm that CD44 is also required for the membrane localization of endogenous LRP6. Unfortunately, staining of endogenous LRP6 with a fluorescently labelled LRP6 antibody did not work in my hands, thus I was not able to

compare the localization of endogenous LRP6 in the absence or presence of CD44 by immunofluorescence microscopy. The analysis of the presence of LRP6 in membrane fractionations of CD44-siRNA transfected cells are currently ongoing in our lab and might provide further evidence for a CD44 dependent membrane localization of LRP6.

Nevertheless, the missing upper (membrane) LRP6 band in the Western-blot (Fig. 16) together with the results of that LRP6-GFP is not found at the cell surface upon loss of CD44 (Fig. 17), provide strong evidence that CD44 is indeed pivotal for a proper membrane localization of LRP6. The mislocalization of LRP6 in the absence of CD44 is most probably the reason why Wnt3a fails to induce phosphorylation of LRP6, as this phosphorylation takes place predominantly at the cell surface (reviewed in Niehrs and Shen, 2010). Moreover, a requirement of CD44 for LRP6 cell surface localization would explain the pivotal role of CD44 for Wnt/β-catenin signaling activation, as the binding of Wnt-ligands to Fz-receptors activates the canonical Wnt-pathway only in combination with LRP6 co-receptors. My hypothesis would also be in agreement with the results of my epistasis experiments, in which I could show that CD44 regulates the Wnt-cascade at the membrane at the level of Wnt and LRP6 (Fig. 12). Indeed, if a knockdown of CD44 suppresses membrane localization of LRP6, Wnt is no-longer able to activate the canonical Wnt-pathway and to trigger TCF/LEF regulated transcription. The same is true for LRP6. As the phosphorylation of LRP6 and the subsequent recruitment of Axin that leads to inhibition of the β-catenin degradation complex, takes place only at the membrane, the ectopically expressed LRP6 can activate the Wnt-pathway only when it is present at the cell surface (reviewed in Niehrs and Shen, 2010).

CD44 might stabilize LRP6 at the membrane

The reason why LRP6 is not present at the membrane in the absence of CD44 might be that CD44 stabilizes LRP6 at the membrane, for example by preventing LRP6 internalization through members of the Dkk-family. Dkks are secreted glycoproteins that bind to LRP6 and mediate LRP endocytosis in cooperation with kremen, a high affinity single-pass transmembrane receptor for Dkk (Niehrs, 2006). This, in turn, strongly reduces the amount of LRP6 at the membrane and blocks the access of Wnt-ligands to LRP6. Dkk is a Wnt-target gene (Gonzalez-Sancho et al., 2005; Niida et al., 2004) and ectopic expression of LRP6 activates TCF/LEF target gene transcription (Fig. 12). Hence, it is possible that ectopic expression of LRP6-GFP activates the expression of Dkk and this in turn would remove LRP6 from the membrane. If CD44 would counteract Dkk mediated endocytosis, a knockdown of CD44 would result in a decrease of membrane located LRP6, the phenotype that was seen in my experiments (Fig. 17). A double knockdown of Dkk together with CD44 and subsequent analysis of LRP6-GFP localization within these cells might unravel whether a repression of Dkk can rescue LRP6 membrane localization in a CD44 loss of function

background. Yet, previous experiments, performed during my Diploma thesis, speak against a role of CD44 in LRP6 inhibition by Dkk. In these experiments I could show that transfection of CD44 in HEK293 cells could not antagonize the inhibition of Wnt-induced TCF/LEF regulated transcription (Mark Schmitt, Diploma thesis).

CD44-mediated regulation of LRP6 membrane-targeting

There is also another hypothesis that might explain the mislocalization of LRP6. Newly synthesized LDL receptor related proteins, like LRP6, reach the plasma membrane via the secretion pathway, a series of steps that are required to bring newly generated proteins from the ER to the plasma membrane. LRPs fold and mature inside the ER. After proper folding, they traffic through the Golgi apparatus to the cell membrane. The export of proteins from the ER is tightly controlled and only properly folded LRP6 proteins can leave the ER. Misfolded proteins stay in the ER and are removed by ER-associated degradation (ERAD) (reviewed in Herz and Marschang, 2003).

Thus, CD44 might be involved in the maturation or trafficking of newly synthesized LRP6 to the membrane. The following observations provide evidence for this hypothesis. In the absence of CD44, ectopically expressed LRP6-GFP is not only absent at the membrane but also accumulating in close proximity to the nucleus (Fig. 17). Here, the endoplasmatic reticulum (ER) is located and it is the ER where the maturation and processing of LRP6 takes place (Hsieh et al., 2003). Counterstaining of CD44-siRNA treated cells with an ER marker confirmed that the ectopically expressed LRP6-GFP is indeed accumulating in the ER (Fig. 20). Within the ER, LRP6 is properly folded and processed. These events are required for LRP6 to reach the plasma membrane (Hsieh et al., 2003). One important protein that promotes the proper maturation of LRP6 and that is required to shuttle LRP6 from the ER to the membrane, is the chaperone Mesd. Indeed, in the absence of Mesd, LRP6 is retained in the ER and fails to reach the cell membrane (Hsieh et al., 2003; Koduri and Blacklow, 2007), whereas ectopic expression of Mesd enhances surface expression of epitope-tagged LRP6 (Li et al., 2006). If it is exclusively the maturation of LRP6 that is affected by the CD44 knockdown, an overexpression of Mesd in a CD44 loss of function background should not lead to a release of LRP6-GFP from the ER. This is for example shown in a study of Jung and colleagues, which describes the inhibition of Wnt-signaling by Mesoderm-specific transcript/paternally expressed gene 1 (Mest/Peg1) (Jung et al., 2011). Mest/Peg1 regulates the glycosylation of LRP6 in the ER. The inhibition of LRP6 glycosylation by Mest/Peg1 leads to an accumulation of LRP6 in the ER, the same phenotype as seen for the CD44 knockdown. Also here, ectopic expression of Mesd could not counteract this accumulation. Another maturation process of LRP6 in the ER is its palmitoylation. Like in the case for the suppression of LRP6 glycosylation by Mest/Peg1, an

inhibition of palmitoylation leads to an accumulation of LRP6 in the ER and an absence of membrane located LRP6 (Abrami et al., 2008). Also here ectopic expression of Mesd could not inhibit the ER retention of LRP6. These two examples demonstrate, that events that affect LRP6 maturation and thereby leading to ER retention of LRP6 cannot be rescued by ectopic expression of Mesd.

Remarkably, in my experiments overexpression of Mesd triggered the release of the retained LRP6-GFP from the ER and increased the amount of LRP6-GFP in the cytoplasm in CD44-siRNA transfected cells (Fig. 20). However, ectopically expressed Mesd could not restore the cell surface expression of LRP6 in CD44-siRNA transfected cells. This indicates that CD44 might rather function in the transport of the processed LRP6 from the ER to the membrane than in the processing or maturation of LRP6. The question is now, which of the steps involved in the membrane transport of LRP6 might be regulated by CD44. Experiments addressing the exact localization of LRP6-GFP in cells transfected with CD44-siRNA in combination with Mesd-cDNA, might answer this question. COP2 (Coat protein complex 2)-coated vesicles mediate the ER-to-Golgi transport of newly synthesized proteins. Thus, a colocalization of LRP6-GFP with a marker for COP2-vesicles would indicate that CD44 is required for the ER-to-Golgi-transport (reviewed in Duden, 2003). Furthermore, a Golgi-marker could be used to detect whether the LRP6-GFP, released by Mesd in the CD44 loss of function background, might now be localized in the Golgi-apparatus. This would indicate that CD44 might be required for LRP6 to leave the Golgi. A colocalization of LRP6-GFP and fluorescently-tagged carrier proteins that are involved in the transport of post-Golgi-vesicles like Conditional aggregation domain-GFP (CAD-GFP) (Jaiswal et al., 2009) might show that CD44 is rather required for the transport of LRP6-containing post-Golgi vesicles to the cell surface.

Indeed, I could show that CD44 is required for a proper localization of LRP6 at the cell surface, most probably regulating the trafficking and/or targeting of newly synthesized LRP6 from the ER to the plasma membrane. Hence, this finding provides strong evidence that CD44 regulates the canonical Wnt-pathway mechanistically by controlling the availability of LRP6 for Wnt-ligands at the membrane. The key question is now, how CD44 might regulate the trafficking of LRP6 to the membrane.

The important function of the intracellular CD44 domain in Wnt/β-catenin signaling

The data obtained during my PhD thesis, demonstrate that the regulation of Wnt/β-catenin signaling by CD44 is highly dependent on its cytoplasmic domain. Indeed, a CD44 mutant that lacked this cytoplasmic domain (CD44ΔCyt) was not able to augment Wnt-induced TCF/LEF regulated transcription (Fig. 9 A). This finding fits to the hypothesis that it is a common feature of all CD44 isoforms that is required for CD44-dependent Wnt-regulation, as the members of the CD44 family differ only in their extracellular part but share the same cytoplasmic domain (Screaton et al., 1992). The question is now which function could the cytoplasmic domain have in the Wnt-pathway?

Intracellular CD44 binding partners are involved in the regulation of Wnt/β-catenin signaling

In my PhD thesis I could show that a functional interaction of CD44 with its intracellular binding partners is crucial for a proper activation of the Wnt-pathway. Indeed, an interference with the binding of endogenous CD44 with its intracellular binding partners by an ectopic expression of a dominant-negative cytoplasmic CD44 domain repressed Wnt3a induced β-catenin activation and TCF/LEF regulated transcription (Fig. 9 B).

The cytoplasmic domain of CD44 interacts with many intracellular proteins, among them linker proteins that link CD44 to the actin-cytoskeleton, like ERMs, Ankyrin, N-WASP or IQGAP1 (Bennett and Stenbuck, 1979; Bourguignon et al., 2005b; Bourguignon et al., 2007c; Kalomiris and Bourguignon, 1988; Legg and Isacke, 1998; Turunen et al., 1994), GTPases of the Rho-family (Bourguignon, 2008; Bourguignon et al., 2007b; Bourguignon et al., 2000) and kinases of the Src-family (Bourguignon et al., 2001) or PKC (Legg et al., 2002). The interaction between CD44 and these proteins is involved in numerous cellular signaling events. The fact that CD44 is not able to regulate the Wnt-pathway in the absence of its cytoplasmic domain strongly indicates that a proper interaction between CD44 and its intracellular binding partner is crucial for the function of CD44 in the Wnt-pathway. Thus, CD44 most probably needs the linkage to the cytoplasmic domain and/or the interaction with GTPases and kinases.

Among the various proteins that bind to the cytoplasmic domain of CD44, the interplay between ERM proteins and CD44 is the best characterized and is often involved in signaling regulation by CD44 (Orian-Rousseau et al., 2007; Tremmel et al., 2009; Tsukita et al., 1994; Yonemura et al., 1998). The Ezrin mediated linkage of membrane bound or transmembrane proteins to the actin-cytoskeleton is involved in the regulation of several important signaling pathways such as RhoA-signaling (Mackay et al., 1997) or Hedgehog-signaling (Molnar and de Celis, 2006). In addition, the collaboration between a specific member of the ERM family, namely Ezrin and CD44 plays an important role in regulation of

signaling from c-Met, VEGFR-2 or Fas (Mielgo et al., 2007; Orian-Rousseau et al., 2007; Tremmel et al., 2009).

In my PhD thesis, I could show that the linkage of CD44 to the actin-cytoskeleton via Ezrin might play a crucial role the Wnt/β-catenin signaling pathway. On the one hand, mutation of the Ezrin binding sites in the dominant-negative cytoplasmic CD44 domain antagonized the inhibition of Wnt3a induced β-catenin activation and TCF/LEF regulated transcription (Fig. 9 B and C). On the other hand, a knockdown of Ezrin (the predominant ERM protein in the cell lines that were used in my thesis) as well as interference with the binding of ERMs to F-actin by ectopically expressed dominant-negative Ezrin strongly repressed Wnt3a to activate TCF/LEF regulated transcription (Fig. 10 B and C).

These results suggest that a proper activation of Wnt-signaling requires the connection of CD44 to cortical F-actin, most probably via ERM proteins like Ezrin.

Most notably, the data obtained during my PhD thesis provide evidence that ERMs, in addition to the connection of CD44 to F-actin, might even have a broader function in the Wnt/β-catenin signaling pathway. Epistasis experiments showed that a knockdown of Ezrin, similar to the knockdown of CD44, suppressed Wnt-pathway activation at the level of Wnt3a and LRP6 (Fig. 13). However, in contrast to the knockdown of CD44, the downregulation of Ezrin expression also interfered with Wnt-target gene transcription when activated at the level of Dsh or β-catenin, even though to a lower extend when compared to the inhibition of Wnt or LRP6 activated signaling (Fig. 13). The observation that Ezrin, in contrast to CD44, regulates Wnt-pathway activation also at the level of Dsh or β-catenin suggests that Ezrin might not exclusively regulate Wnt-signaling via its interaction with CD44.

Indeed, CD44 is not the only protein that is linked to the actin-cytoskeleton by ERMs. Noteworthy, also other proteins like CD43, ICAMs (Intracellular adhesion molecules); Syndecan-2, NHE-1 or -3 (Na+/H+exchanger-1 or -3) and CFTR (Cystic Fibrosis transmembrane conductance regulator) are connected to F-actin by Ezrin. Especially Syndecans as well as CFTR and CD43 have been shown to be involved in Wnt-signaling regulation (Alexander et al., 2000; Andersson et al., 2004; Balikova et al., 2012; Cohen et al., 2008). The fact that Ezrin can collaborate with other regulators of the Wnt-pathway might explain why Ezrin addresses additional steps of the Wnt-cascade when compared to CD44. Particularly, CD43 has been shown to collaborate with the Wnt-pathway at the level of β-catenin (Andersson et al., 2004). The cytoplasmic domain of CD43 contains a nuclear localization signal, can translocate to the nucleus and interact with β-catenin, in order to upregulate Wnt-target gene expression. As the knockdown of Ezrin affected activation of the whole Wnt-cascade down to the level of β-catenin, in particular a potential contribution of the interplay between CD43 and Ezrin should be addressed.

However, how could the linkage of CD44 to the cytoskeleton via Ezrin be important for the Wnt-pathway?

CD44 linkage to F-actin and LRP6-signalosome formation

As previously described, I could demonstrate that CD44 forms a Wnt-inducible complex with LRP6 and thus might take part in the formation of LRP6-signalosomes.
Remarkably, in contrast to full length CD44, a CD44 mutant that lacked the cytoplasmic domain was not able to complex with LRP6 upon induction with Wnt3a (Fig. 15). This suggests that either LRP6 interacts directly with the cytoplasmic domain of CD44 and/or that CD44 requires additional proteins that bind to its cytoplasmic domain to form a complex with LRP6. Remarkably, preliminary data obtained in our lab show that also the prevention of the binding of ERMs to F-actin by ectopical expression of DN-Ezrin inhibits the interaction between LRP6 and CD44 (data not shown). This is a strong hint that CD44 needs to be connected to F-actin via ERMs, in order to interact with LRP6 upon Wnt-induction.

I could show that CD44 complexes with LRP6 upon Wnt-induction even previously to the described formation of LRP6-signalosomes. Thus, CD44 might be required to initiate LRP6-signalosome formation. As CD44 requires its cytoplasmic domain and most probably its linkage to the actin-cytoskeleton to interact with LRP6, this might be a crucial event for LRP6-signalosome formation. Noteworthy, in the case of HGF/Met-signaling it is the proposed model that CD44 has a dual function as a co-receptor. On the one hand, the extracellular part of CD44 can bind to the growth factors HGF and VEGFA most probably in order to present them to their corresponding receptors (Volz et al., in preparation). On the other, hand the intracellular domain is required for the downstream signaling from the activated receptor. Indeed, a CD44v6 mutant that lacked the cytoplasmic domain was sufficient for HGF to trigger the phosphorylation of its receptor c-Met. However the downstream signaling from the receptor via the MEK/Erk pathway occurred only in the presence of full length CD44v6 (Orian-Rousseau et al., 2002). Further studies revealed that it is the binding of CD44 to Ezrin that is crucial for the activation of the downstream signaling from the Met-receptor upon HGF induction (Orian-Rousseau et al., 2007).

Here it is hypothesized that, mechanistically, the linkage of CD44 to F-actin via Ezrin mediates the spatial organization of the cytoskeleton, in order to form a signalosome complex involving i.a. the proteins Ras, Grb2 and Sos (Orian-Rousseau et al., 2007). Indeed, ERM-proteins are known to interact with several RhoGTPases, showing that they have the potential to bring cytoskeletal regulatory proteins in close apposition to the actin-cytoskeleton in order to promote cytoskeletal rearrangements in Hedgehog and Met-signaling (D'Angelo et al., 2007; reviewed in Fehon et al., 2010).

Hence, it is possible that a potential function of ERMs in assembling signalosome complexes via cytoskeletal rearrangements might be important for the activation of canonical Wnt-signaling, even more so as several signaling components of the Wnt-pathway can directly or indirectly bind to actin e.g. β-catenin or APC via IQ domain GTPase-activating protein 1 (IQGAP1) or Dsh via its DIX domain (Capelluto et al., 2002; Kuroda et al., 1998; Tirnauer, 2004). Also actin-cytoskeleton rearrangements have already been shown to have the ability to control Wnt/β-catenin activation in mesenchymal cells (Galli et al., 2012).

Further studies should address whether interference with the binding of CD44 to F-actin via Ezrin can inhibit the formation of LRP6-signalosomes. This would provide further evidence for an involvement of the CD44/Ezrin in this important step of Wnt-signaling activation. Moreover, treatment of cells with Wnt3a in the presence of inhibitors that prevent Actin-polymerization (e.g. Latrunculin or Cytochalasin), thereby abolishing the cortical organization of the actin-cytoskeleton and a subsequent analysis of the consequences on CD44 mediated regulation of Wnt-signaling, might shed light on the involvement cytoskeletal rearrangements in this regulation.

Noteworthy, ERM proteins are probably not the only intracellular CD44 binding proteins that might be involved in the function of CD44 in canonical Wnt-signalling and a possible contribution of CD44 to LRP6-signalosome formation. There are several intracellular proteins that interact with CD44 and which are involved in cytoskeletal rearrangements and the regulation of canonical Wnt-signaling. First, there is IQGAP1, a protein with multiple protein interacting domains. IQGAP1 is known to be able to remodel the membrane linked cytoskeleton via activation of Cdc42. Furthermore, IQGAP1 can directly bind to CD44 upon HA-treatment, in order to activate the MAPK/ERK-pathway (Bourguignon et al., 2005a), which in turn is able to upregulate β-catenin levels via inactivation of GSK3-β (Ding et al., 2005). Moreover, IQGAP1 binds directly to β-catenin, attenuating on the one hand, the binding of β-catenin to E-Cadherin at cell-cell adhesion complexes (Kuroda et al., 1998) and on the other hand, stimulating the co-activator function of β-catenin for TCF/LEF regulated gene transcription (Briggs et al., 2002). Interestingly, IQGAP1 is also able to bind to APC (Watanabe et al., 2004), which plays a role in the regulation of cytoskeletal networks in cell adhesion and migration (Noritake et al., 2004; Watanabe et al., 2004). However, studies of IQGAP1/APC interactions with regard to canonical Wnt-signaling are missing. Altogether, this is a hint that also IQGAP1 might play a role in the regulation of canonical Wnt-signaling by CD44.

A second candidate that interacts with CD44 and in addition plays a role in canonical Wnt-signaling, is Cdc42 (Bourguignon et al., 2005a). Cdc42 controls GSK3-β phosphorylation via modulating PKC-ζ (Wu et al., 2006), a serine/threonine kinase which was

also shown to be a downstream effector of CD44 (Fitzgerald et al., 2000). Absence of Cdc42 leads to a decrease in GSK3-β phosphorylation, this in turn leads to an increase of GSK3-β kinase activity resulting in degradation of β-catenin and reduced canonical Wnt-target gene transcription (Wu et al., 2006). Moreover, activated GSK3-β is able to phosphorylate the cytoplasmic tail of LRP6 (Mi et al., 2006) in order to activate the Wnt-pathway. Thus, it has to be analyzed whether Cdc42 as well as PKC-ζ are involved in the possible regulatory function of CD44 in the Wnt-pathway, namely to establish LRP6-signalosomes. Inhibition of Cdc42 by the selective inhibitor ML141 (Surviladze et al., 2010) in the context of CD44 gain of function might show whether blocking Cdc42 can counteract the augmentation of Wnt-signaling by CD44. Furthermore, this inhibitor could be used to study whether LRP6-signalosomes still form after inhibition of Cdc42.

A third protein that links CD44 and its cytoplasmic domain to the cytoskeleton and canonical Wnt-signaling is N-Wasp. It has been shown that N-Wasp is found in a complex with CD44 and Arp2/3 (actin-realted protein 2/3), a major regulator of actin-polymerization, upon HA treatment (Bourguignon et al., 2007c). HA treatment leads also to ErbB2 recruitment into this complex and stimulates the tyrosine kinase activity of ErbB2. Activated ErbB2 in turn can induce β-catenin tyrosine phosphorylation. This tyrosine phosphorylation suppresses the association of β-catenin with cell adhesion complexes, resulting in cytoplasmic accumulation, nuclear translocation of β-catenin and TCF/LEF-transcriptional co-activation (Bourguignon et al., 2007a; Daniel and Reynolds, 1997). Interestingly, N-WASP can interact with Cdc42 (Aspenstrom et al., 1996) like IQGAP1 and PKC. Cytoskeletal rearrangements might play an important role in LRP6-signalosome formation. Thus, it is likely that actin-regulators, like IQGAP1 or N-WASP, might be also involved in the regulation of Wnt-signaling by CD44, as they can interact with the cytoplasmic domain of CD44, as well as with major Wnt-pathway components.

CD44 linkage to F-actin via Ezrin and LRP6 membrane localization

The results of my PhD thesis, showing that the linkage of CD44 to the actin-cytoskeleton via ERM-proteins is essential for a proper activation of the Wnt/β-catenin signaling cascade, indicate that this linkage might also be crucial for CD44 to regulate LRP6 membrane localization. Indeed, interference with the binding of endogenous CD44 to its intracellular binding partners by ectopic expression of a dominant-negative cytoplasmic CD44 suppressed membrane expression of transfected LRP6-GFP in HeLa cells (Fig. 19). Mutation of the ERM binding sites in this dominant-negative cytoplasmic CD44 domain antagonized this effect. This result suggests that also the localization of LRP6 at the membrane is dependent on the interaction between CD44 and ERM proteins. In addition to this, the transfection of a DN-Ezrin mutant that prevents binding of endogenous Ezrin to F-

actin also suppressed cell surface localization of LRP6-GFP (Fig. 19). Altogether, these results suggest that the connection of CD44 to the cytoskeleton via Ezrin is crucial for a proper cell surface expression of LRP6.

But how might this regulation work and why should the linkage of CD44 to ERMs and via ERMs to the actin-cytoskeleton play a role in the membrane localization of LRP6?

Newly synthesized proteins are packed in vesicles that traffic between different compartments within the cell. For example, COP2-coated vesicles are mediating the ER-to-Golgi transport. Within the Golgi the proteins are sorted into different types of vesicles that are either transported to the lysosome or to the plasma membrane (reviewed in Anitei and Hoflack, 2011). Microtubules and actin-filaments serve as track for these vesicle transports and motorproteins like kinesins or myosins are the driving force that moves the vesicles along these tracks. For example, kinesin-1,-2 and -3 or myosin-1, -2 and -6 have been shown to mediate the transport of vesicles from the Golgi to the plasma membrane (reviewed in Anitei and Hoflack, 2011). Also regulators of actin-polymerization like Cdc42 or Rac1 have been shown to be involved in this transport. Cdc42 for example, regulates actin-polymerization through its direct bindig to N-WASP, which subsequently activates the Arp2/3 complex (Kolluri 1996, Hussain 2001). The Arp2/3 complex binds to existing actin-filaments and initiates the growth of a new filament at a distinctive 70° angle from the existing one. Thus, the Arp2/3 complex controls nucleation of actin-polymerization and branching of actin-filaments in order to form a functional actin-cytoskeleton (reviewed in Pollard, 2007). This functional cytoskeleton is required to bring vesicles from the Golgi to the plasma membrane of the cell.

Remarkably, it has been shown that CD44, as well as Ezrin, can interact with N-WASP and promote actin-assembly via the N-WASP-Arp2/3 machinery (Bourguignon et al., 2007c; Marion et al., 2011). Thus, it is possible that CD44 and the CD44/ERM/actin-interplay triggers the polymerization of actin-filaments at the membrane in order to generate a track that is necessary for the transport of LRP6 to the cell surface.

The working hypothesis: A possible dual function of CD44 in Wnt/β-catenin signaling regulation

The results of my PhD thesis indicate that both, the membrane targeting and the interaction with LRP6 are dependent on CD44 and most probably on its connection to the cytoskeleton via ERMs. However, the interaction between CD44 and LRP6 occurs only in the presence of Wnt-ligands.

Remarkably, the lack of membrane LRP6 upon CD44 knockdown is not Wnt-dependent, as LRP6 was also absent in cells that were not treated with Wnt. Thus, this observation is in contradiction with the hypothesis that LRP6 needs the interaction with CD44 to localize at the membrane.

This contradiction proposes a dual mechanism of CD44 as a positive co-factor in the Wnt-pathway. On the one hand, CD44 and its linkage to the actin-cytoskeleton might be required to target newly synthesized LRP6 to the membrane, thereby making more LRP6 available for Wnt-reception. This can even occur in the absence of Wnt-ligands. On the other hand, the presence of Wnt-ligands might bring CD44 together with LRP6, in order to establish, stabilize or internalize LRP6-signalosomes. If this hypothesis holds true, my PhD thesis identified CD44 not only as a novel essential positive co-regulator of Wnt/β-catenin signaling but also a new mechanism of growth factor signaling regulation by CD44.

5. Conclusions and perspectives

My PhD thesis describes a novel function of CD44 as a positive feedback-regulator of Wnt/β-catenin signaling that might be essentially involved in the fine-tuning of the activity and self-regulation of the canonical Wnt-pathway. In HEK293 and HeLa cell lines, in which CD44 has been described as Wnt-target gene (Li and Zhou, 2011; Sarkar et al., 2011), I could show that CD44 is essential for the Wnt-induced activation and nuclear translocation of β-catenin and furthermore for the transcription of TCF/LEF regulated genes (Fig. 4 A, 5 and 6). Moreover, I could demonstrate an *in vivo* requirement of CD44 for Wnt/β-catenin signaling in CNS development, as demonstrated by the reduced expression of the Wnt-target genes *tcf-4* and *en-2* (Fig.23). The data obtained during my work show that CD44 regulates the Wnt/β-catenin pathway at the level of the Wnt-receptor LRP6 (Fig. 12), most probably by a dual mechanism. On the one hand, CD44 regulates the cell surface localization of LRP6, demonstrated by the lack of membrane located LRP6 in the absence of CD44 (Fig. 17). This cell surface expression is needed for LRP6 to get access to extracellular ligands of the Wnt-family in order to promote downstream signaling from the membrane to intracellular components (Hsieh et al., 2003). On the other hand, CD44 is found in a stable complex with LRP6 upon Wnt-induction (Fig. 14), suggesting a participation of CD44 in LRP6-signalosome formation, stabilization and/or internalization, a prerequisite for proper Wnt/β-catenin pathway activation (reviewed in Niehrs and Shen, 2010). Moreover, my work shows that the regulation of Wnt/β-catenin signaling, LRP6 membrane localization and the CD44/LRP6 interaction is highly dependent on the cytoplasmic domain of CD44 and its linkage to F-actin via Ezrin (Fig. 9, 10 and 19).

It has been extensively shown that CD44 is involved in Wnt-induced tumorigenesis (Wielenga et al., 1999; Zeilstra et al., 2008; Zeilstra et al., 2013). Till now only the aspect of CD44 as a Wnt- target gene has been investigated. In colorectal cancer collaboration between CD44v6 and Met, another Wnt-target gene, might play a role (Orian-Rousseau et al., 2002). This is however only part of the story. Indeed, the finding that CD44 is not only a Wnt-target gene but also a Wnt-pathway regulator sheds new light on the role of CD44 in Wnt-induced tumorigenesis and broadens the mode of action of CD44 in colorectal carcinogenesis.

Especially in cancer stem cells (CSC), the regulation of Wnt-signaling by CD44 might be an important issue. CSCs are believed to be a subpopulation of cells within tumors that are entirely responsible for tumorigenesis (reviewed in Malanchi and Huelsken, 2009), thus finding a way to eliminate these cells might be an important step towards an efficient anti-cancer therapy. Wnt/β-catenin signaling is known to be a main regulator for the self-renewal and maintenance of these CSCs and therefore a promising target of anti-cancer therapy

(reviewed in Reya and Clevers, 2005). CD44 is highly abundant in these cells and therefore often cited as a marker of CSCs (reviewed in Yu et al., 2012). The finding that CD44 is an essential regulator of the canonical Wnt-pathways opens up new perspectives that can lead to a better understanding of the nature and regulation of CSCs. Furthermore, this finding might give rise to new promising therapeutic approaches that specifically target CSCs, which are thought to be the beating hearts of malignant cancers.

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CD44 functions in Wnt signaling by regulating LRP6 localization and activation

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In preparation:

CD44v6 peptide functionalized quantum dot nanoparticles selectively bind on the tumor cells

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