Molecular Dissection of Mouse Wnt3a-Frizzled8 Interaction Reveals Essential and Modulatory Determinants of Wnt Signaling Activity

DISSERTATION

Submitted to the

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Sumit Kumar

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Presented by

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Dedicated to my parents and my wife Shweta

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Table of Contents

1. Summary

Wnt/β-catenin signaling plays a critical role in animal development and adult tissue homeostasis, including regulation of cell fate decisions, axial pattering, organogenesis and stem cell maintenance. Deregulation of Wnt signaling causes many human diseases, including cancer and osteoporosis. Wnt proteins constitute a large family of growth factors characterized by conserved cysteine and a lipid modification at their N-terminal half. Upon transport through a specialized secretary route they act as morphogens in variety of tissues by forming concentration gradient in the extracellular space. The post-translational addition of palmitate or palmitoic acid to Wnts renders them highly hydrophobic and is believed to control their membrane localization, it is unknown, however, how Wnts are recruited from the membrane to signaling complexes and how their signaling range is regulated.

Wnts bind to Frizzled (Fz) seven transmembrane receptors and LRP5/6 co-receptors, which leads to stabilization of the transcription coactivation of β-catenin and activation of target gene. The structural basis of Wnt signaling by receptor binding has long been elusive. Only recently, the crystal structure of the *Xenopus* Wnt8-Frizzled8-CRD complex was solved, but the significance of the interaction sites for signaling has not been assessed. In the present thesis, by using a structure-based mutagenesis approach, I present an extensive structure-function analysis of mouse Wnt3a by *in vitro* and *in vivo* evaluation. Evidence is provided for an essential role of Serine 209, Glycine 210 (site 1) and Tryptophan 333 (site 2) in Fz binding. Importantly, I discovered that Valine 337 in the site 2 binding loop is critical for signaling without contributing to binding. Mutations in the presumptive second CRD-binding site (site 3) partly abolished Wnt binding, suggesting Fz dimerization as a necessary step in signaling. Intriguingly, most site 3 mutations increased Wnt signaling, probably by inhibiting Wnt-CRD oligomerization. In accordance, it was observed that increasing amounts of soluble Fz8-CRD protein modulated Wnt3a signaling in a biphasic manner. Based on these finding, a model was developed, in which a concentration-dependent switch in Wnt-CRD complex formation from an inactive aggregation state to an activated, high mobility state, represents a modulatory mechanism in Wnt signaling gradients.

2. Zusammenfassung

Der Wnt/β-Catenin Signalweg spielt sowohl in der Entwicklung als auch in der Homöostase tierischer Gewebe eine wichtige Rolle. So werden Prozesse wie die Determinierung des Zellschicksals, axiale Musterbildung, Organogenese und die Erhaltung der Stammzellnische durch ihn reguliert. Viele Erkrankungen wie Krebs oder Osteoporose sind in der Fehlregulation dieses Signalwegs begründet. Wnt-Proteine bilden als Wachstumsfaktoren eine große Familie, die durch konservierte Cysteine und eine Lipidmodifikation in ihrer N-terminalen Hälfte charakterisiert ist. Nach dem Transport entlang eines spezialisierten, sekretorischen Wegs bilden sie einen Konzentrationsgradienten in der extrazellulären Matrix, wo sie in verschiedenen Geweben als Morphogene wirken. Palmitat und Palmitinsäure sind post-translationale Modifikationen der Wnt-Proteine, die ihnen einen stark hydrophoben Charakter verleihen und somit die Membranlokalisierung kontrollieren können. Es ist jedoch weder bekannt wie Wnt-Proteine von der Membran zu Signalkomplexen rekrutiert werden, noch wie ihre Signalintensität reguliert wird.

Wnt-Proteine binden an die Transmembranrezeptoren der Frizzled (Fz)-Familie und die Co-Rezeptoren LRP5/6, wodurch β-Catenin stabilisiert und eine Transkription von Zielgenen initiiert wird. Die strukturellen Grundlagen des Wnt-Signalwegs auf der Ebene der Rezeptorbindung blieben lange ungeklärt. Erst kürzlich wurde der *Xenopus* Wnt8-Frizzled8-CRD-Komplex röntgenkristallographisch aufgeklärt, die Bedeutung der Interaktionsstellen für das Signalgeschehen auf biologischer Ebene blieb jedoch weiterhin offen. In der vorliegenden Arbeit wird eine ausführliche Struktur-Funktionsanalyse des murinen Wnt3a-Moleküls vorgelegt, welches durch eine struktur-basierte Mutagenese *in vitro* und *in vivo* charakterisiert wurde. Es wurde gezeigt, dass sowohl Serine 209, Glycine 210 (Region 1) als auch Tryptophan 333 (Region 2) essentiell für die Fz-Bindung sind. Darüber zeigte sich Valin 337 in der Bindungsschleife der Region 2 als entscheidend für den Signalprozess, jedoch nicht für die Bindung. Weiterhin zeigten sich Hinweise darauf, dass die Dimerisierung der Fz-Rezeptoren ein wichtiger Schritt bei der Initiierung des Signalwegs ist, da Mutationen in der präsumptiven, zweiten CRD-Bindungsregion (Region 3) zum Teil die Aktivität von Wnt3a inhibierten. Die meisten Mutationen in der Region 3 verstärkten jedoch die Signalstärke vermutlich durch die Inhibition der Wnt-CRD-Oligomerisierung. Unterstützt wird diese These durch die Beobachtung, dass steigende Konzentrationen an löslichem Fz8-CRD Protein die Wnt3a-Signalintensität biphasisch modulierten. Basierend auf diesen Ergebnissen wurde ein

Modell entwickelt, in welchem ein Übergang des Wnt-CRD Komplexes von einem inaktiven, aggregierten Zustand zu einem aktivierten, hoch mobilen Zustand in Abhängigkeit der CRD-Konzentration postuliert wird, Dieser stellt einen neuen modulatorischen Mechanismus bei der Wnt-Gradientenbildung dar.

3. Introduction

Cell-cell interactions through different signaling pathways play a crucial role in the coordination of embryonic development. In metazoans, at least 17 different types of signal transduction pathways operate, each pathway is used repeatedly during the development of an organism (Gerhart, 1999). The five major cell-cell signaling pathways, which control a vast majority of cell fate decisions during animal development are: the Hedgehog (Ingham & McMahon, 2001) TGFβ (including Nodal/BMP) (Shi & Massagué, 2003), Notch (Bray, 2006) receptor tyrosine kinase (EGF/FGF) (Tan & Kim, 1999) and Wnt (Logan & Nusse, 2004) pathways. Each pathway activates different subsets of target genes in different developmental contexts. Despite the existence of many pathways branches, only the β-catenin signaling branch has been implicated in body-axis specification. The Wnt signaling pathway is the main focus of this thesis and will be discussed in detail.

3.1 The Wnt signaling pathway

The Wnt signaling pathways are highly conserved among metazoans and have been demonstrated to be essential for animal development and tissue homeostasis. The aberrant Wnt signaling leads to varieties of diseases, including cancer, bone diseases, cardiovascular diseases (Cadigan & Nusse, 1997; Clevers, 2006; Logan & Nusse, 2004; MacDonald et al, 2009). Wnt signaling also play an important role in dorsoventral axis specification. The specification of the dorsoventral axis coincides with the formation of an essential embryonic signaling centre known as the Spermann organizer in amphibians, the shield in zebrafish and the node in the mouse (Harland & Gerhart, 1997; Marlow, 2010; Schier & Talbot, 2005). The Wnt signaling pathway is tightly regulated by a large and complex array of proteins, which act as agonists and antagonists of signal transduction modulating Wnt signaling at different levels. There are generally considered to be three major branches of Wnt signaling based on different signaling mechanisms and biological responses.

(1) The β-catenin dependent pathway (canonical pathway), which operates though transcription control of target genes and regulates body axis pattering, cell fate specification, cell proliferation.
(2) The β-catenin independent pathway (non-canonical pathway) also called Wnt/Calcium pathway, which activates Calcium-dependent cellular responses, and it is thought to antagonize Wnt/β-catenin signaling and mostly regulate cell migration.
(3) The planar cell polarity pathway (PCP pathway) mainly involved in cytoskeletal changes and responsible for cell polarity and tissue movements.

Most Fz proteins can activate all three pathways but with different efficiencies. The activation of a certain pathway is particularly dependent on the Wnt ligand, the co-receptor and the cell type.

3.2 Molecular mechanism of β-catenin dependent pathway (Canonical pathway)

Canonical Wnt signaling controls cell specification and proliferation through the regulation of the transcription factor β-catenin, which activates the expression of numerous Wnt target genes (MacDonald et al, 2009; Marlow, 2010). When canonical Wnt signaling is inactive, a group of proteins named the destruction complex promotes β-catenin degradation resulting in the lack of β-catenin-inducible genes. The destruction complex consists of Axin, *Adenomatous polyposis coli* gene (APC), glycogen synthase kinase 3β (GSK3β) and casein kinase (CK1). Serine/threonine kinase CK1 and GSK3β phosphorylate the amino terminal region of β-catenin, which is subsequently ubiquitinated by β-Trcp E3 ubiquitin ligase resulting in its degradation (Liu et al, 2002; Yanagawa et al, 2002). This continual elimination of β-catenin prevents it from reaching the nucleus and Wnt target genes are thereby repressed by the DNA-bound T cell factor/lymphoid enhancer factor (TCF/LEE) (Fig. 1A). A hallmark of the canonical Wnt signaling pathway activation is the elevation of cytoplasmic free β-catenin protein levels. β-catenin is a bifunctional protein and exists in two separate pools in the cell.

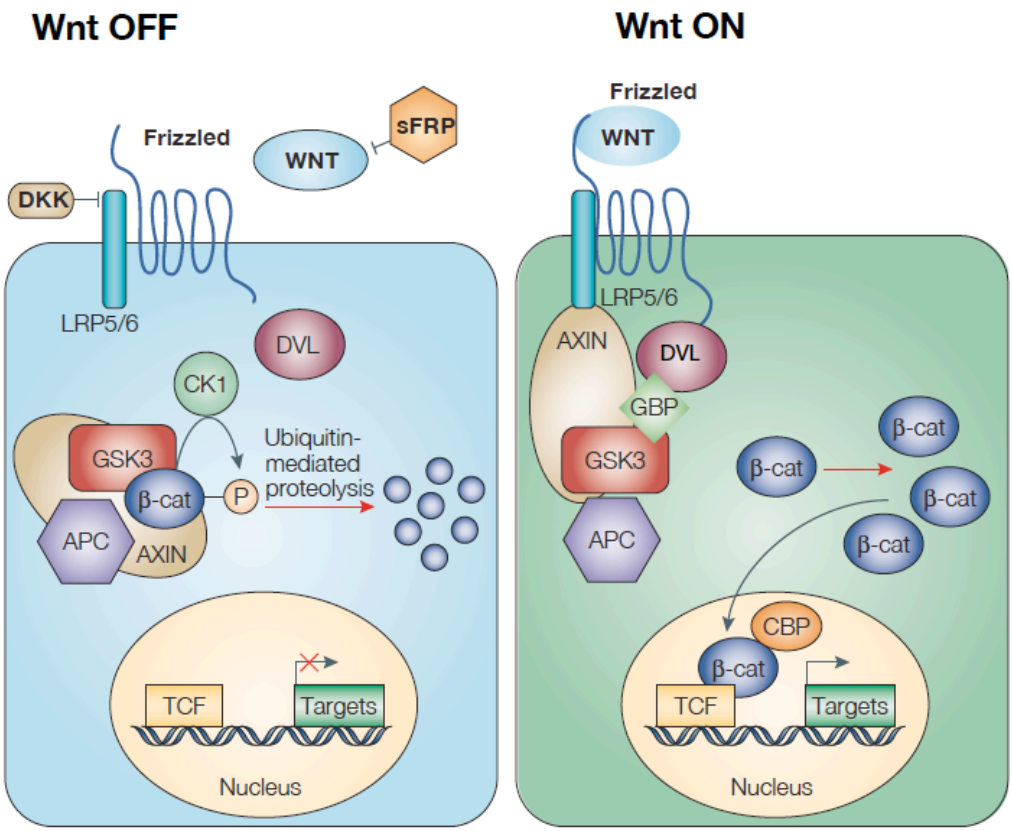

Fig.1. Overview of the Wnt/β-catenin signaling pathway. (A) In the absence of Wnt ligands cytoplasmic β-catenin is constantly degraded and prospective target genes are in a repressed state. **(B)** Binding of Wnt to Frizzled and LRP5/6 receptors induces recruitment of Axin and Dishevelled (Dvl) to the membrane, thereby disrupting the destruction complex. β-catenin can translocate into the nucleus and act as transcriptional co-activator. Picture taken and modified from (Moon et al, 2004).

At the membrane, it facilitates cell-cell adhesion by connecting E-cadherin to the actin cytoskeleton via α-catenin. The cytoplasmic soluble pool of the β-catenin is involved in the transmission of the Wnt signal from the membrane to the nucleus. The Wnt/β-catenin pathway is activated when Wnt ligands bind to seven-pass transmembrane receptor Frizzled (Fz) and its co-receptor, LRP5/6 and leads to the formation of Wnt-Fz-LRP5/6 trimeric complex. The cytoplasmic protein disheveled (Dvl) is recruited, phosphorylated and activated. Activation of Dvl promotes the dissociation of GSK-3β from Axin and leads to the inhibition of GSK-3β.

Phosphorylated LRP5/6, activates and recruits the Axin complex to the receptors, which leads to the inhibition of Axin-mediated β-catenin phosphorylation and thereby to the stabilization of cytoplasmic β-catenin, which accumulates and travels to the nucleus. Nuclear β-catenin is the ultimate effector, binding to Tcf/Lef (T cell factor and lymphoid-enhancing factor) transcription factors that lead to changes in the expressions of different target genes that regulate cell proliferation, differentiation, survival, cell polarity and even angiogenesis (Fig.1B).

3.3 Wnt ligands and their Biogenesis

Wnt ligand genes encode a family of evolutionary conserved secreted glycoproteins. Most mammalian genomes, including the human genome, which harbors 19 Wnt genes, categorized into 12 conserved Wnt subfamilies. Lower metazoans like Hydra and *Nematostella* also have an almost complete set of Wnt genes emphasizing the crucial role of Wnt signaling (Holstein, 2012). Sponges also contain few Wnt genes, whereas single-cell organisms do not have any Wnts, suggesting that Wnt signaling may have been instrumental in the evolutionary origin of the multicellular animals (Petersen & Reddien, 2009).

All Wnt proteins share common characteristics: they are 350-400 amino acids long and have a corresponding molecular weight ~40 kDa and contain 22-26 conserved cysteine residues contributing to proper folding of the ligand (Coudreuse & Korswagen, 2007; Tanaka et al, 2002). Wnt proteins do not contain obvious domains that are shared with other proteins, except the N–terminal signal peptide that targets them to the secretory pathway. The primary amino acid sequence of Wnts contains several charged residues, and predicted to be soluble in aqueous solution, but when Wnt proteins were first isolated, a surprising finding was that they are highly hydrophobic (Bradley & Brown, 1990). The efficient production and biochemical characterization of Wnt proteins remains challenging. Mouse Wnt3a was the first purified and biochemically characterized Wnt (Willert et al, 2003). In addition to N-glycosylation, which is required for Wnt secretion (Komekado et al, 2007), Wnt3a also undergoes two types of lipid modifications that are responsible for the hydrophobicity and poor solubility of Wnt proteins (Hausmann et al, 2007).

The first reported lipid modification was the addition of palmitate to cysteine 77 (Willert et al, 2003). Mutation of Cys77 to Ala had minimal effect on Wnt3a secretion but diminished the ability of Wnt3a to activate Wnt signaling (Galli et al, 2007; Komekado et al, 2007; Willert et al, 2003). The second identified lipid modification was a palmitoleoyl attached to conserve serine 209 and its mutation leads to Wnt3a accumulation in endoplasmic reticulum (ER) and inhibits its secretion (Takada et al, 2006). Wingless (Wg) protein from *Drosophila* is the most investigated Wnt molecule *in vivo* (Hausmann et al; 2007). Porcupine encodes a multiple transmembrane ER protein that contains an O-acyl transferase domain, suggesting a role in Wg lipid modification (Hausmann et al; 2007). Porcupine deficiency results in Wg and Wnt3a accumulation in ER and inhibits Wnt3a palmitoleoylation at Ser 209 (Takada et al; 2006), provides the evidence that Porcupine is responsible for this lipid modification. It has been certainly remains unknown whether Porcupine or a distinct acyltransferase is involved in Wnt3a palmitoylation at cysteine 77.

In addition to this, two other proteins were indentified for Wg/Wnt secretion: Wntless (Wls) also known as Evenness interrupted (Evi) in *Drosophila* and the retromer complex in nematodes (Hausmann et al, 2007). Wls is a multipass transmembrane protein, which is localized to the Golgi, endocytic compartments and the plasma membrane, required for Wg secretion (Banziger et al, 2006; Bartscherer et al, 2006). The retromer complex mediates membrane protein trafficking between endosomes and the Golgi apparatus (Hausmann et al, 2007). Wnt proteins that are glycosylated and lipid modified by Porcupine in the ER and are escorted by Wls from the Golgi to the plasma membrane for secrection. Drosophila WntD protein neither requires Porcupine and Wls for secrection nor is palmitoylated at the conserved cysteine residue (Ching et al, 2008).

3.4 Structure of Wnt proteins

It took almost 30 years to achieve the first high–resolution structure of a Wnt protein. The 3.25 Å crystal structure of *Xenopus laevis* Wnt8 in complex with mouse Fz8-CRD has recently been solved by X-ray crystallography (Janda et al, 2012) (Fig. 2) and yielded important new insights into Wnt biochemistry. In the complex, XWnt8 forms an unusual structure resembling a human hand with a central ‘‘palm’’ that extends a ‘‘thumb’’ plus an index ‘‘finger’’ to grab the Fz8-CRD on two opposite sides, without changing Fz8-CRD configuration. It reveals two-domain structure the NTD mainly containing α-helixes and the lipid-modified thumb. A fatty acid adduct attached to serine 187 at the tip of the thumb, inserts into hydrophobic groove of Fz8-CRD constituting the Wnt8-Fz8-CRD binding site 1 that has extensive hydrophobic interactions between the lipid and apolar residues of Fz8-CRD, in a ‘lipid in groove’ fashion. The remaining site 1 portion contributes to binding by protein-protein interactions between residues of the Wnt8 thumb loop and Fz8-CRD. The core structure of NTD has a saposin-like fold that superimposes well with bacterial atypical saposin-like protein, an ancient class of lipid binding and carrier domains (Bazan et al, 2012).The CTD constitutes the index finger and has two anti parallel β-strands and a long intervening loop, which is stabilized by several disulphide bonds and engages in hydrophobic contacts within a depression of Fz8-CRD, in a ‘knob-in-hole’ fashion. This constitutes the Wnt8-Fz8-CRD interaction site 2. Both site 1 and site 2 are dominated by hydrophobic contacts, which are mostly mediated by conserved residues of Wnt8 and Fz8-CRD, suggesting a possible explanation for broad multiple specificity of Wnt-Fz interactions. A single Wnt can often binds to multiple Fz proteins and vice versa. However, residues of Wnt8 at both site 1 and site 2, also exhibit protein-protein interactions with Fz8-CRD residues that are conserved in some but altered in other Fz proteins, suggesting certain selectivity on top of broad specificity in Wnt-Fz interactions.

XWnt8 contains a region between the thumb and the index finger similar to the palm, where NTD and CTD corresponding to the region with the greatest flexibility among Wnt proteins, in particular, Wg carries a large insert of 80 amino acids not present in other Wnts. This Wg insert has been used to generate arguably the best antisera to any

Wnt protein (van den Heuvel et al, 1989), it also supports the model that the nonconserved linker region is solvent exposed and likely not involved in engagement to the Fz-CRD.Another potential interaction site observed in the crystal structure is called ‘‘pseudo-site 3’’

The residues involved in this interaction are poorly conserved among different Wnts. Pseudo-site 3 was suggested to be responsible for formation of asymmetric dimers in the crystal generating a repeating oligomer. Thus it may account for Wnt-induced receptor clustering and signalosome assembly. However it could also be an artifact due to crystal packing. The physiological relevance of pseudo-site 3 still remained to be examined.

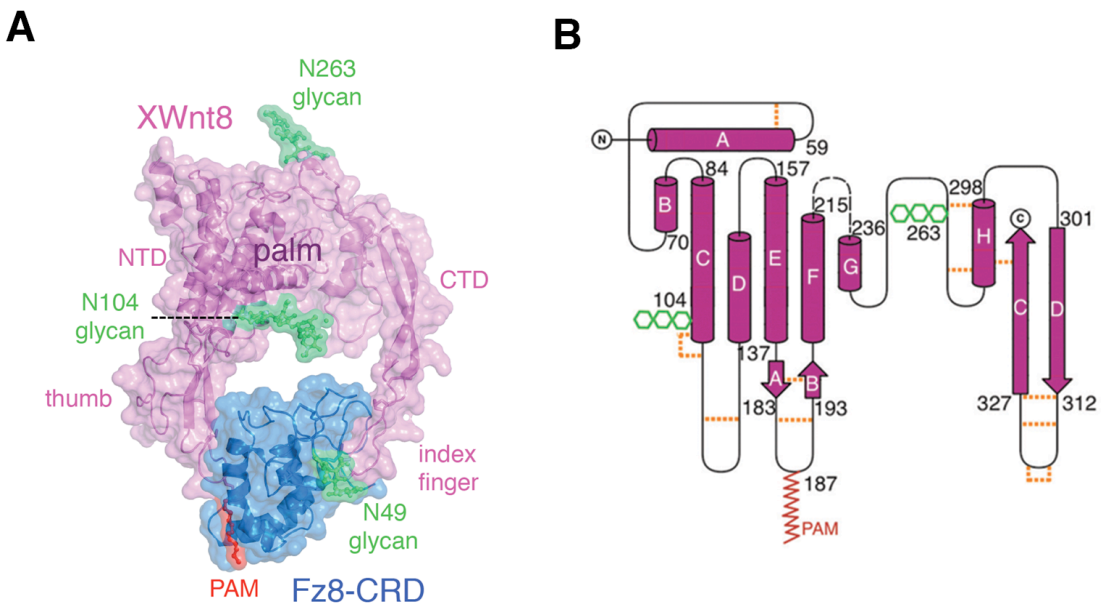

Fig.2. Crystal structure of XWnt8-Fz8-CRD complex (A) Surface representation of XWnt8 bound to mFz8-CRD. N-linked glycosylation of Wnt8 marked with green clusters and Palmitoleic acid (PAM) is shown in red. **(B)** Secondary structure of Xwnt8. The conserved 22-cysteine residues are numbered to indicate the pairs that form disulphide bridge is shown by orange lines. N-linked glycosylation sites are shown by green cartoon. Picture taken from (Janda et al, 2012).

William Weis' laboratory recently reported the 2.5Å resolution crystal structure of a *Drosophila* WntD fragment encompassing the N-terminal domain and the linker that connects it to the C-terminal domain (Chu et al, 2013). There are some differences in the structure of WntD and *Xenopus* Wnt8. Usually all Wnt proteins have a conserved serine residue at the tip of the β hairpin that connects two helices. This residue is modified by lipidation in Wnt3a (Ser209). WntD, however contains a glutamine residue rather than a serine at this position. In the WntD the tip of the hairpin folds back to create a fist-like structure, which is stabilized by a hydrogen bond. However, the tip of the XWnt8 hairpin forms an extended conformation and is stabilized by the lipid contact with the Fz8-CRD. An unusual feature of the WntD structure is the presence of a large solvent filled cavity between helices A C D E, however this cavity is much narrower in XWnt8.

3.5 Frizzled receptors and their interaction with Wnts

Calvin Bridges discovered a recessive *Drosophila* mutant, which he called *frizzled (fz)* and which was responsible for irregularly orientated hairs and ommatidia. The Fz family comprises 10 members in humans, and Fz proteins were the first Wnt receptors identified (Bhanot et al, 1999). The topology of all frizzled receptors is similar to G protein-couple receptors (GPCR) (Vinson et al, 1989). They are the most widespread Wnt receptors and induce both β-catenin-dependent and β-catenin-independent signaling. The identified Fz receptors contain common structural features, which includes a highly conserved cysteines-rich domain (CRD) at the amino-terminus, a seven-transmembane domain and a short cytoplasmic tail at the carboxy-terminus (Fig. 3A). The highly conserved CRD domain (10 cysteine), mediates high-affinity interactions with Wnt proteins (Hsieh et al; 1999). However, recent finding suggest that proteins from other families- sFRPs, R-spondin and Norrin can also directly bind to Fzs and activate downstream signaling cascade (Nam et al, 2007; Rodriguez et al, 2005; Smallwood et al, 2007; Xu et al, 2004). The sFRPs were initially seen as Wnt antagonists that prevented Wnt binding to Fz. However, there are some reports, which suggested that direct binding of sFRPs to Fz-CRDs probably occur by CRD dimerization (Bafico et al, 1999), which leads to the receptor activation....

(Rodriguez et al, 2005). Receptor dimerization through CRD/CRD interaction in particular olimomerization, appears to be important for Fz maturation and proper exposure to the proteins at the cell membrane it has been shown for Fz1 and Fz4 (Kaykas et al, 2004).

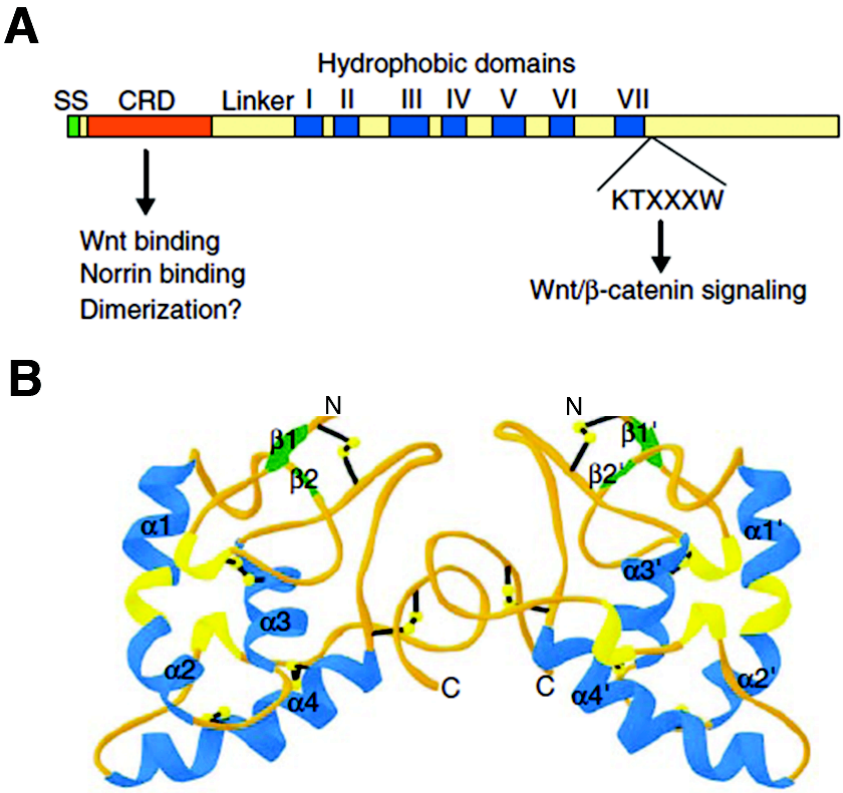

Fig.3. Frizzled structure. (A) Schematic representation of Frizzled protein. SS, signal sequence; CRD, cysteine-rich domain. The CRD is extracellular and binds to Wnts. The C-terminus is intracellular and contains a proximal KTXXXW motif. **(B)** Crystal structure of the mFz8-CRD as dimer, α-helices in blue, 3_{10}–helices in yellow, β-strands in green, coil in gold. Disulphide bonds are shown as ball-and-stick model in yellow and black. Picture taken from (Dann et al, 2001; Huang & Klein, 2004).

The potential Wnt binding sites of the CRD have been mapped using a binding assay that detects direct binding of XWnt8 to the CRD tethered to plasma membrane through glycophosphotidylinositol (GPI) anchor (Hsieh et al, 1999). The CRDs of Fz1

and Fz2 in *Drosophila* and Fz8 in mice have been shown to bind to Wnt proteins with nanomolar affinity (Hsieh et al, 1999; Rulifson et al, 2000; Wu & Nusse, 2002). However, the mutated Fz receptors that omit the CRD domain may still be able to bind Wnt ligands and influence signaling activity, but less efficiently than their wild type counterparts (Chen et al, 2004; Povelones & Nusse, 2005). In addition to this, it has been found that when these mutated Fz receptors are fused with either other Wnt ligand-binding domain or directly linked to other Wnt ligands, they are capable to restore the Wnt signaling activity (Cadigan & Liu, 2006; Chen et al, 2004; Povelones & Nusse, 2005). These findings collectively suggest that the CRD functions as binding domain for Wnt ligands, which is indispensable.

The finding that the CRD of Fz is indispensable for Wnt/-β catenin signaling is not incompatible with the model that Wnt ligands induce the Fz-LRP oligomerization but argues against the idea that Wnt acts as an adaptor between the two co-receptors. It is more likely possible that binding of Wnt to LRP induces a conformational change that makes binding to Fz favorable. Constitutively active Wnt-Fz$^{\Delta CRD}$ fusion proteins could bind to LRP. Indeed, it has been reported that Wnt-Fz$^{\Delta CRD}$ fusion protein requires β-catenin for its signaling activity (Povelones & Nusse, 2005). The role of Fz receptors in Wnt signaling has now clearly been established; however, the specificity of the interaction between the various Wnt ligands and Fz receptors remains elusive. This is probably due to the large number of Wnt ligands and Fz receptors, the potential multitude of Wnt/Fz interactions and because of mutual functional redundancies in both the ligands and receptors (He et al, 2004).

The crystal structure of mouse Fz8-CRD shows that the CRD consists mainly of α-helical stretches and two short sequences forming a minimal β-sheet at the N-terminus representing a novel protein fold (Dann et al, 2001) (Fig. 3B). A ligand-binding surface was predicted from the analysis of the crystal structure integrated by comprehensive mutagenesis. It has been found that the CRD forms a conserved dimer interface, although in solution they appear to exist as monomer. Dimerization of Fz-CRD has a role in activation of Wnt/β-catenin signaling (Carron et al, 2003).

Further regulation of Fz receptors can occur through intracellular binding to cytoplasmic partners, primarily through the Fz cytoplasmic tail or the intracellular domain. The key cytoplasmic partner of Fz is a scaffold protein called Dishevelled (DVL), which interacts with most Wnt co-receptors and transduces all major Wnt subpathways. Fz engages with DVL proteins through a highly conserved KTXXXW motif (Kikuchi et al; 2011). Once ligands binds to Fz, DVL proteins are recruited to Fz receptors at the plasma membrane, and co-recruit other cytoplasmic transducers, such as axin, GSK3 and CK1, presumably along with their partners, to promote β-catenin-dependent signaling. It has been found that DVL itself can form oligomers (Schwarz-Romond et al, 2007), which might be responsible for clustering Wnt-Fz receptor complexes and promote endocytosis and downstream signaling (Bilic et al, 2007).

3.6 Other Wnt Co-receptors: LRP5/6, ROR2 and Ryk

The complexity of Wnt/Fz signaling is further increased by the discovery of co-receptors for Fz and alternative Wnt receptors. In general co-receptors are cell surface proteins that associate with a ligand or its primary receptor to enhance complex formation and downstream signaling. In β-catenin-dependent signaling, Wnt-Fz forms a ternary complex with LRP5/6, whereas in the PCP signaling pathway they interact with ROR or PTK7 (Hikasa et al, 2002; Nishita et al, 2010; Oishi et al, 2003). Frizzled can also engage with RYK to activate β-catenin-dependent, PCP and Wnt-Ca^{2+} downstream pathways (Fig.4).

Members of the low-density lipoprotein receptor related protein family, LRP5 and LRP6, are now well recognized as co-receptors in β-catenin-dependent signaling (He et al; 2004). The extracellular domain (ECD) engages with Wnt and Fz and forming a ternary complex, the ECD contains multiple, independent Wnt-binding sites, which allow different Wnts to bind simultaneously in conjunction with Fz (Bourhis et al, 2010). The main mechanism of regulating LRP6 function is through phosphorylation of its intracellular domain (ICD) which is sufficient to activate β-catenin-dependent signaling (Bourhis et al, 2010; Zeng et al, 2005). The ICD contains five PPPSXP

motives, phosphorylation of which is required for β-catenin-dependent signaling. GSK-3β is the master negative regulator of β-catenin, mediating phosphorylation of the PPPSXP site in response to Wnt signaling and thus plays both a positive and negative role in β-catenin-dependent signaling (Zeng et al, 2005).

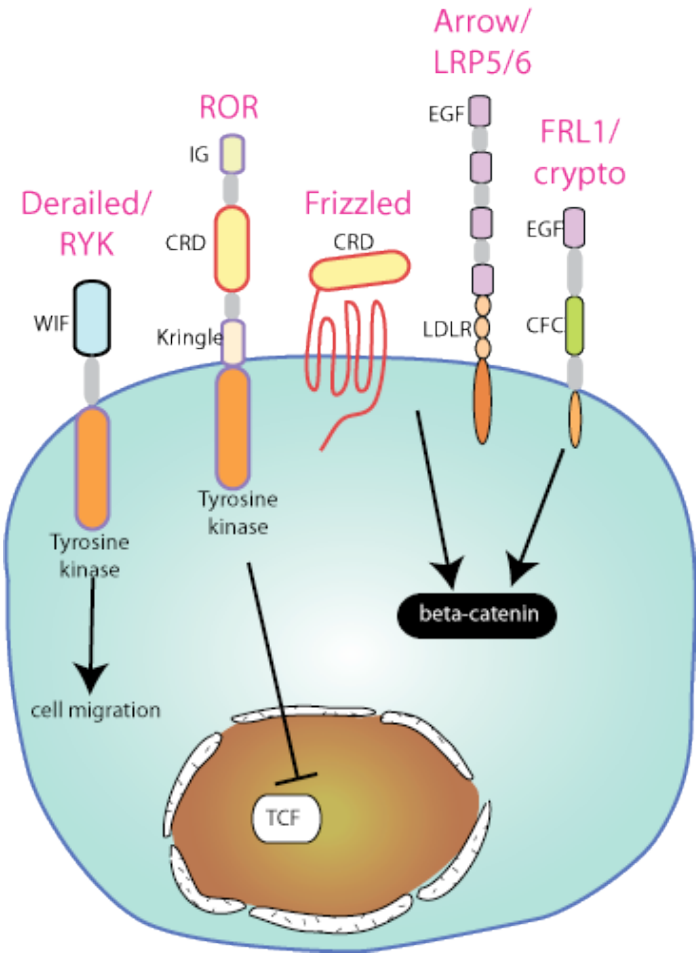

Fig.4. Different Wnt receptors on the cell membrane. Picture taken from Wnt homepage (http://www.stanford.edu/~rnusse/frizzleds/receptors.html).

CKIγ, a member of CKI mediates phosphorylation at adjacent Ser residues (PPPSPXS), which is required for Wnt signaling (Davidson et al, 2005). LRP6 may also be regulated through cleavage by complement component C1q (Naito et al, 2012).

In the presence of C1q, the LRP6 ECD is cleaved, which activates β-catenin-dependent signaling.

Another Wnt binding receptor is ROR, an orphan receptor that is member of the tyrosine kinase family. RORs share the cysteine-rich domain (CRD) with Fz (Kani et al, 2004; Mikels & Nusse, 2006). ROR1 and ROR2 bind Wnt5a and mostly activate PCP signaling in vertebrates (Hikasa et al, 2002; Oishi et al, 2003). Wnt5a is the principal ROR ligand *in vivo*. Wnt5a binding induces homodimerization of ROR2 (Liu et al, 2008) and formation of a ternary complex with Fz (Grumolato et al, 2010; Sato et al, 2010), which leads to the recruitment of Dvl, Axin, GKS3, which ultimately activates the receptor (Grumolato et al, 2010; Nishita et al, 2010; Witte et al, 2010). ROR2 is also activated by forced dimerization (Green et al, 2008).

Apart from ROR, Ryk also called Derailed in *Drosophila* is a member of transmembrane tyrosine kinases. These have a Wnt binding domain similar to the WIF proteins, a group of Wnt binding factors(Hsieh et al, 1999). They play an important role in the central nervous system particularly in axon guidance and neuronal cell fate determination (Clark et al, 2012; Kikuchi et al, 2011).

3.7 Extracellular Regulation of Wnt signaling

Wnt signaling is regulated at different levels by a wide range of effectors. These effectors function as antagonists or agonists and perform their function either intracellularly to regulate components of the signal transduction machinery or extracellularly to modulate ligand-receptor interactions. Antagonist and agonist play critical roles, as they control the fine-tuning of Wnt signaling and are able to inhibit or activate Wnt-regulated developmental processes for example anterior-posterior (AP) axial patterning, angiogenesis, somatogenesis, bones, limbs and eye formation. Aberrant Wnt signaling also leads to many pathological events such as cancer and bone disease. Secreted Frizzled-related proteins (sFRPs) and Wnt inhibitory protein (WIF) bind to Wnt ligands and in the case of sFRPs also to Fz, and therefore they

function as Wnt antagonist for both β-catenin and noncanonical signaling (Bovolenta et al, 2008). Based on biochemical and functional analyses using cultured cells and *Xenopus* embryos it was shown that Frzb/sFRP3 binds to XWnt8 and Wnt1 and inhibits Wnt/β catenin signaling (Leyns et al, 1997; Wang et al, 1997). These results suggest a model wherein sFRPs inhibit Wnt signaling by sequestering Wnt ligands away from active receptor complex (Fig. 5). It has been found that the CRD domain

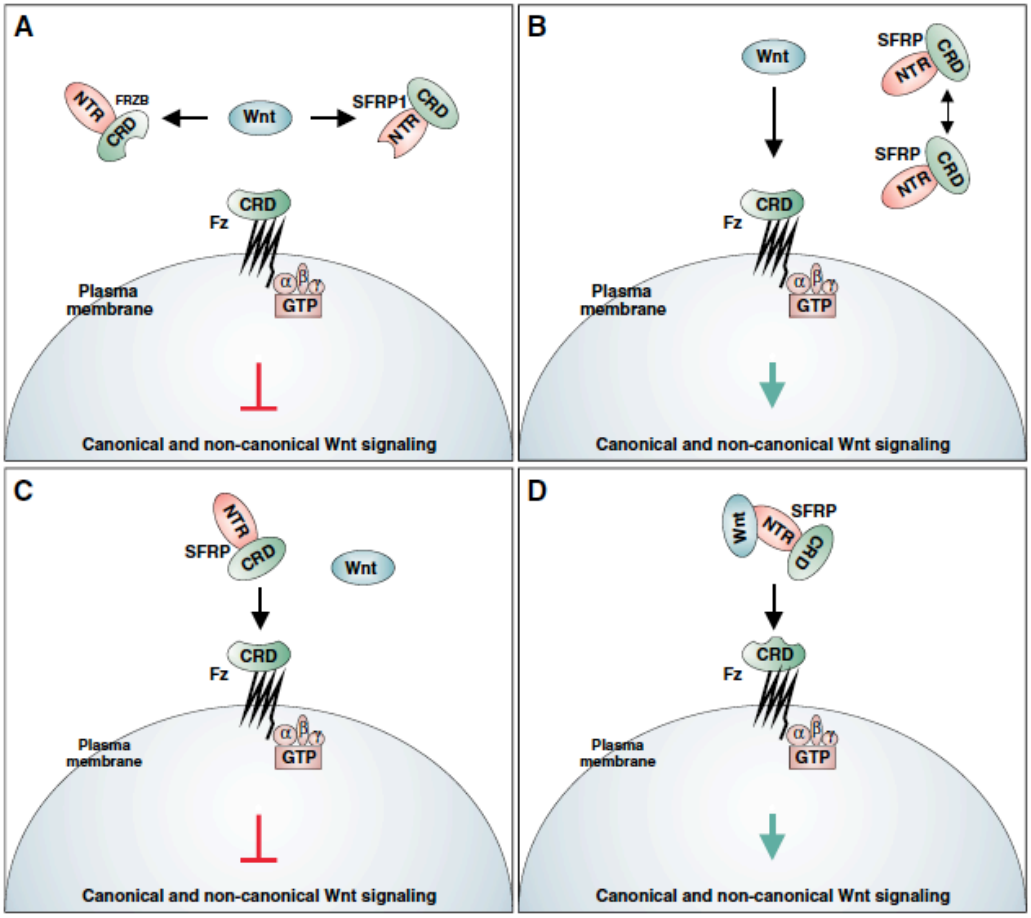

Fig.5. Possible mechanism by which sFRPs could modulate Wnt signaling (A) sFRPs could sequester Wnt either through NTR or CRD domain, therefore acting Wnt antagonists. **(B)** They could dimerized via CRDs and thereby favouring Wnt signaling. **(C)** sFRPs, could form inactive complexes by binding to Fz-CRD thereby preventing signal transduction by Wnt ligands. (D) sFRPs might favour a Wnt-Fz interaction by simultaneously binding to both molecules and promoting signal transduction. Picture taken from (Bovolenta et al, 2008).

of Frzb seemed to be necessary and sufficient for both activities, Wnt binding and inhibition (Lin et al, 1997). In contrast, a mutant form of sFRP1 lacking the CRD domain retained the ability to bind to Wg, whereas deletion of the NTR resulted in a reduction or loss of Wg binding (Uren et al; 2000). Furthermore a recent report on sFRP1 combining biochemical and functional assay in cell culture and medaka embryos showed that its NTR domain that mimics the function of entire molecule in binding Wnt8 and inhibiting Wnt signaling. These apparently contradictory results might imply that sFRPs have multiple Wnt-binding site and sFRP-Wnt pairs associate with different affinities.

The crystal structure of the CRD from mouse Fz8 and mouse sFRP3 revealed the potential for the CRDs to dimerize (Dann et al, 2001). This suggests the ability of sFRP and Fz proteins to form homo- and heteromeric complexes through the CRD domain(Bafico et al, 1999; Rodriguez et al, 2005). These findings suggest an alternative explanation of how sFRPs could inhibit Wnt signaling by forming inactive complexes with Fz receptors (Fig.5C). sFRPs are not only inhibiting Wnt/β signaling but also inhibits noncanonical Wnt/PCP signaling (Li et al, 2008; Matsuyama et al, 2009; Satoh et al, 2008; Sugiyama et al, 2010) which is not surprising, as they bind to both types of Wnts.

The Wnt-binding property suggesting that sFRPs and WIF may also be involved in the regulation of Wnt stability and diffusion/distribution extracellularly beyond just Wnt inhibitors. It was shown that sFRPs could expand a signaling range of Wnt8 despite their molecular function as Wnt inhibitors, suggesting another regulatory mechanism for Wnt signaling (Mii & Taira, 2009).

Two distinct classes of Wnt inhibitors including the Dickkopf (Dkk) family and the WISE/SOST family. Dkk1, which is LRP5/6 ligands/antagonists and considered as specific inhibitors of Wnt/β-catenin signaling. Dkk1 inhibits canonical Wnt signaling by binding to LRP5/6 and another single transmembrane protein Kremen 1 (Krm1) and Kremen 2 (Krm2) (Mao et al, 2002). Krm, Dkk1 and LRP6 form a ternary complex at the cell surface and disrupt Wnt/LRP6 signaling by promoting endocytosis

and removal of the Wnt receptor (LRP6) from the plasma membrane (Mao et al, 2002). Like Dkk1, SOST is also able to disrupt Wnt-induced Fz-LRP6 complex formation *in vitro* (Semenov & He, 2006).

Shisa proteins represent a distinct family of Wnt antagonists that trap Fz proteins in the ER and prevent Fz from reaching the cell surface, thereby inhibiting Wnt signaling (Yamamoto et al, 2005). Other Wnt antagonists like *Xenopus Cerberus* binds to and inhibits Wnt as well as Nodal and BMP signaling (Piccolo et al; 2009). Insulin-like growth-factor (IGF) binding protein-4 (IGFBP-4) also inhibits Wnt signaling via binding to both Fz and LRP6, in addition to modulating IGF signaling (Zhu et al, 2008). Recently Tiki1 was identified by functional cDNA screening as an organizer-specific gene, required for anterior neural development in *Xenopus* (Zhang et al, 2012). Tiki1 inhibits Wnt signaling by removing eight amino-terminal residues from Wnt itself, which leads to the formation of oxidized Wnt oligomers with impaired receptor-binding capability (Zhang et al, 2012).

Norrin and R-spondin (Rspo) proteins are two families of agonists for Wnt/β-catenin signaling. Norrin is a specific ligand for Fz4 and LRP5/6 during retinal vascularization (Xu et al, 2004). R-Spondins (Rspo 1-4) are a small family of secreted growth factors, which in addition to Wnt, potently activate β-catenin signaling. Rspos synergize with Wnts (Kazanskaya et al, 2004; Kim et al, 2005; Kim et al, 2008; Nam et al, 2006) and require the presence of Wnts to activate β-catenin signaling (Binnerts et al, 2007). Rspo binds to the extracellular part of ZNRF3 and promotes the interaction between ZNRF3 and LGR4, which results in ZNRF3 clearance from the membrane. This report suggest a model where in Rspo activates Wnt signaling by inhibiting ZNRF3 in an LGR4-dependent manner, resulting in the accumulation of Wnt receptors at the cell surface.

3.8 Wnt/β-catenin signaling in Cancer and other diseases

Wnt/β-catenin signaling plays a critical role in development and homeostasis. Mutations of the Wnt pathway components are associated with many hereditary disorders, cancer and other diseases (van Amerongen & Nusse, 2009). Mutations in Porcupine (PORCN), various Wnt ligands, agonists and antagonists have shed light on Wnt regulation in human development. Given the diverse phenotypes produced by Wnt knockouts in mice, it is not surprising that loss of Wnts in humans has direct consequences as well. A non-sense mutation in the *Wnt3* gene causes the autosomal recessive disorder terta-amelia, a rare human genetic disorder characterized by complete absence of all four limbs and other anomalies (Niemann et al, 2004).

Recently it has been reported that hypofunctional alleles of *Wnt1* cause autosomal-recessive osteogenesis imperfecta (OI), a congenital disorder characterized by reduced bone mass and recurrent fractures (Keupp et al, 2013). Another recent report on the naturally occurring Wnt1 mutation of valine to phenylalanine at V335 causes osteogenesis imperfecta through impaired Wnt signaling (Fahiminiya et al, 2013). Mutation in *Wnt10A* is associated with Odonto-onycho-dermal dysplasia, which is a rare autosomal recessive syndrome in which the phenotype includes dry hair, severe hypodontia, smooth tongue, hyperkeratosis of skin (Adaimy et al, 2007). A genetic evaluation revealed that loss-of-function mutation in *Wnt4* gene. Wnt4 appears to be important in the development and maintenance of the female phenotype in women (Lauber et al; 2004).

Mutations in either LRP5 or Fz4 are associated with familial exudative vitreoretinopathy (FEVR) (Toomes et al, 2004), which is manifested by defective retinal vascularization, also seen in Norrie disease carrying Norrin mutations, leading to the identification of Norrin as a ligand for Fz4 and LRP5(Xu et al, 2004). One fast growing field involves Wnt/β-catenin regulation of bone mass, as osteoporosis remains a global health problem. This was triggered by the discovery of LRP5 loss-of-function mutations in patients with osteoporosis pseudoglioma syndrome (OPPG), a recessive disorder characterized by low bone mass and abnormal eye vasculature (Gong et al, 2001).

Conversely patients with autosomal dominant high bone mass (HBM) diseases harbor LRP5 missense ('gain-of-function') mutations (Boyden et al, 2002; Little et al, 2002), which are clustered in the LRP5 extracellular domain and render LRP5 resistant to binding and inhibition by the antagonist SOST (Ellies et al, 2006; Semenov & He, 2006) and Dkk1 (Ai et al, 2005). Interestingly mutations in the SOST gene causes Sclerosteosis. Thus LRP5 activity correlates with bone mass likely via regulation of osteoblast) proliferation, whereas SOST and Dkk1, which are specifically expressed in osteocytes, negatively regulate bone mass by antagonizing LRP5. Moreover, deletion of sFRP1 or Dkk1 enhances bone formation further suggesting that Wnt signaling is required for the maintenance of normal bone density (Bodine et al, 2004).

Mutations in intracellular pathway components can also cause dramatic defects. A Finnish family with severe tooth agenesis (oligodontia) and colorectal neoplasia was studied recently and identified the cause to be a nonsense mutation in *AXIN2*. This mutation introduces a premature stop codon to exon 7 of the *AXIN2* gene leading to a truncated protein lacking the C-terminal domain. The *AXIN2* mutation is one of the rare cases in which a mutation simultaneously predisposes to cancer and causes a hypoplastic malformation (Lammi et al, 2004). Mutations that promote constitutive activation of the Wnt signaling pathway lead to cancer. The best-known example is Familial Adenomatous Polyposis (FAP), an autosomal, dominantly inherited disease in which patients display polyps in the colon and rectum. This disease is caused most frequently by truncations in APC (Kinzler et al, 1991; Nishisho et al, 1991) that promote aberrant activation of the Wnt pathway leading to adenomatous lesions due to increased cell proliferation. Mutations in β-catenin and APC have also been found in sporadic colon cancers and a large variety of other tumor types (Giles et al, 2003; Polakis, 2007). Loss-of-function mutations in Axin have been found in hepatocellular carcinomas(Satoh et al, 2000). These examples clearly demonstrate that the uncoupling of normal β-catenin regulation from Wnt signaling control is an important event in the genesis of many cancers.

In the colon, loss of TCF4 or Dkk over-expression promotes loss of stem cells in the colon crypt, indicating that Wnt signaling is required for the maintenance of the stem

cell compartment (Korinek et al, 1998; Kuhnert et al, 2004). Wnt signaling may therefore be a fundamental regulator of stem cell choice to proliferate or self-renew.

4. Aim of Thesis

Wnts are secreted glycoproteins that play critical roles in embryonic development, cell fate determination and stem cell renewal. The Wnt/β-catenin signaling is initiated by binding of Wnt protein to a Frizzed (Fz) receptor and a coreceptor, LRP5/6. The molecular basis of Wnt signaling by the engagement of Frizzled receptors has long been unknown due to the lack of structural data on Wnt ligands. Only recently, the crystal structure of the XWnt8-Frizzled8-CRD complex was solved, but the significance of the interaction sites for signaling was not assessed

The aim of this thesis was to provide functional data on mouse Wnt3a-Frizzled8 interaction to answer the following questions

1. What are the biological significances of sites 1, 2, 3 in Wnt3a-Frizzled8-CRD interactions *in vitro* and *in vivo*?
2. To understand how lipid modified Wnts are recruited from the plasma membrane to the signaling complexe
3. How binding to the Fz-CRD-related proteins regulates the Wnts signaling range in the extracellular space?

To answer these questions, I performed an extensive mutagenesis analysis and tested Wnt isoforms mutated at critical site 1, 2 and pseudo site 3 residues both *in vitro* using Wnt reporter assays and *in vivo* in *Xenopus* and zebrafish embryos.

Further, the influence of the Fz8-CRD on the Wnt3a signaling potential was investigated by applying soluble Fz-CRD-Fc proteins on Wnt producing cells and monitoring the response in a simulated auto/paracrine or endocrine context.

5. Materials and Methods

5.1 Materials

5.1.1 Chemicals

All chemicals that were used in this study are listed in alphabetical order of the company from where they were purchased. The chemical and enzymes were acquired as the highest purity available.

Table 1: Chemicals in alphabetical order of companies

Company	Compounds
AppliChem Darmstadt (Germany)	agar, disodiumm hydrogen phosphate, EDTA, glycerol, glycine, isopropyl alcohol, kanamycin sulphate, magnesium choloride, Nonidet P40, sodium acetate, trypton, X-Gal,
Eurogentec (Köln) Germany)	Wnt3a synthetic peptides
Merck Darmstadt (Germany)	calcium chloride dihydrate, formaldehyde, glacial acetic acid, hydrogen peroxide, imidazole, Triton X-100
Qiagen (Germany)	Ni-NTA Agarose
Roche Diagnostics GmbH, Mannheim (Germany)	Protein A agarose
Roth Karlsruhe (Germany)	acrylamide/bisacrylamide (Rotiphorese Gel 40 (29:1)), agarose,ampicillin, β-mercaptoethanol, DTT, milk powder (blotting grade), sodium dihydrogen phosphate dihydrate, TEMED, Tris, Tween 20
Serva Heidelberg (Germany)	sodium azide, sodium dodecyl sulfate,
Sigma St. Louis, Missouri (USA)	Bovine serum albumin fraction V, CHAPS, ethanol,
VWR International West Chester, Pennsylvania (USA)	ammonium acetate, hydrochloric acid

5.1.2 Equipments

Table 2: List of Technical equipments used in this study

Technical equipment	Company
Benchtop centrifuge	Thermo Fisher Scientific); Waltham, Massachusets (USA)
Centrifuge	Beckman Coulter GmbH Krefeld (Germany)
Centrifuge for tissue culture	Beckman Coulter GmbH Krefeld (Germany
CO2-incubator	Heraeus (Hanau)
Developer machine	Agfa- Gevaert N.V, Mortsel (Belgium)
Electroporation apparatus	BioRad Laboratories GmbH; München (Germany)
Gel documentation system	Intas Science Imaging Instruments GmbH; Göttingen (Germany)
Incubation shaker for cultivation of bacteria	Infors HT; Bottmingen (Switzerland)
Inverted confocal microscope	Nikon Instruments Europe B.V.; Kingston, Surrey (England)
Inverted fluorescence microscope	Nikon Instruments Europe B.V.; Kingston, Surrey (England)
PCR machine MyCycler	BioRad Laboratories GmbH; München (Germany)
Protein blotting cell	BioRad Laboratories GmbH; München (Germany)
Protein gel system	peqlab; Erlangen (Germany)
Multimode microplate reader	Berthold Technologies (Germay)
Nanodrop ND-1000	peqlab; Erlangen (Germany)

5.1.3 Enzymes

DNaseI, Proteinase K, various restriction enzymes, T4 DNA ligase, T4 DNA polymerase, Precisor high-fidelity DNA polymerase, Phusion® High-Fidelity DNA Polymerase Products Suppliers: New England Biolabs, Gibco, Promega, Fermentas

5.1.4 Kits

BCA protein assay kit (Pierce), Dual luciferase assays kit (Promega), mMESSAGE mMACHINE® SP6 *in vitro* transcription kit (Ambion). Wizard Plus SV Minipreps DNA purification system and Wizard SV Gel and PCR Clean-Up system (Promega). QIAGEN plasmid Midi kit (Qiagen).

5.1.5 Antibodies

Table 3: List of Primary and secondary antibodies

Antibody	**Species**	**Dilution**	**Source**
Primary antibodies			
Anti-Wnt3a	rabbit (polyclonal)	1:1000	Abcam
Anti- α-tubulin	mouse	1:1000	Sigma
Anit-Penta-His	mouse	1:1000	Qiagen
Secondray antibodies			
Goat-anti-Mouse HRP	goat IgG (L+H)	1:10000	Jackson
Goat-anti-rabbit HRP	goat IgG (L+H)	1:10000	Jackson
Goat-anti-human IgG	goat (Fc specific)	1:100000	Sigma

5.1.6 Buffer, Antibiotics and Media

1.5 M Tris-HCl pH 8.8: Dissolve 181.71 g Tris base in 900 ml H_2O. Adjust pH to 8.8 with HCl. Adjust volume to 1 litre with H_2O. Sterilize by autoclaving.

1.5 M Tris-HCl pH 6.8: Dissolve 181.71 g Tris base in 900 ml H_2O. Adjust pH to 6.8 with HCl. Adjust volume to 1 litre with H_2O. Sterilize by autoclaving.

10% SDS: Dissolve 10 g SDS in 90 ml H_2O. Adjust volume to 0.1 litre with H_2O. Store at room temperature.

10% APS: Dissolve 0.1 g APS in 1 ml H_2O. Prepare freshly prior to use.

10 x SDS-PAGE running buffer: 10 g SDS, 30.3 g Tris base, and 144.1 g glycine Dissolve in 800 ml H2O. Adjust volume to 1 litre with H_2O.

2 x SDS-PAGE sample buffer: 100 mM Tris-HCl pH 6.8, 20 % Glycerol (v/v), 4 % SDS (w/v), 100 mM DTT (just before use).

5 x SDS-PAGE sample buffer: 250 mM TrisHCl pH6.8, 10% SDS, 30%Glycerol, 0.02% bromophenol blue, 500 mM DTT (just before use)

Gel Staining solution: 10% acetic acid 0.1% Coomassie Brilliant Blue G-250 (w/v).

Gel destaining solution: 20% acetic acid (v/v), 10% ethanol (v/v).

0.5 M EDTA: 186.12 g EDTA in 800 ml H_2O. Adjust pH to 8.0 with NaOH. Adjust volume to 1 litre with H2O. Sterilize by autoclaving.

50 x TAE: 242 g Tris base 100 ml 0.5 M EDTA (pH 8.0) 57.1 ml glacial acetic acid Adjust volume to 1 litre with H2O.

6 x DNA loading dye: 0.09% bromophenol blue (w/v) 0.09% xylencyanol (w/v) 60% glycerol (v/v) 60 mM EDTA (pH 8.0)

10 x PBS: 80g NaCl, 2g KCl, 2g KH2PO4**,** 15.4g Na2PO4.2H_2O. Sterilize by autoclaving.

10 x Transfer Buffer (Blot Buffer): 30.285g Tris, 112.605g Glycin, to make 1X transfer buffer add, 10x methanol.

LB-medium: 10 g tryptone, 5 g yeast extract, 5 g NaCl. Adjust volume to 1 liter with H_2O. Sterilize by autoclaving.

LB-agar plates: 10 g tryptone, 5 g yeast extract, 5 g NaCl, 15 g agar. Adjust volume to 1 liter with H_2O.

SOC-medium: 20 g tryptone, 5 g yeast extract, 0.5 g NaCl, 2.5 mM KCl. Adjust volume to 1 liter with H_2O. Sterilize by autoclaving. Add MgCl2 to a final concentration of 10 mM and glucose to 20 mM.

Ampicillin stock-solution: 100 mg/ml in H_2O. Sterilize by filtration. Store at 20^0C.Working concentration: 100 μg/ml

IPTG (1 M): 1 g IPTG (FW 238.3) resolved in 4.2 ml (~4 ml) H_2O, filter through 0.22 μm filters, aliquot 1 ml in eppendorf. Store at -20^{o}C.

DTT (1 M): 5 g DTT (FW 154.25) resolved in 32.5 ml (~30 ml) 10 mM NaAc (pH 5.2), filter through 0.22 μm filters, aliquot 1 ml in eppendorf. Store at -20^{o}C.

X-gal (20mg/ml): Add 5 ml (~4.8 ml) DMSO into 100 mg X-gal bottom (FW 408.24). Store at -20^{o}C.

5.1.7 Primers

All primers were purchased from Eurofins MWG Operon (Ebersberg, Germany). Primer stock solutions of 100 pmol were stored at -20 ^{0}C.

Table 4: List of primers used in this study

Construct	Forward primer (5'→3')	Reverse primer (5'→3')
pCS2-*mWnt3a*	CGCGGATCCATGGCTCCTCTCGGATAC	CGCCTCGAGCTACTTGCAGGTGTGCAC
pCS2-*mWnt3a*-His	CGCGGATCCATGGCTCCTCTCGGATAC	AAACTCGAGCTAGTGATGGTGATGGTG ATGCTTGCAGGTGTGCACGTCATAG
pCEP-Pu-*mWnt3a*-His	CGCGCTAGCGAGCTACCCGATCTGGTGG	GCGGCCGCCTTGCAGGTGTGCACGTC
pFUSE-hIgG3*01-Fc2 – *mFz8-CRD*	AAAGAATTCGGCTTCGGCCAAGGAGCTGGC	AAAAGATCTGCTGGGCGCGGCCGTGG
pCEP-Pu-–mWnt3a-Fz8-CRD-His	TTTGCGGCCGCAGCTTCGGCCAGGAGCTGGCGTG	GGATCCCTAGTGATGGTGATGGTGATG GCTGGGCGCGGCCGTGGTGAG
pCS2-*Wnt3a-NTD*	TTTGGATCCATGGCTCCTCTCGGATAC	AAACTCGAGCTAGGCCTCGTAGTAGAC CAG
pCS2-*Wnt3a-CTD*	TTTGGATCCATGGCTCCTCTCGGA	AAACTCGAGCTACTTGCAGGTGTGCAC GTC
pCS2-*Wnt3a-NTD*-His	TTTGGATCCATGGCTCCTCTCGGATAC	AAACTCGAGCTAGTGATGGTGATGGGATGGGCCTCGTAGTA
pCS2-*Wnt3a-CTD*-His	TTTGGATCCATGGCTCCTCTCGGA	CACCTGCAAGCATCATCATCAT CAT CAT TAG CTCGAG TTT
Point Mutants		
pCS2-Wnt3a- I45R	CATTCTCTGTGCCAGCAGACCAGGCCTGGTACG	CGGTACCAGGCCTGGTCTGCTGGCACAGAGAATG
pCS2-*Wnt3a*-Y59A	GCTTCTGCAGGAACGCCGTGGAGATCATGCCC	GGGCATGATCTCCACGGCGTTCCTGCAGAAGC
pCS2- *Wnt3a* -V60R	CTGCAGGAACTACAGAGAGATCATGCCCAG	CTGGGCATGATCTCTCTGTAGTTCCTGCAG
pCS2- *Wnt3a* -M63A	CTACGTGGAGATCGCCCCCAGCGTGGCTGAG	CTCAGCCACGCTGGGGGCGATCTCCACGTAG
pCS2- *Wnt3a* -P64A	CTACGTGGAGATCATGGCCAGCGTGGCTGAGGGTG	CACCCTCAGCCACGCTGGCCATGATCTCCACGTAG
pCS2- *Wnt3a* - A67R	ATGCCCAGCGTGAGAGAGGGTGTCAAA	TTTGACACCCTCTCTCACGCTGGGCAT
pCS2- *Wnt3a* -E68A	CATCCCAGCGTGGCTGCCGGTGTCAAAGCGGGC	GCCCGCTTTGACACCGGCAGCCACGCTGGGATG
pCS2-Wnt3a-S92A	CTGCACCACCGTCGCCAACAGCCTGGCC	GGCCAGGCTGTTGGCGACGGTGGTGCAG
pCS2- *Wnt3a* -N93A	GCACCACCGTCAGCGCCAGCCTGGCCATC	GATGGCCAGGCTGGCGCTGACGGTGGTGC
pCS2- *Wnt3a* A96R	GTCAGCAACAGCCTGAGAATCTTTGGCCCTGTT	AACAGGGCCAAAGATTCTCAGGCTGTTGCTGAC

pCS2- *Wnt3a* -F169A	GTCTCTCGGGAGGCCGCCGATGCCAGG	CCTGGCATCGGCGGCCTCCCGAGAGAC
pCS2- *Wnt3a* -R173A	TTTGCCGATGCCGCCGAGAACCGGCCGG	CCGGCCGGTTCTCGGCGGCATCGGCAAA
pCS2- *Wnt3a* -N175A	GCCGATGCCAGGGAGGCCCGGCCGGATGCCCGC	GCGGGCATCCGGCCGGGCCTCCCTGGCATCGGC
pCS2-*Wnt3a*-K204A	CACCTCAAGTGCGCCTGCCACGGGCTATCT	AGATAGCCCGTGGCAGGCGCACTTGAGGTG
pCS2- *Wnt3a* -L208A	AAATGCCACGGGGCCTCTGGCAGCTGT	ACAGCTGCCAGAGGCCCCGTGGCATTT
pCS2- *Wnt3a* -S209A	AAATGCCACGGGCTAGCCGGCAGCTGTGAAGTG	CACTTCACAGCTGCCGGCTAGCCCGTGGCATTT
pCS2- *Wnt3a* -G210R	CACGGGCTATCTAGAAGCTGTGAAGTG	CACTTCACAGCTTCTAGATAGCCCGTG
pCS2- *Wnt3a* -W218A	GTGAAGACCTGCGCCTGGTCGCAGCCG	CGGCTGCGACCAGGCGCAGGTCTTCAC
pCS2- *Wnt3a* -F331A	TGCCACTGTGTTGCCCATTGGTGCTGC	GCAGCACCAATGGGCAACACAGTGGCA
pCS2- *Wnt3a* -W333A	CACTGTGTTTTCCATGCCTGCTGCTACGTCAGC	GCTGACGTAGCAGCAGGCATGGAAAACACAGTG
pCS2- *Wnt3a* -C335A	GTTTTCCATTGGTGCGCCTACGTCAGCTGCCAG	CTGGCAGCTGACGTAGGCGCACCAATGGAAAAC
pCS2- *Wnt3a* -Y336A	TCCATTGGTGCTGCGCCGTCAGCTGCCAGGA	TCCTGGCAGCTGACGGCGCAGCACCAATGGA
pCS2- *Wnt3a* -V337A	CATTGGTGCTGCTACGCCAGCTGCCAGGAGTGC	GCACTCCTGGCAGCTGGCGTAGCAGCACCAATG
pCS2- *Wnt3a* -V337R	CATTGGTGCTGCTACAGAAGCTGCCAGGAGTGC	GCACTCCTGGCAGCTTCTGTAGCAGCACCAATG
pCS2- *Wnt3a* -E68A/F169A	GTCTCTCGGGAGGCCGCCGATGCCAGG	CCTGGCATCGGCGGCCTCCCGAGAGAC
pCS2- *Wnt3a* -K204A/L208A	AAATGCCACGGGGCCTCTGGCAGCTGT	ACAGCTGCCAGAGGCCCCGTGGCATTT
pCS2- *Wnt3a* -F331A/W333A	CACTGTGTTGCCCATGCCTGCTGCTACGTCAGC	GCTGACGTAGCAGCAGGCATGGGCAACACAGTG

5.1.8 Plasmids

pCS2+ is a mammalian expression vector and was used as vector for cloning WT Wnt3a and mutant constructs. It contains a strong promoter/enhancer region (simian CMV IE94), polylinker and SV40 viral polyadenilation signal. The gene for the WT Wnt3a protein was inserted via the restriction site BamHI and NcoI in the mutiple-cloning site of vector. The SP6 viral promoter allows in vitro transcription of sense polyadenilated mRNA for microinjections. The T7 viral promoter allows *in vitro* transcription of antisense RNA for *in situ* hybridization.

pCEP-PU-is an episomal mammalian expression vector that uses the cytomegalovirus (CMV) immediate early enhancer/promoter for high level transcription of recombinant genes inserted into the multiple cloning site. pCEP-PU contains the puromycin resistance gene for stable selection in transfected cells. WT mouse Wnt3a protein and Wnt3a-Fz8-CRD fusion protein was cloned in this vector (Invitrogen).

pFUSE-hIgG3*01-Fc2- is a family of plasmid developed to facilitate the construction of Fc-fusion protein by fusing the effector region of a protein to the Fc region of an immunoglobulin G (IgG). They contain the IL2 signal sequences (IL2ss) for the generation of Fc-Fusion proteins derived from proteins that are not naturally secreted. This vector was used to clone mouse Fz8-CRD. They also contain a Zeocin resistance gene for stable selection in transfected cells (Invivogen).

pBluescript- is a phagemid vector usable for DNA cloning. It contains phage f1 intergenic region required for packaging of DNA into bacteriophage particles (Fermentas).

5.2 Methods

5.2.1 Molecular Biology Methods

5.2.1.1 Polymerase chain reaction (PCR)

The DNA fragments were amplified by PCR, from a plasmid, which is used as a DNA template. The primers contained restriction sites and 3 extra flanking bases at their 3´-ends. The following composition was used to afford 50 µl sample volume for the PCR reaction.

Table 5: Strandard PCR reaction mixture

Volume	Components	Stock solution
1 µl	Template DNA	20 ng/µl
10 µl	1X HF buffer	5X
1.25 µl	dNTP	10mM
4 µl	Forward primer	10 pmol/µl
4 µl	Reverse primer	10 pmol/µl
0.5 µl	Preciso rhigh-fidelity polymerase	250U
	H_20	
50 µl	Total	

The PCR reactions were carried out in the Mycycler^{TM} Thermal cycler from BIO-RAD (Munich, Germany), using following program.

Table 6: Standard PCR conditions for DNA amplification

Step	Temperature (^{0}C)	Time
Melting	95	2 min
Melting	95	30 sec
Annealing	55-56	30 sec
Elongation	72	1 min
Elongation	72	10 min
Finish	4	∞

5.2.1.2 Plasmid Construction

Mouse Wnt3a coding sequences were PCR amplified and cloned into pCS2+ expression vector using *BamHI* and *XhoI* restriction sites. All point mutations were introduced into full-length pCS2-Wnt3a using PCR. All DNA fragments derived from PCR were automatically sequenced to confirm the presence of the desired mutation and to rule out spurious mutations. The N-terminal domain (1-276) and C-terminal domain (277-352) of mouse Wnt3a were cloned into pCS2+ using the same restriction sites as above. An expression plasmid encoding the mouse Wnt3a-His was constructed by introducing a histidine tag at the C-terminus of Wnt3a in the pCEP-PU vector. To generate a C-terminal His-tagged version of mouse NTD and CTD, a histidine tag was introduced by PCR-amplification and cloned in pCS2 vector. An expression construct for Wnt3a-Fz8-CRD-His fusion protein was generated in the pCEP-PU vector (Invitrogen). A short alanine linker was included between C-terminal Wnt3a fragment and the N-terminal Fz8 CRD lacking the native signal sequence. To produce Fz8-CRD-Fc fusion protein, the CRD (*i.e.* the 135-amino acid region extending from the first to the tenth conserved CRD cysteine) was inserted between *EcoRI* and *BglII* in the pFUSE-hIgG3*01-FC2 vector (InvivoGen, USA), which contains an IL-2 signal sequence and a cytomegalovirus immediate-early gene 1 enhancer and promoter, followed upstream by the hinge region of human IgG heavy chain.

5.2.1.3 Site-directed mutagenesis

In order to create point mutations, site-directed mutagenesis was performed. The primers were designed as having the desired mutation site in the middle and amplify of the whole vector plus insert. Amplification of plasmid DNA was achieved by polymerase chain reaction (PCR). The following composition was used to afford 50 µl sample volume for the PCR reaction.

Table 7: 50 µl Reaction mixer for site directed mutagenesis

Volume	Component	Stock solution
1.0 µl	Template	40 ng/µl
2.4 µl	Sense primer	5 pmol/µl
2.4 µl	Anti-sense primer	5 pmol/µl
1.2 µl	dNTP	10 mM
1.0 µl	Phusion® High-Fidelity DNA Polymerase	2 U/µl
10 µl	Phusion® HF buffer	5 x
1.0 µl	MgCl2	50 mM
31.5 µl	Water	

The PCR reactions were carried out in the Mycycler™ Thermal cycler from BIO-RED (Munich, Germany), using following program

Table 8: PCR program f for site directed mutagenesis

Step	Temperature (^{0}C)	Time
Melting	95	2 min
Melting	95	30 sec
Annealing	55-56	30 sec
Elongation	72	1 min
Elongation	72	10 min
Finish	4	∞

- 40 µl of PCR reaction sample was digested with DpnI for 2h at 37^0 C. This enzyme cuts only methylated DNA, so all templates are removed.

Precipitaton of Dpn I digested product

- Mix sample (50 µl) with 0.1 volume (e.i 5 µl) of Sodium acetate (3M) and 2.5 volume (125 µl) of cold ethanol (96%) incubate for 15 min at -20 ^{0}C.
- Centrifuge at 12000g for 15 min at 4 ^{0}C

- Pellet was washed (transparent) with 70% ethanol
- Centrifuge at 12000g for 5min.
- Supernatant was discarded and pellet air-dried.
- DNA was resuspended in 5 µl H_2O
- 3µl of the digest was transformed into 60µl electro-competent XL1 blue *E. coli* cells and plated on ampicillin agar plates. Incubated overnight at 37˚C.
- Usually, picking 5-6 colonies was enough. All plasmids digested with appropriate enzyme to diagnose the presence of the desired mutations, then sequenced.

5.2.2 Standard DNA manipulation techniques.

5.2.2.1 Digestion with restriction endonucleases.

Cleavage by restriction enzymes (or restriction endonucleases) plays a key role in all the gene manipulation procedures. These enzymes, part of the bacterial restriction/modification (or restriction/methylation) systems, allow bacteria to monitor the origin of incoming DNA and to destroy the molecules recognized as foreign. In particular, the enzymes termed type II restriction endonucleases are important in all aspects of molecular biology. They recognize specific sequences (usually 4 – 6 bp in length) and of specific base composition, and cleave the DNA into fragments in a defined manner. The sequences recognized are usually palindromic, that is they read the same in both directions on each strand, and when cleaved, they leave a cohesive-ended fragment. The ends produced from different molecules but by the same enzyme are complementary (also 'cohesive' or 'sticky') and consequently, anneal to each other. The standard reaction mixture contained DNA to be cut, the appropriate enzyme, enzyme buffer (from 10 × stock) and sterile distilled water. All digests were done at 37°C. To double digest pCS2, pCEP-PU vector for cloning PCR products, 5-7 µg of vector DNA was digested for 5-6 hours in a total reaction volume of 100 µl. Both digested vector backbone and PCR products were gel purified after excising desired band and used for setting up the ligation reaction.

5.2.2.2 Dephosphorylation.

To reduce the background in cloning experiments, phosphatases which remove the 5'-terminal phosphate groups from a nucleic acid strand are used. By dephosphorylating the restricted cloning vector they prevent its re-ligation, eliminating any possibility of dimer formation or re-circularization of the vector. The restricted, linearized vector DNA was treated with calf intestine alkaline phosphatase before ligation. For dephosphorylation of restricted vector, 1-3 µg of DNA was treated with 0.25-0.5 units of enzyme for 30 min at 37°C in the final volume of 50 µl.

5.2.2.3 Ligation

The cohesive ends produced by certain restriction enzymes anneal and can be joined covalently by DNA ligase to create artificially recombinant molecules. The ligase forms a covalent bond between the 5'-phosphate at the end of one strand and the 3'-hydroxyl of the adjacent one. Ligation of the specific DNA fragment with a specialized DNA carrier molecule (vector) is necessary in order to be able to propagate the DNA of interest. Ligation was performed using T4 DNA ligase (Promega) as recommended in the manufacturers instructions. Briefly, 1 µl (5 units) of T4 DNA ligase was used and the vector/insert ratio of 1:5 for all constructs (total DNA about 50 ng) in a final volume of 10 µl was used. A control ligation reaction with vector but no insert DNA present was set up at the same time to assess the likelihood of vector re-ligation (reaction background). The ligation was performed either for 2-3 hours at 37^0C or overnight at 16^0C. The ligated fragments were then transformed into *E.coli* XL1 blue strain.

5.2.2.4 Transformation of electrocompetent bacteria

One aliquot of electrocompetent cells was thawed on ice and mixed gently with 1 ng - 200 ng of plasmid DNA. The DNA and cells were transferred to a prechilled electroporation cuvette. An electric pulse (25 µF, 2.5 kV, 200, field strength: about 12,5 kV/cm) was applied. Usually a time constant of 4.8 - 5.2 ms indicated that the procedure was successful. After transformation, the bacteria were mixed with 1 ml SOC-medium immediately and incubated at 37 ^{0}C in a shaker for 30 - 60 minutes. Different volumes of cells were either plated on LB-plates or the cells were directly used to inoculate a liquid culture.

The appropriate antibiotics were added to the medium or plates to select for the desired transformants.

5.2.2.5 Transformation of chemocompetent bacteria

The chemocompetent cells were thawed rapidly and mixed gently with up to 200 ng of plasmid DNA. The DNA and cells were cooled on ice for 15 min. The cells were heat shocked for 50 seconds at 42 ^{0}C. After transformation, the bacteria were chilled on ice for 2 minutes and mixed with 1 ml SOC-medium. The bacteria were incubated at 37 ^{0}C in a shaker for 30 - 60 min. Different volumes of cells were either plated on LB-plates or the cells were directly used to inoculate a liquid culture. The appropriate antibiotics were added to the medium or plates to select for the desired transformants.

5.2.2.6 Purification of plasmid DNA

DNA has been amplified in bacteria as 'high copy' (300 >1000 copies per cell) or 'low copy' (5-20 copies per cell) extrachromosomal plasmid DNA. The plasmid DNA was isolated by alkaline lysis. The bacteria were lysed by the addition of sodium hydroxide. The cell membranes were dissolved and the proteins denatured. Neutralization led to precipitation of the denatured proteins and bacterial chromosomal DNA. The plasmid DNA, which stayed in solution, was isolated.

For the purification of plasmid DNA in a small scale, the Wizard Plus SV Minipreps DNA Purification System from Promega was used according to the manufacturer´s instructions.

For the purification of plasmid DNA on large scale, Qiagen Midi prep DNA purification system was used according to the manufacturer's instructions.

5.2.2.7 Extraction of nucleic acids by phenol-chloroform

The phenol-chloroform extraction method is used to remove proteins from nucleic acids solutions. Proteins are denatured by phenol and accumulate in the organic phase and at the interphase between the organic and the aqueous phase. Chloroform helps to separate the two phases and isoamyl alcohol prevents the formation of foam. An equivalent volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added to the DNA solution.

The solutions were vigorously mixed by shaking for 1 minute. The organic and the aqueous phase were separated by centrifugation at top speed in a tabletop centrifuge for 5 minutes at room temperature. The upper, aqueous phase was transferred to a new tube and an equal volume of chloroform-isoamyl alcohol (24:1) was added. Again the solutions were mixed by shaking and the phases were separated by centrifugation. The aqueous phase contains the nucleic acids.

5.2.2.8 Precipitation of DNA

Ethanol precipitation was used to purify and concentrate DNA from aqueous solutions. A mixture of salt and ethanol forces the DNA to precipitate out of solution by decreasing its solubility in water. To an aqueous solution of DNA 1/10 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of 100% ethanol were added. The DNA was centrifuged at top speed in a tabletop centrifuge for 20 minutes at room temperature. The pellet was washed with 70% ethanol. Finally the pellet was air-dried and dissolved in an appropriate amount of H2O or TE-buffer.

5.2.2.9 Quantification of DNA

The concentration of DNA was determined by spectrophotometry using the Nanodrop spectrophotometer from Peqlab. 1-2 µl of DNA was used for analysis at absorption of 260 nm. An OD_{260} of 1.0 equals a DNA concentration of 50 µg DNA per ml. (For RNA, an OD_{260} of 1.0 equals an RNA concentration of 40 µg RNA per ml) Conclusions on the purity of the DNA were made by examining the ratio of OD_{260} to OD_{280}. A value between 1.8 and 2.0 represents a high-quality DNA sample.

5.2.2.10 Analysis of DNA by gel electrophoresis: agarose gel electrophoresis (AGE)

The quality and size of DNA was analyzed by agarose gel electrophoresis. Plasmid DNA preparations and PCR reactions were analyzed on 1% agarose gels using 1 x TAE. To check the size of the DNA, additionally a DNA size marker has been loaded onto the gel. Electrophoresis was carried out at 100 mV in the same buffer, which was used for the preparation of the gel. The DNA was visualized by staining for 10 minutes in an ethidium bromide solution and detection at 254nm using a transilluminator.

The gels were documented using the INTAS gel documentation system.

5.2.3 Preparation of mRNA for embryos injection

Capped mRNAs for injections were prepared from linearized template using the mESSAGE mACHINE® SP6 *in vitro* transcription kit (Ambion)

Linearization of DNA template

- Plasmids (pCS2+) encoding the DNA template were linearized with restriction enzyme *NotI* (NEB) for 5-6 hours.
- Linearized plasmids were purified by adding equal volume of buffer saturated Phenol:chlorophorm:Isoamyl alcohol (1:1). And further traces of phenol were removed by adding equal volume of chlorophorm to the aqueous layer.
- After chloroform extraction upper phase was precipitated with 1/10 of Na-acetate and 2.5 volume of cold absolute ethanol was mixed at RT for 30 min.
- Precipitate was pelleted at max. speed for 15 min, washed with 75% ethanol, dried and resuspended in 15 μL nuclease free water.

RNA synthesis by in vitro transcription

- The RNA Polymerase Enzyme mix was placed on ice, vortexing of the 10X reaction buffer and the 2X NTP/CAP was done till they are completely in soultion (RT). Once thawed, store the ribo-nucleotide (2X NTP/CAP) was placed on ice, but 10X reaction buffer was kept at room temperature while assembling the reaction.
- Transcription reaction was assembled at room temperature because the spermidine in the 10X reaction buffer could coprecipitate the template DNA if the reaction was assemled on ice. The 10X reaction buffer was added after the water and the ribonucleotide was already in the tube.

Table 9: 20 µl transcription reaction

Linear DNA template	2-4 µl
2XNTP/CAP	10 µl
10X Reaction buffer	2 µl
Enzyme mix	2 µl
Nuclease free water	to 20 µl

- Samples were incubated at 37 ^{0}C for 3-5 hours, and subsequently treated with 1 µl DNase I at at 37 ^{0}C for 15 minutes.

Purification of RNA transcripts

- To stop the reaction 125µl Nuclease- free water and 15 µl Ammonium acatate were added to samples, and mixed throroughly.
- Samples were extracted with an equal volume of Phenol/ Chloroform and then with an equal volume of Chloroform. The aqueous phase was removed and transferred to a new tube.
- RNA was precipitated by adding 1 volume of Isopropanol. Samples were kept least 30 min at -20°C.
- Precipitates were pelleted at 4°C for 30 min at 15000 rpm.
- The supernatant was removed with a pipette, and the pellets were washed once with 70% ethanol and dried briefly
- Dried pellets were dissolved in 20 µl nuclease free water, concentrations were quantified photometrically, and RNA quality was checked on an 1% agarose gel.

5.3 Embryological Methods

5.3.1 *Xenopus laevis* stem culture

Adult wild type specimens of *Xenopus laevis* were obtained from Xenopus Express (Le Bourg 43270, France). They were kept in groups of 20 to 30 animals in tap water at 19°C and fed 2-3 times a week with chopped beef heart or pellets (special clawed

frog food, Provimi Klima, Switzerland).

5.3.2 *In vitro* fertilization of *Xenopus laevis* oocytes

Adult *Xenopus laevis* males were sacrificed to surgically remove their testes, which were then stored at 4°C in testis buffer (80% MMR, 20% fetal bovine serum (FBS), and 50 µg/ml Gentamycin). Adult *Xenopus laevis* females were stimulated to start oogenesis by hypodermal injection of 500 IU of human chorionic gonadotropin (hCG, Sigma-Aldrich) the evening before microinjection. Oocytes were collected the next day by gently squeezing the animals. *Xenopus* sperm suspension was prepared by macerating a small piece of testis in 1 ml of 1X MBS-H (88 mM NaCl, 1 mM KCl, 2.4 mM Na2HCO3, 0.82 mM MgSO4, 0.33 mM Na(NO3)2, 0.41 mM CaCl2, 10 mM HEPES pH 7.4, 10 µg/ml Streptomycin sulfate, and 10 µg/ml Penicillin). Oocytes were fertilized with 400 µl of sperm suspension and covered with deionized water. Fertilization was successful when eggs reoriented in relation to gravity with their pigmented animal pole facing upwards. Embryos were dejellied by treatment with 2% Cysteine (pH 8) for 3-4 minutes followed by several washing steps with deionized water, and were then transferred to 1X MBS-H.

5.3.3 Zebrafish maintenance and stages of embryonic development

Zebrafish embryos (*Danio rerio*) were obtained from the fish facility of Centre for organismal studies (COS). The fish were maintained according to the method described by Westerfield (Westerfield, 1995). Zebrafish AB wild-type line was staged as described previously (Kimmel et al, 1995).

Table 10. List of constructs for mRNA injection in *Xenopus* embryos

Plasmids	Linearization	Transcription	Source
pSC2-m*Wnt3a*	NotI	SP6	This study
pSC2-m*Wnt3a-NTD*	NotI	SP6	This study
pSC2-m*Wnt3a-CTD*	NotI	SP6	This study

Table 11. List of constructs for mRNA injection in zebrafish embryos

Plasmids	Linearization	Transcription	Source
pSC2-m*Wnt3a*	NotI	SP6	This study
pSC2-m*Wnt3a*-V60R	NotI	SP6	This study
pSC2-m*Wnt3a*-E68A	NotI	SP6	This study
pSC2-m*Wnt3a*-F169A	NotI	SP6	This study
pSC2-m*Wnt3a*-S209A	NotI	SP6	This study
pSC2-m*Wnt3a*-G210R	NotI	SP6	This study
pSC2-m*Wnt3a*-F331A	NotI	SP6	This study
pSC2-m*Wnt3a*-W333A	NotI	SP6	This study
pSC2-m*Wnt3a*-C335A	NotI	SP6	This study
pSC2-m*Wnt3a*-V337R	NotI	SP6	This study
pSC2-m*Wnt3a*-E68A/F169A	NotI	SP6	This study
pSC2-m*Wnt3a*-F331A/W333A	NotI	SP6	This study

5.3.4 *mRNA* microinjections in frog (*Xenopus*) and zebrafish (*Danio rerio*) Embryos

Capped mRNAs coding for wild type mWnt3a, NTD and CTD were synthesized from linearized pCS2+ plasmids using the mESSAGE mACHINE Kit (Ambion). Embryos for the axis duplication assay were injected at the ventral marginal zone at 4 cell-stage with the 3, 30, 300 pg of indicated mRNAs and screened for axis-duplication after 48 hours with an Olympus SZX12 stereomicroscope and a Soft Imaging System CC-12 digital camera.

Zebrafish AB wild-type line were raised and staged as described previously (Kimmel et al, 1995). For zebrafish injection experiments, a series of 1.5, 3, 33, 100 and 300pg of wild type mWnt3a mRNA was first injected and dose dependence evaluated. 3 pg of mWnt3a or more, caused very severe abnormalities at 1dpf, therefore 2 pg of each mRNA was microinjected into 1-cell stage embryos in experiments. Embryos were incubated in E3 medium (with additional PTU after 20hpf) at 28 °C and examined at 1, 1.5 and 3dpf. Zebrafish embryos were either photographed live or fixed with 4% PFA and stained with Alexa-488 conjugated Phalloidin (Molecular Probes), mounted in 1% low melting point agarose and imaged on A1R (Nikon) confocal microscope using 40x LWD water immersion objective.

5.4 Cell Culture Methods

5.4.1 Maintaining cells in culture

Human embryonic kidney 293T cells and mouse L-cells were routinely maintained in Dulbecco's modified Eagle's medium, containing 10% fetal bovine serum and 1% penicillin/streptomycin in 5% CO_2 at 37 ^{0}C. The media for LWnt3a cells were also supplemented with 0.4mg/ml G418 (Invitrogen). In order to replenish nutrients and avoid the potentially harmful metabolic by products and dead cells, the cells were passaged every 2 or 3 days according to the confluency of the cells.

Table 12: List of Cell culture media and solutions obtained from PAA if mentioned otherwise.

Product	Ingredients and Usage
DMEM with L-Glutamine	Glucose (4.5 g/l) with L-Glutamin for cell cultivation
DMSO	Cell cryopresenvation
FCS, Fetal calf serum	Additive for cell culture medium
Pencillin-Steptomycin antibiotic solution	10,000 U/ml Pencillin and 10mg/ml Streptomycin as an antibacterial in cell culture medium
G 418 (Invitrogen)	Maintaining LWnt3a cells
Trypsin EDTA solution	0.05% trypsin/ and 0.02% EDTA, for cell passage
jetPRIME^{TM} (Polyplus)	Cell transfection
Advanced low serum DMEM/F-12	For protein expression

5.4.2 Passaging of the cells

In order to passage the cells, the medium was aspirated and the adherent cells were gently washed once by 10 ml of 1X PBS. PBS was then aspirated and 2 ml of 0.05% trypsin/0.02% EDTA was added onto cells and distributed all over the plate for the detachment of the cells from the plate. The Trypsin-EDTA was aspirated and the cells were incubated in 37°C incubator for 5-10 minutes. A 10 ml of appropriate medium

was added onto cells to resuspend and collect the cells. For continuation of the cells in culture, 1/10 dilution was done.

5.4.3 Freezing and thawing of the cells

For freezing, the cells were harvested at 1000 rpm for 2 minutes and washed once with 1X PBS. After removal of the PBS, the cells were resuspended in freezing medium, which contains 10% DMSO and 90% of serum. DMSO was used as a cryoprotectant to protect the cells from rupture by the formation of ice crystal. 1 ml of the suspension was added into each cryo-tube. In general, from one 10 cm dish with 80% confluency, 2 ml of cell suspension was generated. The tubes containing the cells were then placed in -80°C freezer overnight. For longer storage, the cells were transferred to the liquid nitrogen tanks. For thawing, the cells taken from liquid nitrogen were placed into 37°C water bath. In order to avoid the toxic effect of DMSO, this process should be performed quickly. As soon as the cells were thawn, they were transferred into 15 ml falcon tubes containing the appropriate medium. In order to get rid of residual DMSO, the cells were washed with the medium for two times. After the last wash, they were resuspended in medium and transferred into 10 cm dishes and placed in 37°C incubator.

5.4.4 Transient transfection of cells

Cells were split one day prior to transient transfection and grown to 60-70% confluence. The HEK293T cells were transfected using jetPRIME^{TM} reagent (Polyplus transfection, USA) according to the manufacturer's protocol. After transfection, cells were kept in DMEM and harvested after 24-48 hours, depending on the experiment.

Table 13: Transfection of HEK293T cells with jetPRIME™

Culture Vessel	Volume of jetPRIME® Buffer (µl)	Amount of DNA (µg)	Volume of jetPRIME® reagent (µl)	Volume of growth medium (ml)
24-well	50	0.5	1	0.5
12-well	100	1	2	1
6- well	200	2	4	2
Flask 75cm^2	500	10	20	10

Table 14: List of constructs used for transfection of HEK293T cells

Plasmid	Source
pSC2-m*Wnt3a*	This study
pSC2-m*Wnt3a*-I45A	This study
pSC2-m*Wnt3a*-Y59A	This study
pSC2-m*Wnt3a*-V60R	This study
pSC2-m*Wnt3a*-M63A	This study
pSC2-m*Wnt3a*-P64A	This study
pSC2-m*Wnt3a*-A67R	This study
pSC2-m*Wnt3a*-E68A	This study
pSC2-m*Wnt3a*-S92A	This study
pSC2-m*Wnt3a*-N93A	This study
pSC2-m*Wnt3a*-A96R	This study
pSC2-m*Wnt3a*-F169A	This study
pSC2-m*Wnt3a*-R173A	This study
pSC2-m*Wnt3a*-N175A	This study
pSC2-m*Wnt3a*-K204A	This study
pSC2-m*Wnt3a*-L208A	This study
pSC2-m*Wnt3a*-S209A	This study
pSC2-m*Wnt3a*-G210R	This study

pSC2-m*Wnt3a*-W218A	This study
pSC2-m*Wnt3a*-F331A	This study
pSC2-m*Wnt3a*-W333A	This study
pSC2-m*Wnt3a*-C335A	This study
pSC2-m*Wnt3a*-Y336A	This study
pSC2-m*Wnt3a*-V337A	This study
pSC2-m*Wnt3a*-V337R	This study
pSC2-m*Wnt3a*-E68A/F169A	This study
pSC2-m*Wnt3a*-K204A/L208A	This study
pSC2-m*Wnt3a*-F331/W333A	This study
pSC2-m*Wnt3a*-His	This study
pSC2-m*Wnt3a*-NTD-His	This study
pSC2-m*Wnt3a*-CTD-His	This study
pCEP-PU-m*Wnt3a-His*	This study
pCEP-PU-m*Wnt3a-Fz8-CRD*-His	This study
pFUSE-hIgG3*01-Fc2 – *mFz8-CRD*	This study
pFUSE-hIgG3*01-Fc2	Invivogen
pTK-Ranilla	Provided by M. Boutros
TOPFLASH	Provided by M. Boutros

5.5 Dual luciferase reporter gene assay

Luciferase reporter assays were carried out in HEK293T cells in 384 well plates. HEK 293T cells were seeded 24 hours prior to transfection. Cells were transiently transfected with the reporter construct TCF-firefly (10ng), actin- *Renilla* (10ng) and Wnt3a (5ng) or mutant isoforms, and balanced with empty expression vector (pRL-CMV) using TransIT (Mirus Bio LLC, USA) according to the manufacturer's protocol. Firefly luciferase and *Renilla* luciferase activities were measured using the dual luciferase assay kit (Promega, USA) and detected using a Mithras LB940 Multimode Microplate Reader Luminometer (Berthold Technologies, Germany). *Renilla* luciferase activity was measured as an internal control for transfection

efficiency. The Wnt-induced relative luciferase activity is depicted as the ratio of the activity of the firefly to *Renilla* luciferase. All assays were performed in triplicate and repeated in independent experiments.

5.5.1 Peptide library generation and epitopes screening

Peptides covering the complete mouse Wnt3a sequence with a 5 amino acid overlap were synthesized by Eurogentec (Seraing, Belgium) using standard solid-phase Fmoc chemistry. All peptides were analyzed by Reverse phase HPLC (RP-HPLC) for purity assessment. Molecular masses of all peptides were confirmed by MALDI Mass Spectrometry. Binding epitope screening was done using a double luciferase Wnt reporter assay. The HEK293T cells were seeded in a 384-well plate 24 hours prior to transfection. Cells were transiently transfected with reporter construct TCF/Firefly, actin-*Renilla* luciferase and wild type Wnt3a construct using TransIT (Mirus Bio LLC, USA) according to the manufacturer's protocol. After 24 hours of transfection peptides were added at a final concentration of 1μM. After 24 hours firefly luciferase (Wnt reporter) activity and *Renilla* luciferase activity were measured according to the manufacturer's protocol (Promega, USA). For assaying possible agonistic effects of the peptides transfection of the Wnt3a construct was omitted. The peptide concentration of 1μM was chosen to have an optimal readout of antagonistic effects without toxic effects on the cells.

5.6 Biochemical methods

5.6.1 SDS-PAGE

SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) is a technique, which is used to separate proteins by size in a discontinuous gel system under denaturing conditions. Due to binding of SDS, the proteins are charged identically in proportion to their mass. The proteins are negatively charged and migrate to the anode during electrophoresis. For protein separation, 12% resolving gels and 6% stacking gels have been cast between two glass plates. After polymerization, the protein samples and a prestained molecular weight marker 'PageRuler Prestained Protein Ladder' from Fermentas or 'Prestained Protein Ladder,

Broad Range' from NEB) have been loaded onto the gel. The molecular weight marker was used to monitor the progress of the electrophoretic run and to calculate the molecular weights of the samples.

Table 15: Solution mixture for polyacrylamide gel preparation

Components	Separating Gels (16 ml) 12%	(Stacking gel (10 ml) 6%
dH_2O	6.9 ml	5.6 ml
40% Acrylamide	4.8 ml	1.5 ml
1.5 M Tris pH 8.8	4 ml	-
0.5 M Tris pH 6.8		2.5 ml
10% SDS	160 µl	100 µl
10% APS	160 µl	100 µl
TEMED	16 µl	10 µl

Protein samples for SDS-PAGE were prepared by addition of 5X sample buffer (10% w/v SDS, 300 mM DTT, 20% v/v glycerol, 0.2 M Tris-HCl, pH 6.8, and 0.05% bromophenol blue) and heating at 95°C for 5 min. Samples were electrophoretically separated on a 12% polyacrylamide gel. Electrophoresis was done in 1X running buffer (25 mM Tris, 200 mM glycine, and 0.1% (w/v) SDS) at a constant current of 20 mA using the Mini Protean 3 system (Bio-Rad). Afterwards, gels were stained with Coomassie brilliant blue R-250 or used for western blotting.

5.6.2 Western blotting (semi-dry)

Proteins were transferred from polyacrylamide gels to a PVDF transfer membrane (Immobilon™-P, Millipore). Membranes were activated in 100% Methanol for 15 seconds and washed in deionized H_2O for 1 min. Polyacrylamide gels, activated membranes, and 6 sheets of Whatman® chromatography paper were equilibrated in 1X blotting buffer (25 mM Tris, 150 mM glycine, and 10% (v/v) methanol) and assembled in a Bio-Rad Trans-Blot SD semi-dry transfer cell. Transfer was done at a constant voltage of 12V for 1 hour using a Bio-Rad PowerPac™ HC power supply. Afterwards, membranes were blocked in 5% BSA/PBS Tween 0.2% for 1 hour

followed by incubation with primary antibody diluted 1:1000 in 1% BSA/PBS Tween 0.2% over night at 4°C on a rotator. The next day, membranes were washed 3x 5 min in PBS Tween 0.2% and incubated with peroxidase-conjugated secondary antibody in 5% BSA /PBS 0.2% Tween for 1 hour at room temperature. Finally, membranes were washed 3x 10 min with PBS 0.2% Tween and 2x 10 min with PBS. To detect the proteins by chemiluminescence, membranes were incubated for 1 min in a 1:1 mixture of ECL solution 1 (2.5 ml of 1M Tris pH 8.5, 200 µl of luminol, and 88 µl of cumaric acid to a final volume of 20 ml) and ECL solution 2 (2ml of 1 M Tris pH 8.5 and 12 µl of H_2O_2 to final volume of 20 ml). In a darkroom, an autoradiographic film (Amersham Hyperfilm ECL, GE Healthcare) was exposed to the membranes for different time point and developed in a Curix 60 processor (Agfa).

5.6.3. BCA protein assay for peptides concentration determination

The Protein concentration of Wnt3a synthetic peptides was determined according to standard protocol of Pierce™ BCA Protein Assay Kit (Thermo Scientific). BCA Protein Assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantization of total protein. This method combines the well-known reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+1}) using a unique reagent containing bicinchoninic acid. The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 562nm that is nearly linear with increasing protein concentrations over a broad working range (20-2000µg/mL). For each measurement a complete calibration curve with BSA-standards raining from 20-2000 µg/ml protein has been generated. The BSA standards for calibration were measured in triplicate. For the determination of the peptide concentration, a total of 10 µl sample mixed with 200 µl working reagent (WR). Samples were incubated for 30 min at 37^0C in water bath, further cooled to room temperature. The absorption of sample was determined at 562 nm using BCA program in nanodrop.

5.6.4 OD280 measurement

The protein concentration of purified proteins and cell extracts was determined by measuring the absorbance at 280 nm in a spectrophotometer versus a suitable control. An absorbance of 1.0 at 280 nm equates a protein concentration of 1 mg/ml. This method provides a rough approximation of the protein concentration.

5.6.5 Rapid Coomassie staining

The dye Coomassie Brilliant Blue binds unspecifically to proteins and can be used to stain proteins (Wilson, 1983). For staining of proteins in polyacrylamide gels, the rapid Coomassie staining described by Sasse and Gallagher (2009) has been used. After SDS-PAGE, the polyacrylamide gel was soaked in fixation solution (25% isopropanol, 10% acetic acid) and incubated for 30 minutes at room temperature. The gel was transferred to the staining solution (10% acetic acid, 0.006% Coomassie Brilliant Blue G-250), and staining was performed until distinct protein bands became clearly visible on the gel. The gel was documented using the INTAS gel doc system.

5.6.6 Preparation of total protein lysate

To detect Wnt3a proteins in cell lysates, HEK 293T cells were transfected with wild type Wnt3a or its mutant isoforms. The cells were harvested after 48 hours of transfection, washed with ice cold 1X PBS and disrupted in lysis buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1% NP-40, 10 mM EDTA protease inhibitor cocktail complete (Roche)]. The resulting supernatant was transferred to a fresh tube. Proteins were analyzed by Western blot using anti-Wnt3a antibody. For loading control the membranes were probed with anti-α-tubulin.

5.6.7 Production of mouse Wnt3a and mutant isoforms in HEK293T cells

To collect conditioned medium of wild type mouse Wnt3a and indicated mutants, HEK293T cells were transiently transfected with the corresponding cDNAs under control of a cytomegalovirus immediate early gene enhancer and promoter. For protein expression, cells were incubated at 37 °C for 48 hours in DMEM containing 10% fetal bovine serum and 1% penicillin/streptomycin. Then conditioned media were harvested, centrifuged at 2000 g for 10 min, and directly used or stored in

aliquots at -80 ^{0}C. Control medium was prepared from untransfected HEK 293T cells following the same protocol. For the solution-binding assay conditioned media containing wild type Wnt3a or mutant isoforms were produced in low serum (3% FBS) condition. To detect the secretion levels of wild type Wnt3a and mutant isoforms, equal amounts of conditioned media were resolved on 12% SDS-PAGE and analyzed by Western blot using anti-Wnt3a antibody.

For production of pCS2-Wnt3a-NTD-his, pCS2-Wnt3a-CTD-his, pCEP-Pu-Wnt3a-His and pCEP-Pu-Wnt3a-Fz8-CRD-his conditioned medium, HEK 293T cells were transiently transfected with 10 μg of the respective plasmids in 75-cm2 flasks. One day after transfection, cells were transferred to advanced low serum DMEM/F-12 (GIBCO, Invitrogen) and secreted proteins were harvested after additional 48 hours. Proteins were in addition purified by incubating conditioned medium with Ni-NTA agarose beads (Qiagen) for 1 hour at 4^{0}C. Bound proteins were eluted with elution buffer (300 mM Imidazole pH 8.0, 250 mM NaCl, 1X PBS). Purified proteins were resolved on 12% SDS-PAGE and analyzed by western blotting using anti-his antibody.

5.6.8 Production and purification of Fz8-CRD-Fc fusion protein from HEK 293T cells

The mouse Fz8-CRD-Fc and Fc were produced in HEK293T cells that were transiently transfected by using jetPRIME^{TM} reagent (Polyplus transfection, USA). One day after transfection, cells were transferred to advanced low serum DMEM/F-12 (GIBCO, Invitrogen corporation), and secreted protein was harvested after additional 48 hours. Control conditioned medium was obtained from untransfected 293T cells. For purification of Fz8-CRD-Fc and Fc conditioned medium was incubated with Protein G Agarose (Roche Diagnostics GmbH, Mannheim, Germany) for 3 hours at 4^{0}C, and washed three times with wash buffer (10 mM Tris-Cl pH 7.8, 0.2 mM EDTA, 250 mM NaCl, 0.1% Tween). Proteins were eluted in low pH elution buffer (0.1 M Glycine-HCl pH 2.5, 0.1 %Tween) and immediately neutralize with 1 M Tris-HCl, pH 9.0). Purified protein was resolved on SDS-PAGE and analyzed by Western blot and Coomassie blue staining.

5.6.9 Solution binding Assay

To investigate the binding of Wnt3a mutants with Fz8-CRD-Fc we used solution-binding assay. Twelve micrograms of recombinant mouse Fz8-CRD-Fc or Fc alone as negative control were pre-incubated with Protein G Agarose (Roche Diagnostics GmbH, Mannheim, Germany) for 3 hours at 4°C, then beads were washed with 2 times with cold 0.01% Triton X-100, 1X PBS, and two times with cold 1X PBS. The beads were then incubated with 1 ml of Wnt3a conditioned medium for 3-4 hours. The beads were separated by centrifugation and washed 4 times with cold 0.01% Triton X-100, 1X PBS buffer and boiled in 2X SDS sample buffer containing beta-mercaptoethanol. The supernatants were analyzed by Western blot analysis using anti-Wnt3a primary antibody. The membranes were further re-probed with anti-human IgG antibody for loading control of Fz8-CRD-Fc and Fc.

5.6.10 Modulation of Wnt3a activity and solubility by soluble CRD protein

To investigate the effect of recombinant Fz8-CRD protein on Wnt solubility, HEK293T cells were transfected with wild type 1 µg Wnt3a plasmid in 24 well plates and cultivated in 500 µl DMEM medium supplemented with 10% FCS. After 24 hours the indicated amount of recombinant Fz8-CRD-Fc or Fc alone as negative control was added on Wnt3a producing cells and further incubated for 16-20 hours. Supernatant containing Wnt3a protein was analyzed by Western blot using the anti-Wnt3a antibody. For dual chamber luciferase assay HEK293T cells were seeded on thinCerts TC chambers with a pore size of 8.0 µm (Greiner, Germany), transfected with 0.25µg CMV-b-Galactosidase and 0.4 µg Super TOPFlash by Ca++-phosphate precipitation and cultivated in 1 ml DMEM medium supplemented with 10 % FCS. 40 hours before analysis, the inlets with the transfected cells were transferred on mouse Wnt3a producing cells (ATCC: CRL-2647) seeded in 500µl DMEM medium supplemented with 10 % FCS in 12 well plates. The recombinant Fz8-CRD protein was added 16 hours before analysis to the Wnt producing cells and relative luciferase activity was measured.

5.7 Bioinformatics

5.7.1 Modeling of Wnt3a structure

We performed a homology modeling based on the most recently determined crystal structure of the XWnt8-Fz8-CD complex (Janda et al, 2012) (pdb-code: 4F0A). Both, ligand and receptor gather homology scores of >75%. For subsequent modeling steps, two criteria's were important: the e-value as returned by BLASTP against PDB dataset and the 3D-Jury score from the metaserver which represents an approximation to the number of Ca atoms within 3.5Å from the PDB template as compared to the current theoretical model. Since the secondary structure coverage of the PDB corresponded to the secondary structure elements predicted for Wnt3A by PSIPRED, DSSP, STRIDE, and PROFSEC, it seemed reasonable to try a homology modeling protocol to the PDB from XWnt8-Fz8-CRD as templates using alignment information provided by the metaserver. Modeller9v2's (http://salilab.org/modeller/) script for automated model generation using homology modeling was run with successive molecular dynamics simulated annealing refinement (refine.slow) and repeated optimization (4 times) to get the first impression of the model. Finally, the model was optimized with variable target function method (VTFM), which tries to minimize constraint violations (e.g. torsion angles) with a maximum of 300 iterations.

6. Results

6.1 Identification of binding epitopes in mouse Wnt3a for Fz receptor engagement

Wnt ligands bind to Fz receptors and activate the Wnt/β-catenin signaling pathway. However, it is unknown which epitopes of Wnt bind to Fz. To gain insight on this issue, an unbiased screen was performed to identify the binding epitopes in mouse Wnt3a protein that are significant for signaling via receptor (Frizzled) binding. I have designed a peptide library covering the complete sequence of the mature mouse Wnt3a protein. The 33 different peptides comprised a length of 15 amino acids with a 5-residue overlap were synthesized (Fig. 6A). All peptides were screened for their biological activity in a Wnt reporter assay. HEK 293T cells were transiently co-transfected with the reporter construct TCF/firefly, actin-*Renilla* luciferase in presence and absence of wild type Wnt3a. The Peptides were added to the cells at 1μM concentration (Fig. 6B).

From the Wnt reporter assay it was found that peptides from the N-terminal half of the Wnt3a sequence (1-14, residues 1-163) reduced signaling by Wnt3a only moderately (Fig. 6C), indicating a minor contribution of this region to signaling. Peptides 19 and 20, which harbor the lipid attachment site Ser209 (site 1) reduced the relative luciferase activity by up to 70% as compared to Wnt3a alone. A significant reduction of reporter activity was also observed for peptides 22-27, which harbor predicted LRP5/6 binding sites. From the site 2 containing peptides only peptide 31 showed a pronounced inhibitory effect, although peptide 32 contained the complete set of site 2 residues. This might be due to the formation of non-native disulfide bonds in peptide 32. Interestingly we observed that none of the applied peptides showed an agonistic effect in the absence of full-length Wnt3a (Fig. 6C, red columns) suggesting a synergistic contribution of the binding sites to effective signaling. It was found 1μM peptide concentration optimal for this peptide screen without having any toxic effect on cells.

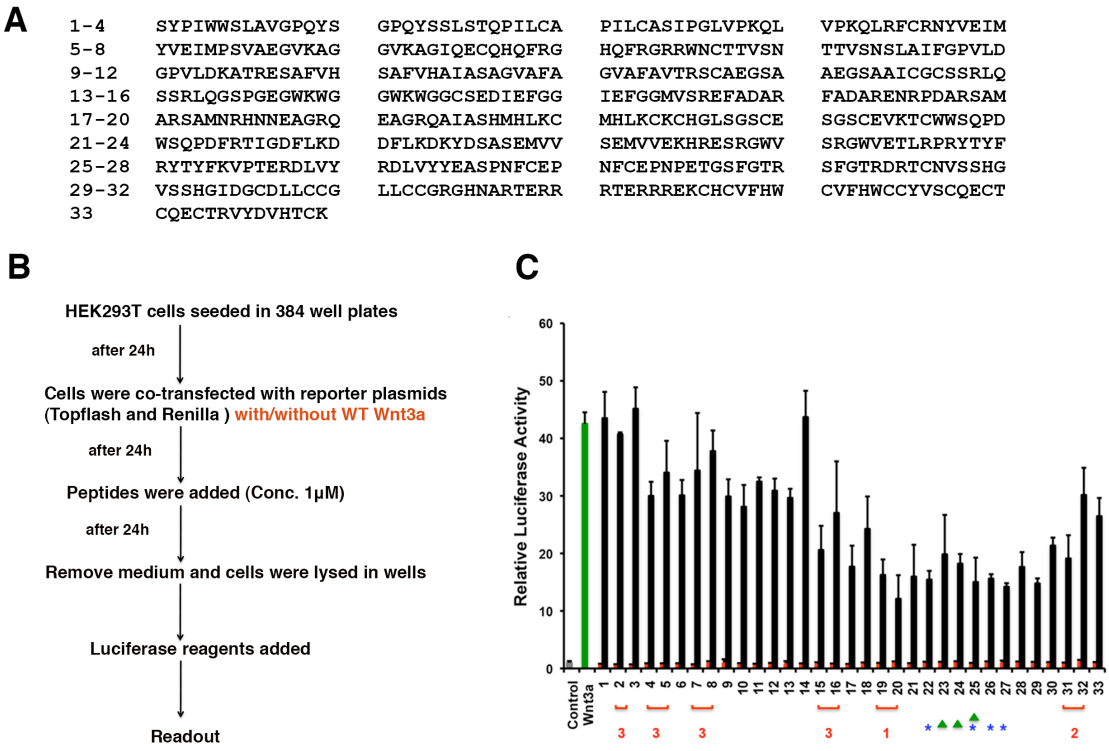

Fig.6. Identification of binding epitopes in mouse Wnt3a for Fz binding (A) Library of 33 synthetic linear peptides covering the whole mouse Wnt3a sequence. Each peptide was designed to have a length of 15 amino acids with a 5-residue overlap. **(B)** Workflow of peptide screen done in HEK 293T cells for Wnt reporter assay **(C)** Binding screen of peptides at 1µM was performed using a standard Wnt reporter assay in HEK293T cells in the presence (black column) or the absence (red column) of mouse Wnt3a. Peptides encompassing site 1, 2 or 3 residues are marked accordingly. Peptides harboring potential binding sites for LRP5/6, Ryk and Ror2 co-receptors are marked with asterisks. LRP5/6 binding sites according to (Chu et al, 2013) are marked with green triangles.

6.2 Structure analysis of mouse Wnt3a protein

In order to predict the critical sites in mouse Wnt3a required for Fz8-CRD binding, I have generated a structural model on the mouse Wnt3a-Fz8-CRD complex (Fig. 2B) adopting the recently published X-ray structure of *Xenopus* Wnt8 complex with Fz8-CRD (Janda et al, 2012). Mouse Wnt3a protein has 83% sequence identity with XWnt8 (Fig. 7A). The overall structure of Wnt3a in complex with Fz8-CRD is similar to a hand with a thumb and an index finger projecting from a central palm domain to interact with the Fz8-CRD at two distinct binding sites within the N-terminal and C-terminal domains designated as site 1 and site 2 respectively (Table 17). The N-terminal domain is primarily a α-helix bundle structure with two β-strands hairpin extensions packed against each other. The site 1 interaction is dominated by the lipid group attached to a conserved serine 209 (S209) at the tip of the thumb, which is directly involved in binding to Fz8-CRD. Sites 2 is located at the conserved hydrophobic loop (C329-V337) in the CTD of Wnt3a and interact with Fz8-CRD via highly conserved residues (Appendix 9.2). Some of the contact points on the Fz8-CRD are substituted in other Fz-CRDs, thus providing a possible mechanism to influence Wnt-Fz binding specificities.

Another potential interaction was observed in the crystal structure at pseudo-site 3. The residues involved in this interaction were poorly conserved among the different Wnt proteins (Appendix 9.2) but residues on the Fz8-CRD contacting Wnt3a in this site are mostly conserved. Pseudo-site 3 mediates formation of asymmetric Wnt/Fz dimer in the crystal and can generate a repeating oligomer, it may account for Wnt-induced receptor clustering and signalosome assembly.

However, it is remained unknown whether and how site 1, 2, 3 residues impact on biological Wnt function under relevant physiological conditions.

A

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Xwnt8   --------MQNTTLFILATLLIFCPFFT---ASAWSVNNFLMTGP-----------KAYL 38
Mwnt3a  MAPLGYLLVLCSLKQALGSYPIWWSLAVGPQYSSLSTQPILCASIPGLVPKQLRFCRNYV 60

Xwnt8   TYSASVAVGAQNGIEECKYQFAWERWNCPESTLQLATHN-GLRSATRETSFVHAISSAGV 97
Mwnt3a  EIMPSVAEGVKAGIQECQHQFRGRRWNCTTVSNSLAIFGPVLDKATRESAFVHAIASAGV 120

Xwnt8   MYTLTRNCSMGDFDNCGCDDSRNGRIGGRGWVWGGCSDNAEFGERISKLFVDGLETGQDA 157
Mwnt3a  AFAVTRSCAEGSAAICGCSSRLQGSPG-EGWKWGGCSEDIEFGGMVSREFADARENRPDA 179

Xwnt8   RALMNLHNNEAGRLAVKETMKRTCKCHGISGSCSIQTCWLQLAEFRDIGNHLKIKHDQAL 217
Mwnt3a  RSAMNRHNNEAGRQAIASHMHLKCKCHGLSGSCEVKTCWWSQPDFRTIGDFLKDKYDSAS 239

Xwnt8   KLEMDKRKMRSGNSADNRGAIADAFSSVAGSELIFLEDSPDYCLKNISLGLQGTEGRECL 277
Mwnt3a  EMVVEKHRESRG-WVETLRPRYTYFKVPTERDLVYYEASPNFCEPNPETGSFGTRDRTCN 298

Xwnt8   QSGKNLSQWERRSCKRLCTDCGLRVEEKKTEIISSCNCKFHWCCTVKCEQCKQVVIKHFC 337
Mwnt3a  VSSHGIDGCDLLCCGRGHNARTERRREK-------CHCVFHWCCYVSCQECTRVYDVHTC 351

Xwnt8   ARRERDSNMLNTKRKNRGHRR 358
Mwnt3a  K-------------------- 352
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B

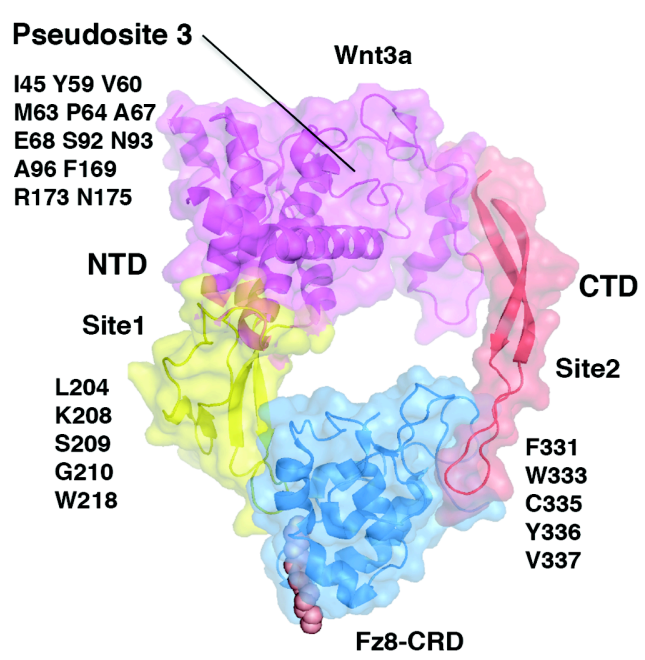

Fig.7. 3D structure of mouse Wnt3a-Fz8-CRD complex (A) Sequence alignment of *Xenopus* Wnt8 and mouse Wnt3a protein. Binding sites interacting with Fz8-CRD are marked, site 1 (green), site 2 (blue) and site 3 (red). **(B)** 3D model of mouse Wnt3a complex with Fz8-CRD showing the different binding sites. NTD, N-terminal domain, CTD, C-terminal domain.

Table 16: Interaction of site 1, site 2, site 3 residues with Fz8-CRD in Wnt3a-Fz8-CRD complex structure.

Site 1

MWnt3a residues	Fz8-CRD residues
Lys 204	Asn 58 Glu 64 His 59
Lue 208	Phe 127
Ser209	Gln71 Phe 72
Gly210	Glu 68 Arg 132
Trp 218	Asn 58

Site 2

MWnt3a residues	Fz8-CRD residue
Phe 331	Leu 104 Leu 147 Cys 148 Met 149 Asp 150
Trp 333	Phe 86 Asp 150 Ala 151 Ala 152
Cys 335	Gly 47 Tyr 48 IIe 95 Leu97
Thr 33	Leu 97
Val 337	Leu 97 Asp 99 Tyr 100 Leu 147

Site 3

MWnt3a residues	Fz8-CRD residues
IIe 45	Phe 127 Gly 126
Tyr 59	Tyr 125 Phe 127
Val 60	Gln 71 Phe 127
Met 63	Pro 74
Pro 64	Pro 74
Ala 67	IIe 78
Glu 68	Trp 73
Ser 92	IIe 78
Asn 93	IIe 78 Gln 79
Ala 96	Leu 121
Phe 169	Tyr 125
Arg 173	Gln 124 Tyr 125
Asn 175	Gln 124

6.3 Mutational analysis of *in vitro* Wnt3a activity

6.3.1 Effect of Site 1, site 2 and site 3 point mutants on Wnt signaling activity

In order to investigate the importance of point mutants in Wnt3a responsible for Fz8-CRD engagement, the corresponding interacting residues mutated to alanine whereas glycine, alanine and valine residues were mutated to arginine. Each Wnt3a mutant was analyzed for its biological activity in Wnt reporter assay. HEK 293T cells were transiently co-transfected with TCF-firefly, actin *renilla* luciferase reporter for normalizing and WT Wnt3a or mutant isoforms plasmid. For control Wnt3a plasmid transfection was omitted.

It was observed that site 1 interaction was dominated by the lipid group attached to S209 in Wnt3a, while other mutations introduced into site 1 reduced signaling activity only moderately, with the exception of G210R, which revealed a dramatic loss in its signaling activity (Fig. 8A). As expected, mutation of the lipid attachment site S209 completely abolished Wnt signaling activity. The other three mutants K204A, L208A, W218A had moderate effect on Wnt signaling activity. A combined mutation of K204A and L208A, which has only mild effect as individual mutant, the signaling activity loss was more pronounced (Fig. 9C). Mutations in site 2 generally showed a more pronounced effect on β-catenin-dependent transactivation of a luciferase reporter plasmid (TOPflash). In particular, C335A and V337R mutations resulted in a complete loss of the relative luciferase activity (Fig. 8C). Interestingly, alanine substitution of V337 did not alter the signaling efficiency of Wnt ligand (Fig. 9E). Mutant Y336A had no effect on signaling activity (Fig. 8C). While F331A and W333A single mutations still showed some residual signaling activity (Fig. 8C), but the double mutant F331A/W333A showed signaling activity comparable to vector control (Fig. 9C). Interestingly, it was observed that co-transfection of Wnt3a and V337R mutant, inhibit the Wnt signaling activity by 50%, suggesting there might be a competition between these two mutants for receptor binding.

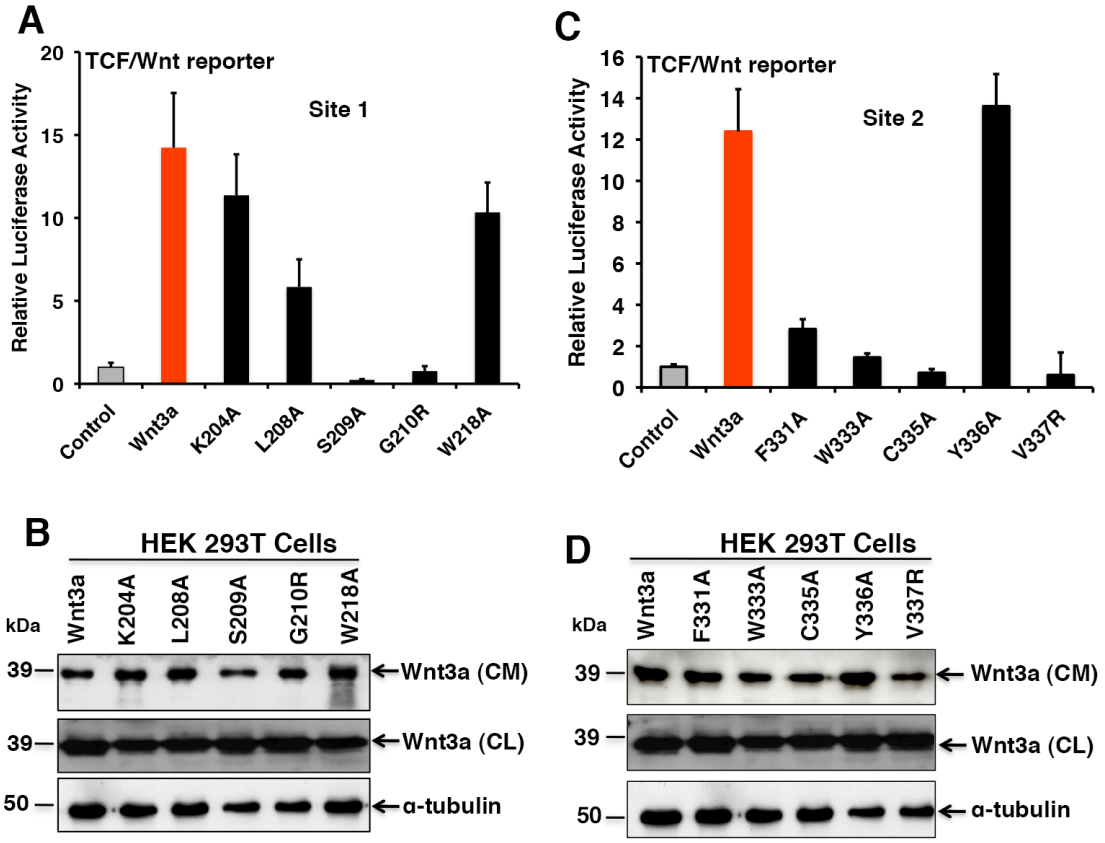

Fig.8. **Effect of Wnt3a site 1 and site 2 point mutations on signaling activity**. **(A, C)** Wnt reporter assay for the activity of site 1 and site 2 mutants and compared with WT Wnt3a. HEK 293T cells were transiently cotransfected with luciferase bases TCF/Wnt and Actin *Ranilla* reporter with 5ng WT Wnt3a or indicated mutant isoforms. **(B, D)** Secretion level of Wnt3a mutants analyzed by Western blotting. HEK 293T cells transfected with WT Wnt3a or indicated mutant constructs. Conditioned medium (CM) and cell lysates (CL) were analyzed 48 hours after transfection. Membranes were probed with anti-Wnt3a and anti-tubulin (loading control) antibodies. Error bars represent the standard deviation.

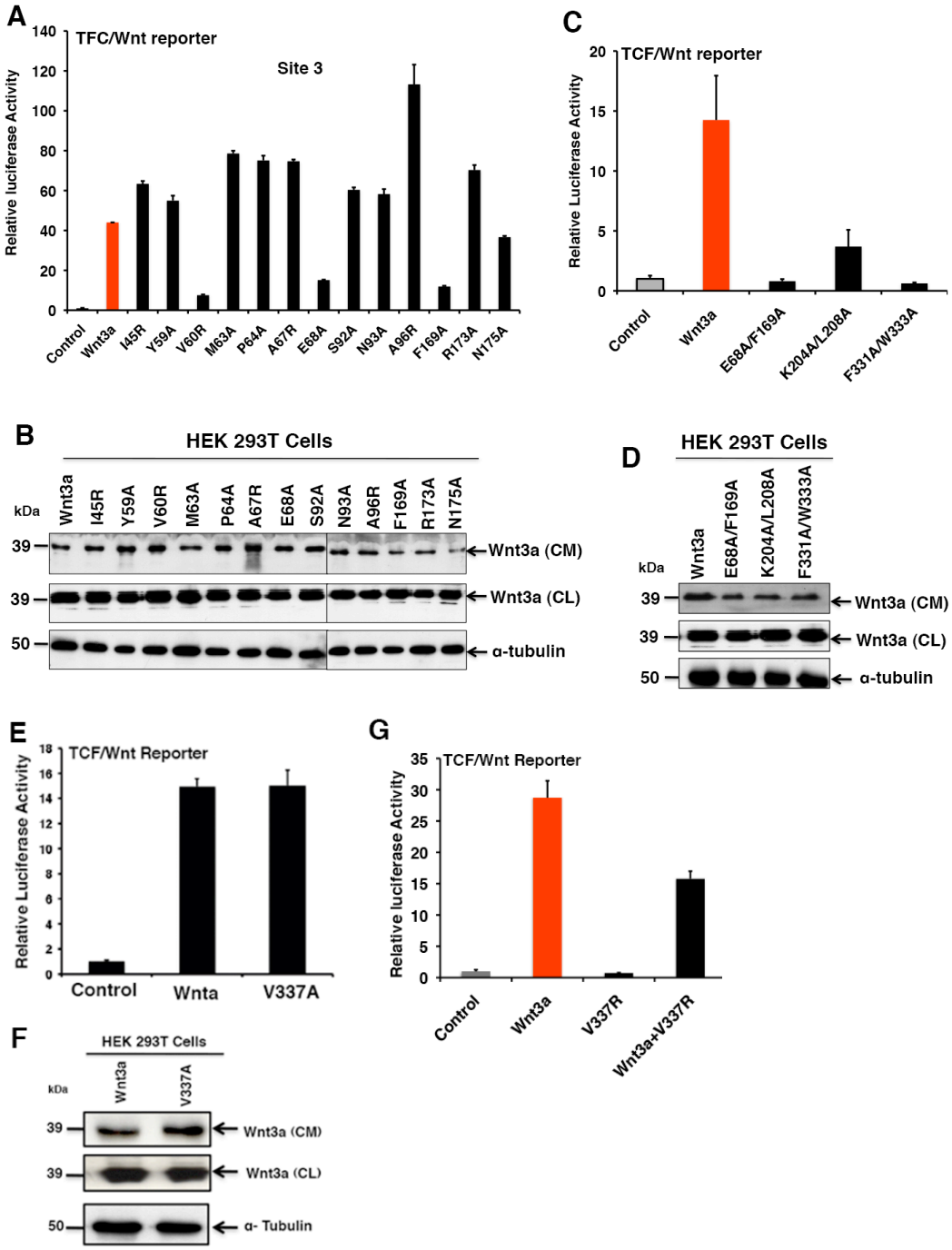

Fig.9. **Effect of Wnt3a site 3 and double mutants on signaling activity**. **(A)** Wnt reporter assays for the activity of Site 3 mutants **(B, D, F)** Secretion level of Wnt3a mutants analyzed by Western blotting. **(C)** Effect of double mutants on Wnt signaling activity. **(E)** The alanine substitution at V337 position (V337A) had no effect on activity in Wnt reporter assay. **(G)** Competitive binding result of Wnt3a and V337R in Wnt reporter assay. Error bars represent the standard deviation.

I further asked whether the predicted interaction residues in pseudo-site 3 were also involved in Wnt3a signaling activity. As a result it was found that site-directed mutations at site 3 positions, mutants V60R, E68A, F169A, significantly decreased signaling activity in the Wnt reporter assay (Fig 9A). The E68A/F169A double mutant did not show any residual activity (Fig. 9C). However in contrast to site 1 and site 2 mutations, the majority of the mutations introduced in the site 3 augmented Wnt signaling as compared to wild type Wnt3a (Fig. 9A). It was observed that A96R showing the most pronounced effect on signaling activity.

Taken together these results lead to the conclusion, that Wnt3a CTD which harbor the binding site 2 has more pronounce effect on Fz binding, compared to NTD which comprise binding site 1. I have also observed that site 3 relevant for Wnt signaling, precluding a 'pseudo' contact likely caused by a crystal artifact (Janda et al, 2012).

6.3.2 Secretion level of mutant Wnt proteins in HEK 293T cells

To exclude the fact that observed activity loss resulted from impaired secretion of the mutant proteins, I analyzed the respective mutant protein levels in cell supernatants and cell lysates. For this, I transiently transfected HEK293T cells in 6 well plates with 2 μg of each plasmid. For protein expression cells were grown in low serum medium for 48 hours. 20 μl of conditioned medium and 10 μl of cell lysates were submitted to SDS PAGE and analyzed by Western blotting using anti-Wnt3a primary antibody, alpha-tubulin was used as loading control in all experiments. The secretion levels of all mutant proteins from site 1, site 2 and site 3 did not grossly deviate from wild type Wnt3a protein (Fig. 8B, D) and (Fig. 9B, D, F) indicate that the mutations introduced did not significantly alter protein folding and secretion. The lowest levels were observed for S209A, which can be explained by impaired secretion due to the lack of lipid modification.

6.4 Impact of Wnt3a mutations for direct binding to Fz8-CRD

In order to investigate whether the introduced Wnt3a mutations affected Wnt-Fz binding. A pull-down assay was performed using the recombinant mouse Fz8-CRD-Fc fusion protein. As a negative control the Fc protein was applied. I purified recombinant Fz8-CRD-Fc and Fc protein from HEK293T cells (Fig. 10A). Cells were transiently transfected with 10 μg of corresponding plasmids. Conditioned medium was collected after 2 days of transfection and incubated with Protein G agarose for 3 hours at 4^0C. Proteins were eluted with low pH elution buffer.

As a result, it was observed that at least 80-90% of wild type Wnt3a is competent to specifically bind Fz8-CRD-Fc (Fig. 10B), but the binding of site 2 mutant W333A to the Fz8-CRD-Fc was nearly abolished when compared to the wild type, while C335A still had some residual binding activity (Fig. 10B). More surprisingly F331A and V337R mutations although lacking the signaling activity in Wnt reporter assay, showed full Fz8-CDR binding. The double mutant F331A/W333A binding was comparable to the W333A single mutant in the pull down assay (Fig. 10C). Site 1 mutants, S209A and G210R, showed a complete loss of binding however, the site 1 double mutant K204A/L208A showed no significant diminution in binding, confirming the previous results obtained from the Wnt reporter assay. Site 3 mutants V60R and E68A showed only minimal binding to Fz8-CRD, whereas mutant F169A and double mutant E68A/F169A completely failed to bind the receptor (Fz8-CRD) in the pull-down assay (Fig. 10C). In summary, our binding data indicate site 2 residues, F331 and V337, as being crucial for signaling without contributing Fz8-CRD binding.

Taken together the results obtained from Wnt reporter assay of mutants and direct binding assay between Wnt and Fz, collectively elucidate the specific contribution of Wnt residues from different sites in Fz receptor signaling (Fig. 11). It was found that mutants from site 1, S209A and G210R contribute to Wnt signaling activity and direct Fz binding. In site 2, mutants W333A, C335A, contribute to both Wnt signaling activity and Fz binding, whereas F331A and V337R are crucial for signaling without contributing to the binding energy to the Fz8-CRD. In site 3, mutants V60R, E68A and F169A are critically important to both signaling activity and Fz binding.

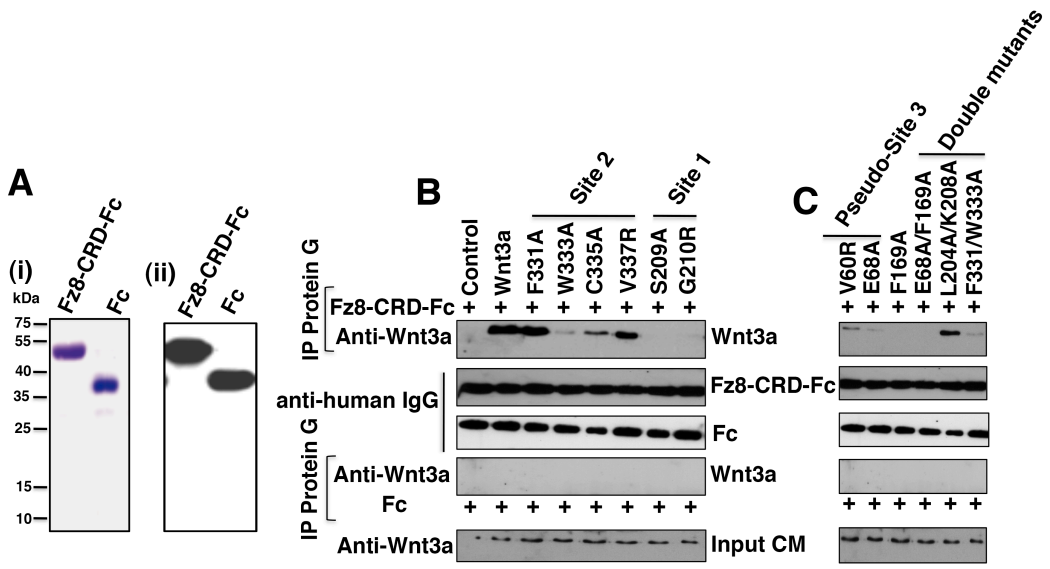

Fig.10. Binding of Wnt3a mutants to Fz8-CRD-Fc. (A) Purification of Fz8-CRD-Fc and Fc protein (i) Coomassie-stained gel with Fz8-CRD-Fc and Fc fusion partner alone secreted from HEK 293T cells and purified with Protein G agarose. (ii) Immuno blot of purified protein with anti-human-IgG (Fc specific) anitbody **(B)** Binding efficiency of respective mutants from site 1 and site 2 was analyzed by precipitating from conditioned medium with recombinant Fz8-CRD-FC or Fc alone pre-bound to Protein G agarose beads. Bound protein fractions were analyzed by immunoblotting using anti-Wnt3a antibody. **(C)** Binding analysis of site 3 and double mutants to Fz8-CRD-Fc.

A

Wnt3a mutants	TOPflash activity	Binding to Fz8-CRD-Fc
Site 1		
K204A	-	ND
L208A	-	++
S209A	---	--
G210R	---	--
W218A	-	ND
Site2		
F331A	--	++
W333A	--	+
C335A	---	+
Y336A	+ +	ND
V337R	-- -	++
Pseudo site 3		
I45R	+ + +	ND
Y59A	+ + +	ND
V60R	--	-
M63A	+ + +	ND
P64A	+ + +	ND
A67R	+ + +	ND
E68A	--	-
S92A	+ + +	ND
N93A	+ + +	ND
A96R	+ + +	++
F169A	--	-
R173A	+ + +	ND
N175A	-	ND

B

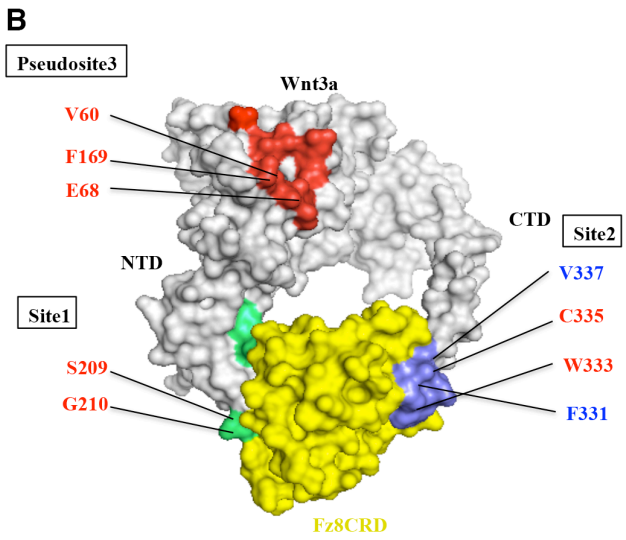

Fig.11. Effect of different Wnt mutants in TCF/Wnt-reporter assays and Fz binding. **(A)** -, --, --- represent intermediate, low and no signaling activity. ++, +, - ND, represent full binding, low binding, no binding and binding not determined **(B)** Surface diagram of mouse Wnt3a-Fz8-CRD representing the residues of Wnt3a specific for Fz engagement. Residues highlighted in red are critical for both signaling activity and binding. Residues highlighted in blue responsible for signaling activity without contributing to Fz binding.

6.5 Individual Wnt3a protein domains do not show independent signaling activity

It was suggested that a mini-Wnt comprising the *Xenopus* Wnt8 CTD has similar binding specificity to Fz as the full-length Wnt ligand (Janda et al, 2012). However it is not known whether the mouse Wnt3a subdomains have an independent signaling property. To investigate this issue, I designed two expression construct Wnt3a NTD (1-276) and Wnt3a CTD (277-352) which, bear the native signal peptide (Fig. 12A) and evaluated their activity *in vitro* and *in vivo*.

For *in vitro* activity a Wnt reporter assay was used. As a result, it was found that constructs expressing Wnt3a subdomains i.e. Wnt3a NTD and CTD, did not activate Wnt reporter assay, neither alone nor in combination. (Fig. 12B). However, when these single domains were co-transfected with full-length Wnt3a, both of them showed a moderate inhibition of the Wnt reporter activity likely by competing for receptor binding, with the CTD having a stronger inhibitory effect than the NTD. I have also observed the additive effect by combining both Wnt3a domains in triple co-transfection experiments with full-length Wnt3a (Fig. 12B).

To exclude the possibility that the loss of Wnt3a signaling activity resulted from abnormal cellular synthesis or secretion Wnt domain fragments Wnt3a NTD and CTD were expressed as His-tagged fusion protein in HEK293T cells. I further partially purified these proteins by incubating equal amounts of conditioned medium containing Wnt3a NTD and CTD proteins with Ni-NTA agarose and analyzed by Western blot using anti-his antibody. The calculated molecular masses are 31.4 kDa for Wnt3a NTD and 11.6 kDa for the CTD, respectively. An unspecific serum protein at about 17kDa was co-precipitated in all samples including vector control. The second band in the CTD lane at about 15kDa likely represents a different conformational state due to partial disulfide bonding. Taken together, it showed that both domains could be detected at similar level indicating sufficient structural integrity of the Wnt domains for passing the cellular protein folding control of the secretory pathway (Fig. 12C).

6.5.1 Mouse Wnt3a NTD and CTD did not induce double axis in *Xenopus* embroys

It has been known that mouse Wnt3a is a potent inducer of Wnt signaling in *Xenopus*, which itself provide a powerful system to characterize activation of Wnt signaling pathway. I performed double axis assay taking the advantage of the fact that ectopic expression of Wnt ligands can establish an additional organizer, leading to formation of a secondary dorsal axis (Sokol et al, 1991).

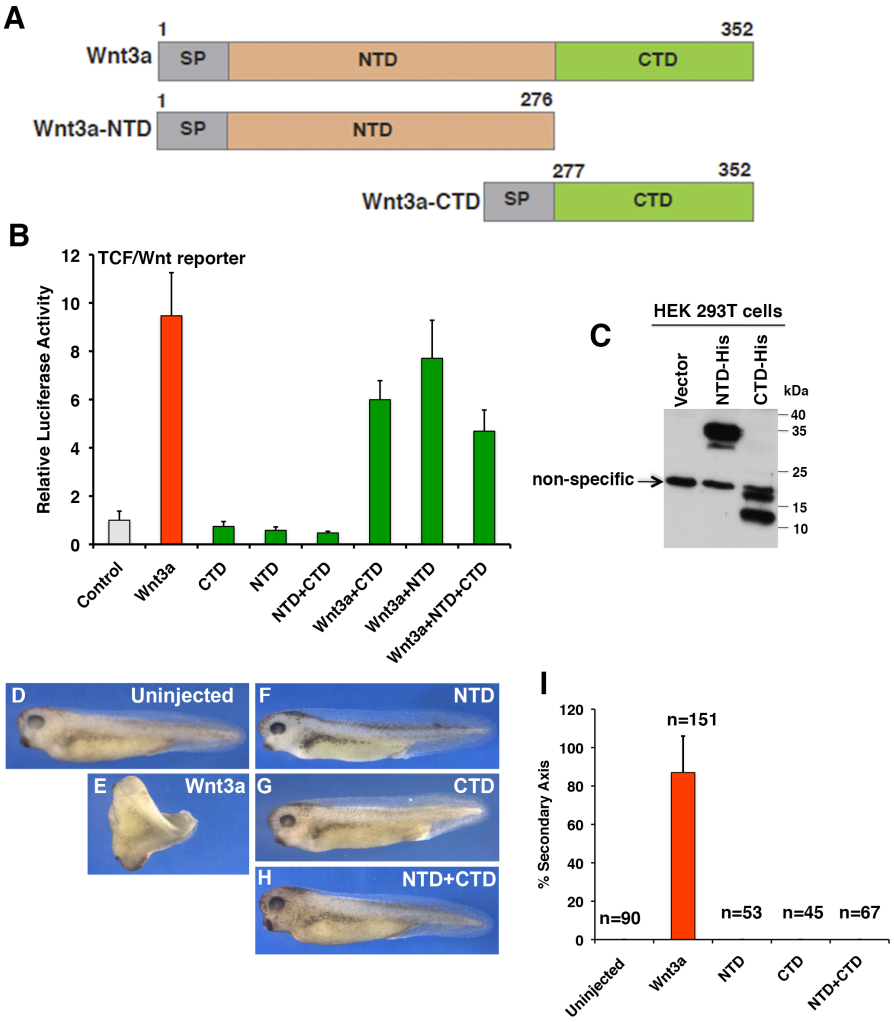

Fig.12. *In vitro* and *in vivo* activity of Wnt3a NTD and CTD domains. (A) Schematic representation of applied mouse Wnt3a NTD and CTD constructs. (B) Wnt reporter assay in HEK293T showing the effect of Wnt3a NTD and CTD on signaling activity. (C) Western blot analysis showing the secretion level of Wnt3a NTD and CTD protein in HEK293T cells. (E-H) *Xenopus* embryos at 4-cell stage were injected into the ventral-marginal-zone with 3pg mRNA encoding (E) Wild type Wnt3a (F) NTD (G) CTD (H) NTD+CTD. SP= signal peptide, NTD= N-terminal domain, CTD=C-terminal domain. (I) Statistical overview of mRNA injection experiment.

To perform this experiment, I synthesized the capped mRNA from pCS2-Wnt3a NTD and CTD by linearzing the plasmid. For double axis experiment 3 pg of Wn3a NTD and CTD capped mRNA were injected in embryos at 4 cell-stage, injection of wild

type Wnt3a mRNA served as positive control. Embryos injected with 3 pg of wild type Wnt3a mRNA very effectively induced secondary axes, while the separate Wnt domains did not show a comparable effect, even at a ten or hundred-fold access of mRNA (Table 17). Consistently, a combined injection of equimolar mRNAs encoding for the Wnt3a domains also did not induce an *in vivo* activity (Fig. 12 D-H). This leads to conclude us that Wnt domains can fold independently but are not able to bind to Fz receptor effectively in an independent manner in the *in vivo* context.

Table 17: The effect of Wnt3a NTD and CTD mRNAs on secondary axis development in *Xenopus* embryos. Numbers represent total numbers of embryos. In combined injections the mRNAs were applied at equimolar concentrations.

mRNA injection	Normal body axis	Secondary axis	Total
Uninjected	90	0	90
Wnt3a (3pg)	19	132	151
NTD			
3pg	53	0	53
30pg	48	0	48
300pg	44	0	44
CTD			
3pg	45	0	45
30pg	31	0	31
300pg	38	0	38
NTD+CTD			
3+1 pg	67	0	67
30+10 pg	71	0	71
300+100 pg	53	0	53

6.6 Physiological relevance of Wnt3a point mutations in zebrafish embryos

To determine whether mouse Wnt3a point mutations have any effect on the Wnt3a function *in vivo,* I evaluated the activity of Wnt3a mutants that were found to be critical *in vitro* by their expression in the zebrafish embryo. Ectopic expression of Wnt antagonists can promote head formation, whereas ectopic activation of Wnt signaling during gastrulating blocks head formation (Niehrs, 1999). Furthermore, it

has been previously shown that over expression of zebrafish Wnt3 can activate the canonical Wnt pathway within the zebrafish embryo and block the head formation (Clements et al, 2009). To investigate mouse Wnt3a mutants *in vivo,* I injected 3pg mouse Wnt3a capped mRNA per embryo, which causes a very specific and robust effect on specifying the balance of anterior-posterior (A-P) cell fates along the neural axis in the zebrafish, resulting in severe posteriorization of the anterior nervous system (Fig. 13A, B). Using the optical sectioning, it was found that 2 pg of Wnt3a mRNA per embryo leads to the loss of the eye field, forebrain and partially also midbrain structure (Fig. 14B). These abnormalities were specific to the cranial part of the embryo, whereas the posterior part of the embryos showed no gross abnormalities. Injection of higher amounts of Wnt3a mRNA leads to severe abnormalities during gastrulating (Fig. 13) that were specific to high Wnt3a expression level only.

If the point mutations in mouse Wnt3a were deleterious for their activity *in vitro,* then similar levels of the Wnt3a mutants would be expected to have no effect on A-P neural axis establishment. To investigate the above point I injected a set of Wnt3a mutant capped mRNAs at 2 pg per embryo and quantified the effects on the embryo development at 1.5 day post fertilization (dpf). Similar to wild type mouse Wnt3a, the mutants F331A, W333A, C335A, V60R, E68A, A96R and F169A lead to severe defects in the formation of anterior most neural system. They were characterized for lack of telencephalon and the eye field structure and occasionally also led to diminished mesencephalic structure when compared to the non-injected or the EF1 α control injected embryos (Fig. 14A). Interestingly, in the A96R mutant, the effects were most severe among all expressed constructs, where very frequently the complete cranial part of the embryonic axis was missing. In 24.8% of G210R expressing embryos the eyes were either not present or appeared to be significantly smaller in size. I was very intrigued by the finding that when the V337R mutant was expressed, 81.3% of the injected embryos were normal and not distinguishable from the controls. Interestingly, from all injected constructs, each carrying a single mutations in Wnt3a, only S209A mutant mRNA injected embryos (94.7%) did not have any effect, neither on the formation of forebrain/midbrain nor on the eye field, suggesting S209A mutation as deleterious for *in vivo* function of Wnt3a. Double mutants, E68A/E169A and F331A/W333A, did not show any reversal of the phenotype and their effects were

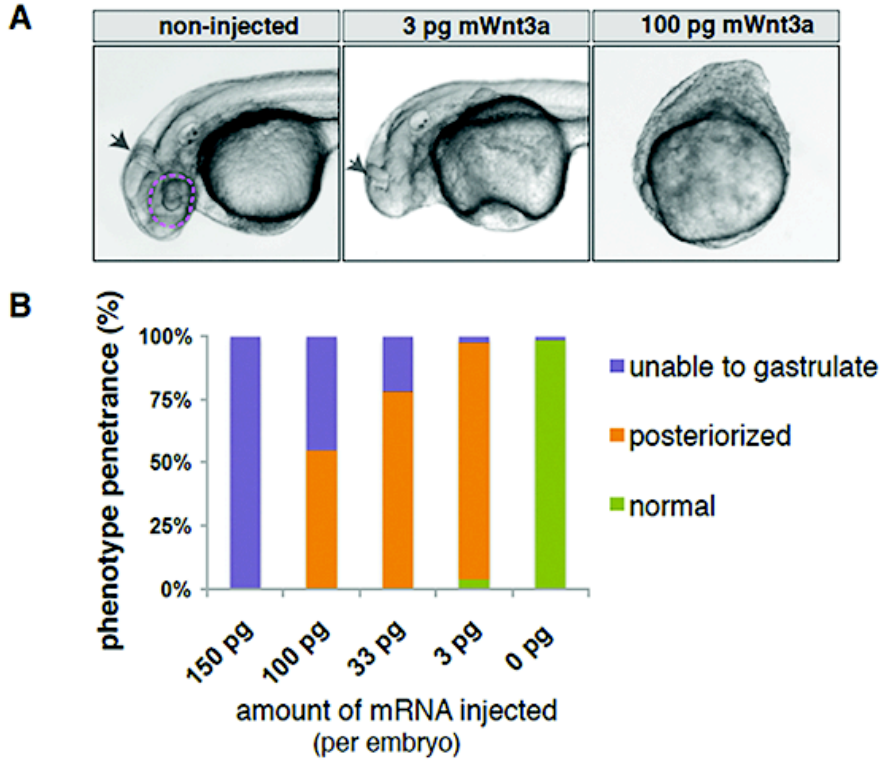

Fig.13. Effect of ectopic mouse Wnt3a capped mRNA injections on zebrafish embryonic development. (A) Pictures of live embryos in lateral view. Anterior is to the left and posterior to the top. Note the effect of 3pg mouse Wnt3a mRNA injection on loss of eye field, and forebrain. Midbrain-hindbrain barrier (arrows) is still present. Upon injection of 33pg mouse Wnt3a mRNA or more, the embryos are not able to gastrulate properly. **(B)** Quantification of the effect of full-length wild-type mouse Wnt3a mRNA on zebrafish embryonic development. Number of embryos analyzed for each condition: non-injected n:65, 3pg n:81, 33pg n:82, 100pg n:93, 150pg n:74.

not significantly different from each single mutant at gross morphological level. Altogether, these results reveal that the mutations in F331A, W333A, C335A, V60R, E68A, A96R, F169A residues do not significantly impair Wnt3a *in vivo* signaling activity in this context. Similar to the experiments from cultured HEK 293T cells, the S209A mutant completely lost the activity on zebrafish embryogenesis, suggesting its critical role in zebrafish embryo development. Interestingly, in this analysis V337R was indentified as the most essential residue required for signaling.

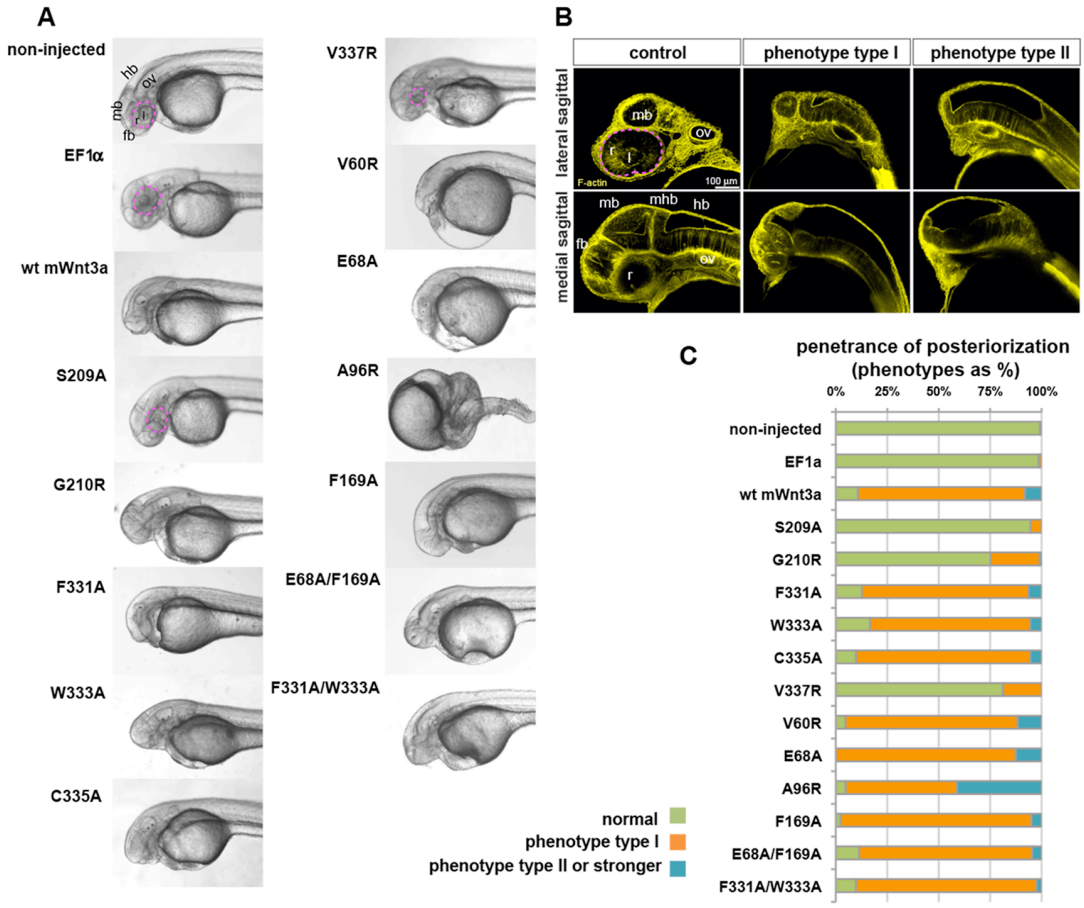

Fig. 14. Expression of mouse Wnt3a mutants in zebrafish embryos. (A) Lateral view of 1.5 dpf old zebrafish embryos upon injection of capped mRNA encoding wild type and mutant mouse Wnt3a. Note lack of forebrain, eye field and midbrain structures in lateral view. **(B)** Posteriorizing effect of Wnt3a overexpression on zebrafish development as analyzed at 1.5 dpf. Pictures represent single optical sections either at lateral (upper panel) or medial (lower panel) sagittal sections in staining for F-actin marking cell outlines and thereby general nervous system morphology. Weaker phenotype with loss of retina and forebrain, whereas remnants of midbrain structures might be present termed as phenotype type I. Stronger phenotype (with lack of forebrain, retina, lens, midbrain) determined as phenotype type II. **(C)** Penetrance of the dorsalized phenotype in zebrafish embryogenesis upon wild type and mutant mouse Wnt3a expression, quantified as percentage. Type I (weaker) and type II (stronger) respond to the phenotypes as shown in Fig. 11B. Total number of embryos (n) from three independent experiments: non-injected:403, EF1a: 76, wild type mouse Wnt3a:224, S209A:113, G210R:96, F331A:79, W333A:54, C335A:91, V337R:139, V60R:86, E68A:64, A96R:124, F169A:82, E68A/F169A:88, F331A/W333A:82. In all panels: anterior is to the left and dorsal is up. Eyes are outlined with dashed pink line. Fb = forebrain, mb = midbrain, hb = hindbrain, r = retina, l= lens, ov = optic vesicle.

6.7 Quantification of mouse WT Wnt3a and mutants protein in HEK 293T cells

The amount of Wnt3a protein secreted into the medium was quantified by comparing it with various amount of purified recombinant mouse Wnt3a protein (R&D system). 20 µl of the condition medium (CM) contained, ≈ 12 ng of the mouse Wnt3a protein, indicating that the concentration of Wnt3a protein was 0.6 µg/ml in the condition medium (Fig. 15).

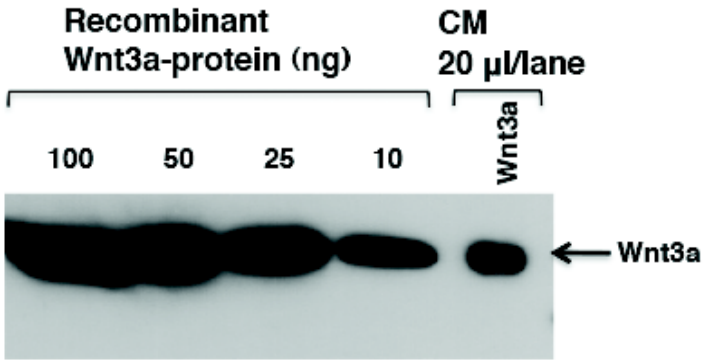

Fig.15. Estimation of mouse Wnt3a concentration in conditioned medium of HEK293T cells. 20 µl of conditioned medium from HEK 293T cells expressing wild type mouse Wnt3a were blotted together with increasing amounts of commercial recombinant mouse Wnt3a using anti-Wnt3a antibody. The intensity of the Wnt3a band in conditioned medium was found to be between the intensities of the 10 and 25 ng bands.

6.8 Soluble Fz8-CRD protein influences Wnt3a signaling in a biphasic manner

The *in vitro* data obtained from TCF/Wnt reporter and pull down assay on site 2 mutants W333A and C335A strongly suggested a major role of the Wnt3a CTD in Fz binding, leads to concluded that receptor engagement of the membrane-anchored Wnt is a two-step process, in which the initial interaction with the Wnt CTD facilitates membrane detachment of the lipid modified NTD. Soluble CRD proteins might therefore be able to augment Wnt signaling by the increasing local concentration of membrane-detached Wnt ligands at cell surface. To investigate this hypothesis, I performed a Wnt reporter assay in HEK 293T cells with wild type Wnt3a in presence

of Fz8-CRD-Fc protein at increasing concentration. HEK293T cells were transiently co-transfected with reporter constructs TCF-firefly, actin *Renilla* and wild type Wnt3a, after 24 hours post transfection recombinant Fz8-CRD-Fc protein at indicated concentrations was added on these Wnt3a producing cells. It was observed that soluble Fz8-CRD applied at micromolar concentrations inhibited Wnt signaling (Fig. 16A). In contrast, CRD concentration that were in the range or slightly above the Wnt3a concentration in the cell supernatant (~ 1 ng/µl) increases Wnt reporter activity by three-fold as compared to the Wnt3a alone, while Fc protein alone did not influence Wnt reporter activity.

To confirm that the increase in Wnt reporter activity was due to a higher amount of soluble Wnt3a ligands, I transfected HEK 293T cells with wild type Wnt3a plasmid. After 24 hours of post-transfection cells were incubated with different amount of Fz8-CRD-Fc protein and further monitored for Wnt3a protein levels in the cell supernatants by western blotting. The amount of soluble Wnt3a protein was to a large extent correlated with the applied Fz8-CRD concentration, while Fc protein alone did not induce Wnt3a detachment from the cell surface (Fig 16B).

To provide further evidence that the observed increase in Wnt signaling activity by Fz8-CRD protein was due to a higher mobility of the Wnt ligands, a double chamber luciferase assay was performed, in which the LWnt3a transfected cells were separated from the responder cells, which were transfected with reporter plasmid by a porous membrane (Fig. 16C). The Fz8-CRD-Fc protein was applied at increasing concentration to the Wnt-producing cells. As shown in Fig. 16D it was found that the luciferase activity of the responder cells was modulated by the soluble CRD protein in a comparable manner as in Fig. 16A, suggesting that the increase of signaling activity at nearly equimolar concentrations of Wnt3a and Fz8-CRD is independent from direct cell-cell contacts. Taken together, these results clearly indicate that, Wnt3a activity was modulated by the presence of soluble CRD protein positively by increased solubility at low CRD concentrations and negatively at high CRD concentration.

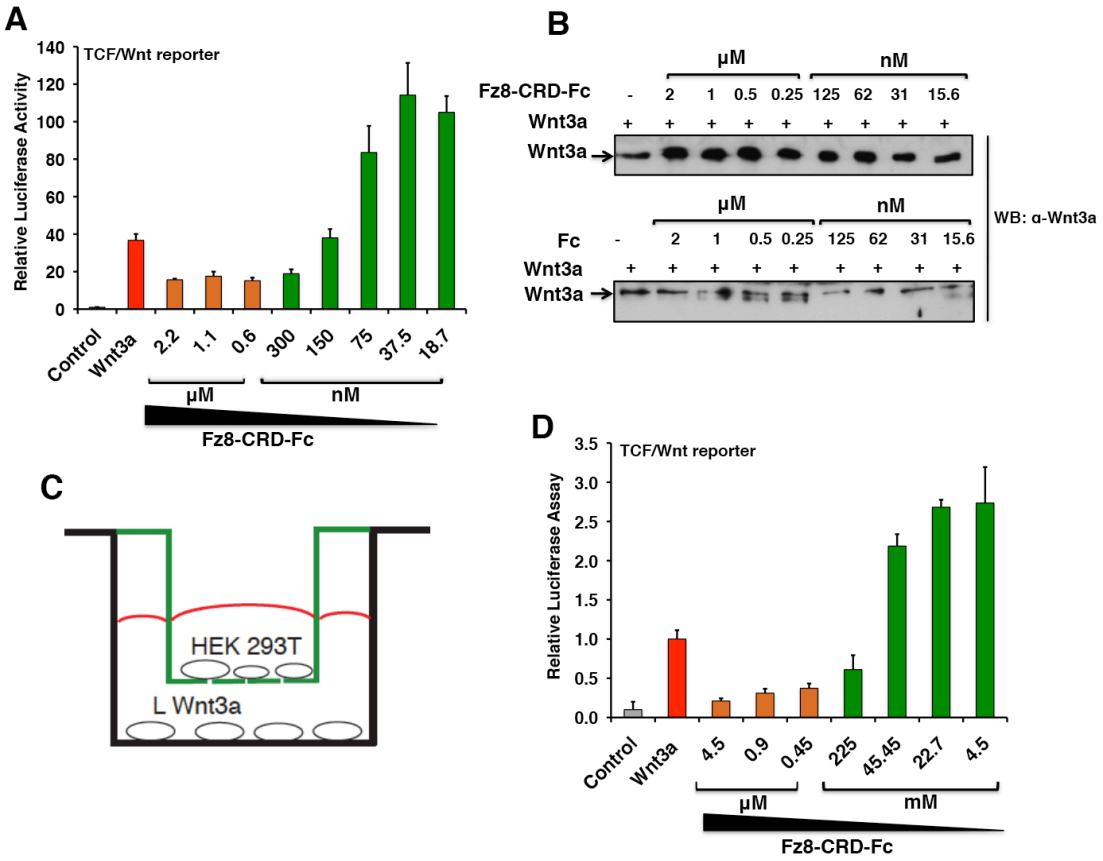

Fig. 16. Modulation of Wnt signaling by soluble Fz8-CRD-Fc (A) Wnt reporter assay showing the influence of soluble mouse Fz8-CRD-Fc on Wnt signaling in a dose-dependent manner **(B)** Immunoblot depicting the influence of recombinant Fz8-CRD-Fc or Fc as negative control on the concentration of soluble Wnt3a protein in the cell supernatant. **(C)** Schematic representation of set up for double chamber luciferase assay in which responder cells were separated by Wnt producing cells via porous membrane. **(D)** Wnt reporter assay readout in which CMV-b-Galactosidase and Super TOPFlash reporter were transfected in HEK 293T cells. The Fz8-CRD-Fc protein was applied at increasing concentrations to the Wnt producing cells. Luciferase activity was monitored in Wnt3a responder cells. Experiments were carried out in triplicate. Error bars depict standard deviation.

7. Discussion

Although the Wnt/Fz signaling pathway has been studied extensively at the cellular level, structure-function properties of Wnt ligands are poorly understood. In the present study, I demonstrate an *in vitro* and *in vivo* functional characterization of mouse Wnt3a-Fz8 binding interface. The mutations that were introduced in the Wnt3a sequence were directed by a model of the mouse Wnt3a-Fz8-CRD complex structure on the basis of the recently published X-ray structure (Janda et al, 2012) (Fig. 7B). The binding epitopes screen using the synthetic Wnt3a peptide library indicated several critical sites in accordance with those suggested by the crystal structure. Interestingly, a peptide comprising the lipid attachment residue showed the strongest effect although, lacking the lipid modification. This indicates that site 1 binding is not exclusively dependent on the presence of the lipid modification. The strong effect of G210R mutation underscores the importance of protein-protein interactions apart from the lipid-CRD interaction in site 1. The fact that none of the peptides induced a complete inhibition of Wnt signaling in cell culture competing for binding site with the full-length ligand argues against linear Wnt peptide fragments as potent inhibitors. This is in contrast to a study using peptide from the LRP6 binding domain of Dkk-1 (Gregory et al, 2005) and might reflect a higher complexity of the Wnt-Fz interface.
When I mutated residues in mouse Wnt3a responsible for site 1 interaction (Fig. 8) S209 mutation abolished signaling activity in the Wnt reporter assay completely, due to its function as lipid attachment site. The importance of the lipid modification in Wnt signaling has been repeatedly reported (Doubravska et al, 2011; Takada et al, 2006; Tang et al, 2012; Willert et al, 2003). Interestingly, Ser209 mutation also led to a complete loss in binding to soluble Fz8-CRD (Fig. 10B), although the C-terminal Wnt domain was reported to be sufficient for binding in the absence of site 1 (Janda et al, 2012). In concert to this, it was also found that S209 to be crucial for Wnt3a activity in injected zebrafish embryos *in vivo*. Interestingly, S209 is highly conserved among members of Wnt family except *Drosophila* WntD. It can be concluded that the Fz interaction with the Wnt lipid might be necessary for the correct positioning of the ligand in the signaling complex and its stability.

In particular, LRP5/6 binding sites in the linker between sites 1 and 2 might be sensitive to lacking conformational restrictions imposed on this region (Chu et al, 2013). Alternatively, the lack of lipid modification might induce protein aggregate formation precluding site 2 accessibility. It was found that G210R mutation also leads to a complete loss both in binding and activity, however alanine substitution at this position (G210A) has no effect on Wnt activity, suggesting that charged amino acids might disrupt interaction between Wnt and Fz. In zebrafish embryos G210R was still partially active, suggesting that the presence of the lipid attachment at site 1 alone is likely insufficient for full Wnt3a activity. Apparently, a distortion of the ‘‘thumb’’ loop interaction with Fz, can also lead to a largely dysfunctional ligand. The G210 position is conserved among all Wnt ligands with the exception of medaka and zebrafish Wnt8-like, where it is substituted to glutamic acid (Yokoi et al, 2003). Considering the findings that substitution is supposed to result in a Wnt ligand with attenuated activity, which might have a distinct developmental function. Other conserved site 1 residues reported to contribute secondarily to Fz interaction (K204, L208, W218) in *Xenopus* Wnt8 showed moderate reduction in signaling activity of mouse Wnt3a, both as single mutants and in double mutant (K204/L208).

As predicted by the structural data, site 2 mutations had more pronounce effect on signaling in the Wnt reporter assay (Fig. 8C). In particular mutations in residues F331, W333, C335 and V337 that engage in van der waals interactions at the tip of the Wnt ‘‘index finger’’ in the complex structure showed a dramatic loss of ligand activity when mutated to alanine. C335, which showed the strongest effect, forms a disulphide bond with Cys334. From the above result, it can be presumed that its mutation besides attenuating CRD interaction has an additional impact on the overall structure of the binding loop. When I tested these mutants in the physiological context of zebrafish embryogenesis, F331, W333 and C335 showed only mild deviations from the wild type Wnt3a, retaining most of their signaling capacity, which might be due to compensatory effects by a different receptor context or higher sensitivity of the responder tissue. The V337R substitution, which had the most severe effect on signaling *in vitro* and *in vivo*, probably induces a stronger conformational distortion of the binding loop due to the bulky Arg side chain. Accordingly, an alanine substitution at this position did not alter the signaling efficiency of the Wnt ligand. Interestingly,

V337R mutation did not significantly inhibit binding to Fz8-CRD in solution (Fig 10B). It can be concluded that this site can be considered as primarily essential for signaling without contributing to binding. This result further strengthened by a very recent report on a naturally occurring Wnt1 mutation of valine to phenylalanine at position V337 (V335) causing osteogenesis imperfecta (OI) through impaired Wnt signaling (Fahiminiya et al, 2013). It was identified that residue V337, as being essential for Wnt3a activity during zebrafish embryogenesis is novel. I therefore concluded that the insertion of W333 into the hydrophobic pocket of the Fz receptor is a critical binding event in the Wnt CTD might well be independent from the signaling competence of the ligand-receptor complex.

Among site 3 mutants that abolished signaling, F169 showed the most pronounce loss in binding, which is in accordance with the results of the peptide screen (Fig. 6C). Here, peptide 15, which harbors F169 as a central residue showed the strongest inhibitory effect as compared to peptides comprising other site 3 amino acids. It was found that several site 3 mutants are inactive Wnt signaling strongly suggests CRD dimer formation as a necessary step in receptor signaling as was already suggested by previous reports (Carron et al, 2003). Interestingly, position F169 in Wnts is highly conserved and the interacting site in Fz CRDs and sFRPS is either phenylalanine or tyrosine suggesting stacking interactions of the aromatic residues (Appendix 9.3). As F-F and F-Y pairs exhibits different binding energies (Chelli et al, 2002) this interaction might be relevant for ligand-receptor specificity.

The present study also showed that separate domains of Wnt3a could be secreted but are unable to activate Fz receptor signaling in an independent manner in Wnt reporter assay and *Xenopus* embryos. Previous reports also showed that Drosophila Wg independent domains fold properly, but did not obtain evidence for the separate function (Wu & Nusse, 2002).

The lipid modification of Wnts (Willert et al, 2003) most probably acts as membrane anchor brining the C-terminal domain into a membrane-distal position. I proposed a model that suggests a sequential binding of Wnt to Fz-CRD protein, in which the first contact to the Fz-CRD and other CRD related proteins might therefore be realized via

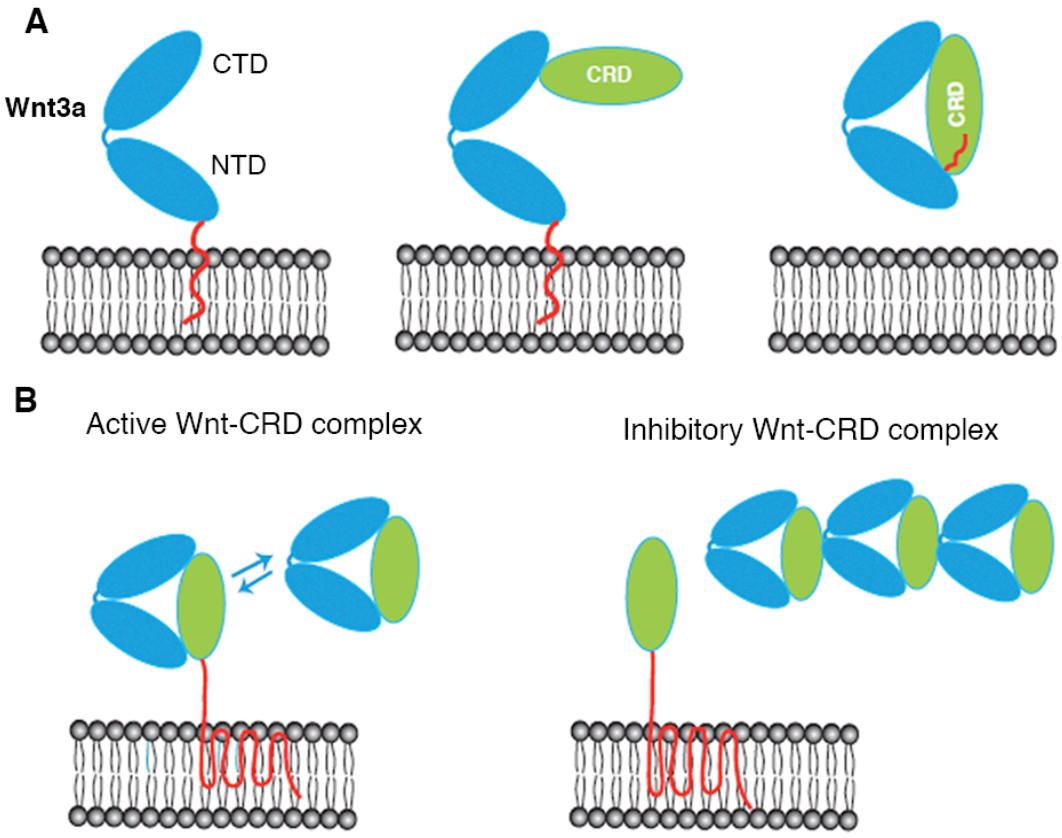

Fig.17. Model for Wnt-CRD complex formation. (A) Sequential binding of membrane-associated Wnt ligands to CRDs. The first binding step is realized between the membrane-distal Wnt CTD and Fz-CRD or other CRD-related proteins via site 2 interaction. This facilitates membrane detachment of the Wnt NTD and a switch of the lipid chain into the site 1 binding pocket. **(B)** Biphasic modulation of Wnt activity by CRD proteins. At low concentrations of CRD proteins active Wnt-CRD complexes are formed by Wnt mobilization and transfer to Fz receptors. At high CRD concentrations oligomerization is induced via site 3 interaction, leading to trapping of Wnt ligands in inactive Wnt-CRD aggregates.

site 2, which in the second step facilitates membrane detachment and a switch of the lipid chain into the site 1 binding pocket, (Fig. 17A). Soluble CRD-related proteins should therefore effectively increase the amount of cell-detached Wnt ligands. In accordance with this, the spatial signaling range of Wnt was increased significantly by sFRP *in vivo* (Mii & Taira, 2009). Our data on the modulatory activity of Fz8-CRD-Fc proteins on Wnt signaling clearly support such mechanism.

In particular, by using the Wnt reporter assay with separated chambers for Wnt producing and responding cells, I was able to demonstrate that the presence of soluble CRD proteins is able to promote Wnt activity by facilitating mobility. This hypothesis was additionally investigated by designing fusion protein in which the Fz8-CRD was covalently linked to the N or C-terminus of Wnt3a via a flexible linker.

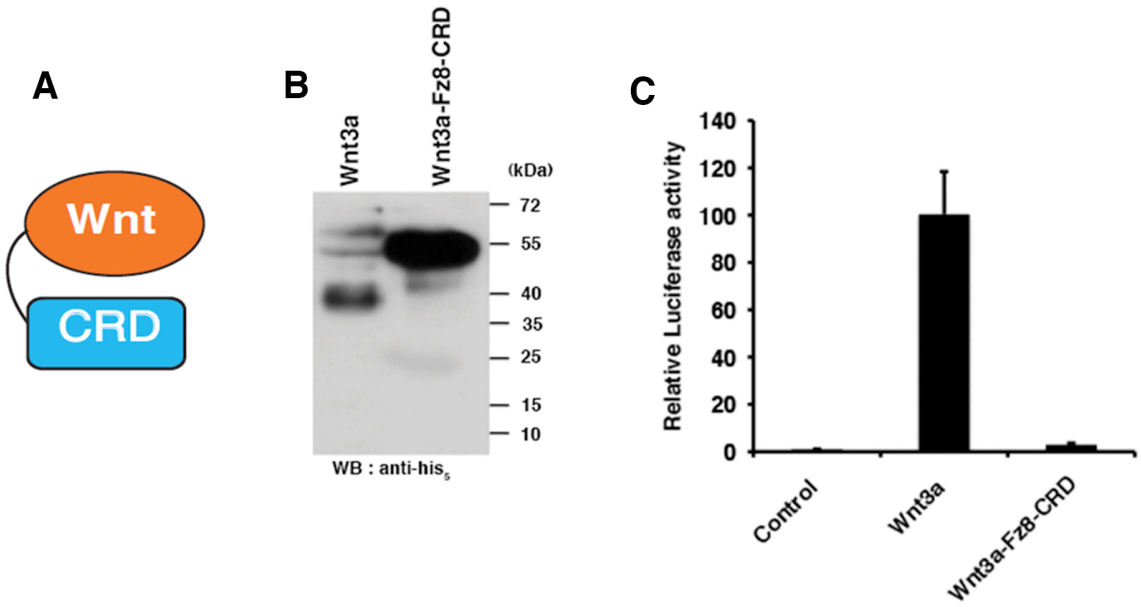

Fig.18. Secretion and activity of a Wnt3a-Fz8-CRD fusion protein. (A) Schematic representation of Wnt3a-Fz8-CRD fusion protein. **(B)** Secretion levels of His-tagged Wnt3a and Wnt3a-Fz8-CRD fusion protein. Precipitated proteins from equal amounts of conditioned medium were analyzed by Western blot using anti-his antibody **(C)** Wnt3a-Fz8-CRD fusion protein did not induce Wnt signaling activity in Wnt reporter assay. Error bars depict standard deviation.

The level of these fusion proteins in conditioned medium were highly increased compared to Wnt3a alone indicating enhanced solubility by the presence of the CRD moiety (Fig. 18B). Mutants that inhibit this process would accordingly increase the concentration of active Wnt-CRD complexes, which facilitate efficient receptor engagement. The severe effect of the A96R mutation in the zebrafish embryo together with its pronounced activity in the Wnt reporter assay strongly support such a model and suggest this mutant as a possible new tool for the field representing a hyperactive Wnt ligand.

7.1 Conclusions and Outlook

In summary, a comparison of our results obtained from *in vitro* and *in vivo* analysis provides novel important insights into structure function relationships of the Wnt3a-Fz8 binding interface and its modulation by soluble CRD-related proteins. The dissection of Wnt3a residues into sites essential for binding and for signaling will clearly suggest new approaches to tackle the problem of ligand-receptor specificity in the Wnt field. This could help in the design of potent Wnt agonists and antagonist that mimic the ability of Wnt to bind to multiple biologically relevant sites. This may have therapeutic implication in treating Wnt related diseases. In particular, our analysis of site 3 residues that likely induce CRD homo or hetero dimers might improve our understanding of different signaling in the context of different receptors and soluble CRD-related factors.

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9. Appendix

9.1 Abbreviations

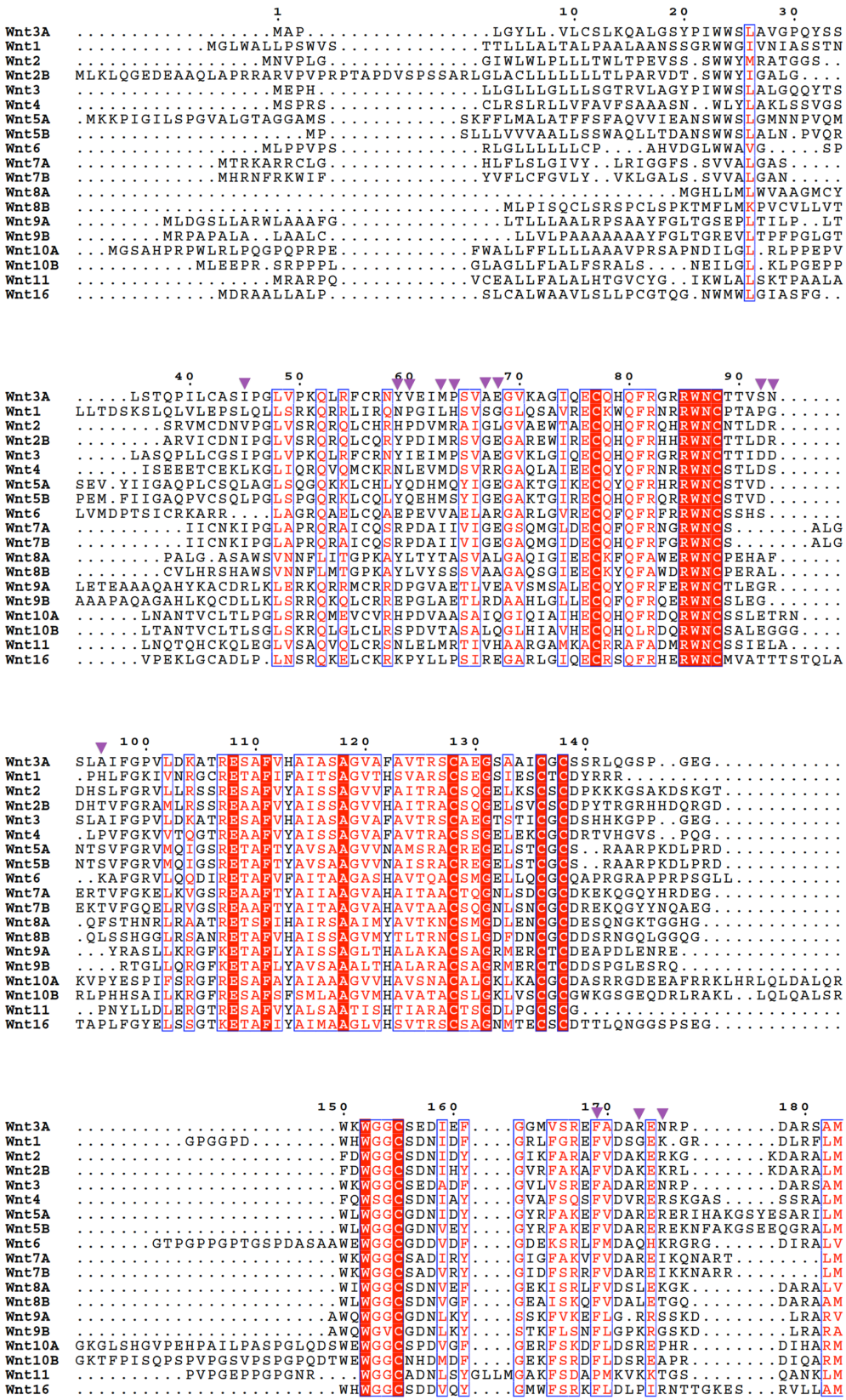
a-p	Antero-posterior
APC	Adenomatous polyposis coli
APS	Ammonium Persulphate
BMP	Bone morphogenetic protein
BSA	Bovine Serum Albumin
bp	Base pairs
C. elegans	*Caenorhabditis elegans* (roundworm)
CKI	Casein Kinase I
CoIP	Co-immunoprecipitation
D. melanogaster	*Drosophila melanogaster* (fruit fly)
Dkk	Dickkopf
DNase	Deoxyribonuclease
nNTP	Deoxyribonucleoside triphosphates
DTT	1,4- Dithiothreitol
d-v	Dorso-ventral
Dvl	Dishevelled
EDTA	Sodium ethylendiaminotetraacetate
FCS	Fetal calf serum
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
FGF	Fibroblast growth factor
Fz	Frizzled receptor
GK3β	Glycogen synthase kinase 3
kDa	kiloDalton
Krm	Kremen
LB	Luria-Bertani
LDLR	Low density lipoprotein receptor
LRP	Lipoprotein receptor-related protein

M	Molar
min	Minutes
mRNA	messenger RNA
µl	microliter
µg	microgram
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PDB	Protein data bank
PCP	planar cell polarity
PCR	polymerase chain reaction
RT	Room temperature
SDS	Sodium dodecyl sulphate
sFRP	Secreted Frizzled-related protein
TCF	T-cell factor
TEMED	N,N,N',N'-tetramethyethylenediamine
Tris	Tris(hydroxymethyl)-amino-methane
UV	Ultraviolet
WT	Wild type
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
X. laevis	*Xenopus laevis* (African clawed frog)

Amino Acids

A	Ala	alanine	I	lIe	isoleucine	R	Arg	arginine
C	Cys	cysteine	K	Lys	lysine	S	Ser	serine
E	Asp	aspartic acid	L	Leu	leucine	T	Thr	threonine
D	Glu	glutamic acid	M	Met	methionine	V	Val	valine
F	Phe	phenylalanine	N	Asn	asparagine	W	Trp	tryptophan
G	Gly	glycine	P	Pro	Proline	Y	Tyr	tyrosine
H	His	histidine	Q	Gln	glutamine			

9.2 Multiple sequence alignment of mouse Wnts



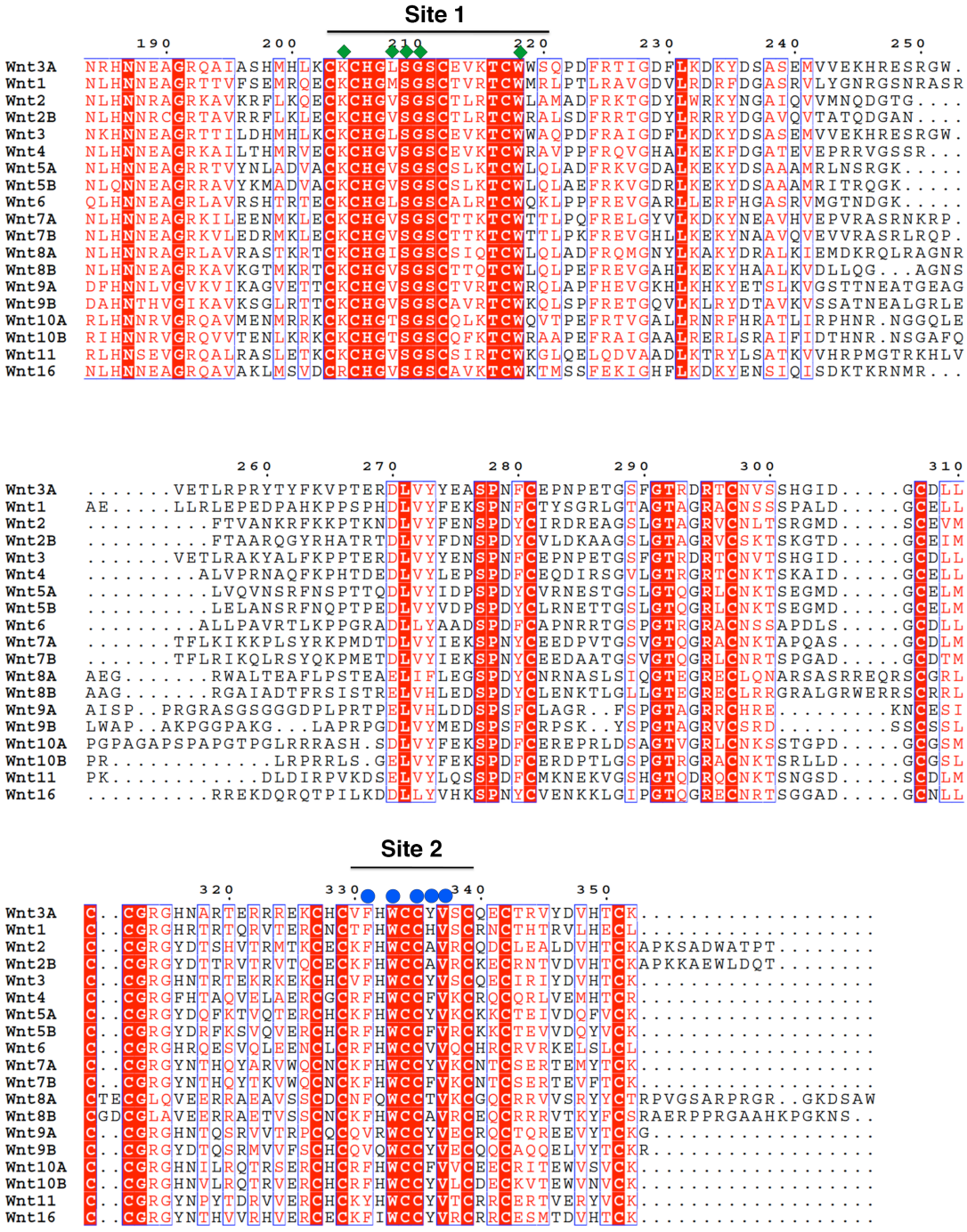


Fig.19 Multiple sequence alignment of mouse Wnts. Sequences were aligned with Clustal W (Chenna et al, 2003) and ESPript (Gouet et al, 2003). Invariant residues are shown as white text in red boxes. Conserved residues are shown in red text in white boxes. Interacting residues from different binding sites are marked with different colors. Site 1 interacting residues marked with green square, Site 2 residues are marked with blue circle and site 3 residues are marked with violet triangle.

9.3 Multiple sequence alignment of Fz8-CRD and sFRPs from different organisms

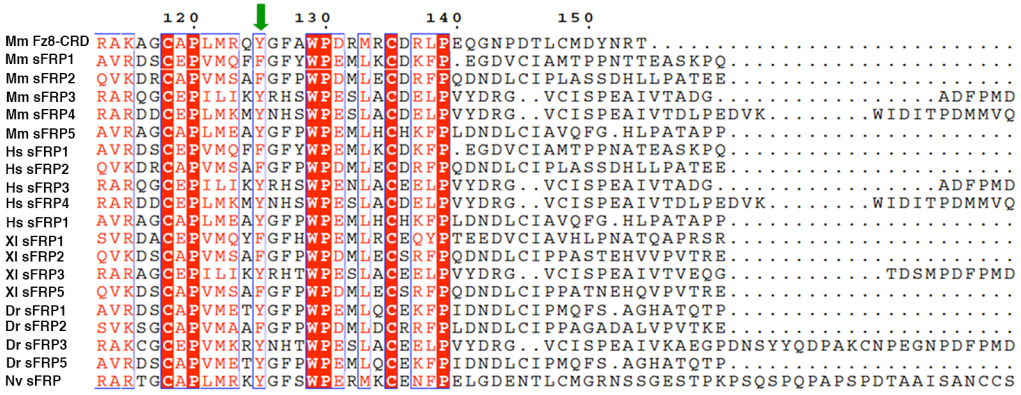


Fig.20. Multiple sequence alignment of Fz8-CRD and sFRPs. Sequences were aligned with Clustal W (Chenna et al, 2003) and ESPript (Gouet et al, 2003). Invariant residues are shown as white text in red boxes. Conserved residues are shown in red text in white boxes. The F169 residue from site 3 either which is highly conserved in all Wnts interact with and interacting site in Fz CRDs and sFRPs is either phenylalanine or tyrosine (marked with green arrow).
Mus musculus (Mm), *Homo sapiens* (Hs), *Xenopus laevis* (Xl), *Danio rerio* (Dr), *Nematostella vectensis* (Nv).

9.4 List of figures

9.5 List of tables