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DISSERTATION

Titel der Dissertation

Interactions between Lef/Tcf and β -catenin for transcriptional
activation in Wnt signaling
and antisense function of modified peptide nucleic acids
in Medaka fish

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1. Summary

The canonical Wnt signaling pathway is involved in elementary cell processes like proliferation, polarity, survival, apoptosis, and motility and during embryonic development cell fate determination, tissue homeostasis and axis specification in all metazoan organisms. It fulfills the transcriptional control of target genes in the nucleus over a cascade of events. The activation occurs via the binding of the Wnt proteins, which represent cysteine-rich secreted ligands, to characteristic receptors, leading to high amounts of free β -catenin in the cytoplasm. Finally this main signal transducer protein interacts in the nucleus with the transcription factors Tcf/Lef for the upregulation of numerous prominent genes. Exactly the regulation of some of these genes can be disordered by some known mutations, leading to several human diseases and the development of human cancer.

In this study the mechanism of the interaction between the key factor β -catenin and the DNA binding transcription factors Tcf/Lef, which is essential for the transcriptional control of the Wnt target genes, was examined in detail. The formation of the β -catenin-Tcf/Lef complex is primarily carried out by the N-terminal binding domain of Tcf/Lef. Based on a study of Daniels and Weis (Daniels and Weis, 2005), in combination with recently performed cell culture experiments using a luciferase assay (Dworak, 2006; Winkler, 2008), a potential existence of a second interaction domain of Lef1, Tcf3 and Tcf1, representing the HMG box, which is responsible for DNA binding, was indicated. I could confirm this interaction with a functional assay in cell culture cells. In this assay an improved DNA binding of Tcf/Lef proteins can be shown due to β -catenin interaction. In addition, this second weak interaction was also seen between Lef1 and Plakoglobin, a β -catenin related protein with controversial functions. In further experiments, based on the usage of deletion mutants, the Armadillo repeats 10 to 12 of β -catenin were mapped as the most important region for this additional Tcf/Lef interaction, affecting the DNA binding of Tcf/Lef. In additional experiments the biological role for this β -catenin effect was indicated by the application of reporters with specific low affinity Tcf/Lef binding motifs, derived from the enhancers and promoters of the Wnt target genes *Otx2*, *Axin2*, *c-myc* and *naked cuticle*. The improved DNA binding of Tcf/Lef, caused by β -catenin, seems to gain importance in case of weaker Tcf/Lef- DNA interactions for an efficient gene upregulation.

In a further study the antisense function of modified peptide nucleic acids (PNAs) on Wnt interacting genes for efficient knock down experiments could be successfully demonstrated in Medaka fish. PNAs represent artificially synthesized polymers with N-(2-aminoethyl)-glycine units linked by peptide bonds with various purine and pyrimidine bases. Performing injection experiments in Medaka embryos the effect of PNAs could be strongly improved by modifying length, backbone and end groups leading to a block of the genes *Tcf3* and *Six3* which are anteriorly expressed in the the neural tube during gastrulation. Usually these genes antagonize the active Wnt signaling pathway to create an gradient increasing from anterior to posterior, resulting in the normal development of the forebrain including the eye field. By doing a knock down of *Six3* and *Tcf3*, the formation of the tel- and diencephalon, together with the prominent eye anlagen was strongly inhibited in dependence of the affected target gene. These results showed that PNAs may represent a powerful tool for switching off genes in biological research and medical treatments.

2. Zusammenfassung

In allen Metazoa steuert der Wnt-Signaltransduktionsweg elementare Zellprozesse wie Proliferation, Polarität, Überleben, Apoptose und Motilität und während der Embryonalentwicklung Differenzierung, Gewebemöostase und die Festlegung der Körperachsen. Eine Hauptaufgabe ist auch die transkriptionelle Steuerung seiner Zielgene durch eine Vielzahl von Prozessen. Die Aktivierung dieses Signaltransduktionsweges erfolgt über die Bindung der Wnt Proteine, welche Cystein-reiche Liganden darstellen, an spezifische Rezeptoren, was in großen Mengen an frei verfügbaren β -catenin im Cytoplasma resultiert. Schließlich interagiert β -catenin, das Schlüsselprotein für den Signaltransduktionsprozess, mit den Transkriptionsfaktoren Tcf/Lef im Zellkern, um die Expression zahlreicher Gene herbeizuführen. In einigen Krankheiten und bei der Krebsentwicklung können die Expressionsaktivitäten der Gene durch Mutationen fehlgeleitet sein.

In diesem Projekt wurde der detaillierte Mechanismus der Interaktion von β -catenin mit den DNA bindenden Transkriptionsfaktoren Tcf/Lef, welcher die Transkription der Wnt-Zielgene kontrolliert, untersucht. Die Komplexbildung von β -catenin und Tcf/Lef erfolgt primär über die N-terminale Bindungsdomäne von Tcf/Lef. Eine Studie von Daniels and Weis (Daniels und Weis, 2005), in Kombination mit kürzlich durchgeführten Zellkulturexperimenten, in denen die Luciferaseinduktion gemessen wurde (Dworak, 2006; Winkler, 2008), deuten auf eine Existenz einer zweiten Interaktionsdomäne von Lef1, Tcf3 und Tcf1 innerhalb der DNA bindenden HMG box hin. In meinen Zellkulturexperimenten konnte ich diese Interaktion bestätigen. Wie mit einem funktionellen Luciferase assay gezeigt werden konnte, scheinen die DNA-Bindungen von Tcf/Lef durch eine Interaktion mit β -catenin verstärkt zu werden. Außerdem konnte auch eine zweite schwache Interaktion zwischen Plakoglobin, einem mit β -catenin verwandten Protein mit kontroversiellen Funktionen, und Lef1 gezeigt werden. In weiteren Experimenten mit β -catenin Deletionsmutanten konnten die Armadillo-Sequenzen 10 bis 12 als wichtigsten Bereich für diese zusätzliche Tcf/Lef Interaktion, die auch die DNA-Bindung zu beeinflussen scheint, identifiziert werden. Außerdem wurde die biologische Rolle dieses „ β -catenin Effekts“ mit Luciferase Reportern mit spezifischen und schwachen Tcf/Lef DNA-Bindungsstellen, die von Enhancern und Promotoren von den Wnt Zielgenen *Otx2*, *Axin2*, *c-myc* und *naked cuticle* herrühren, untersucht. Dabei hat sich herausgestellt, dass die verbesserte DNA-Bindung von Tcf/Lef durch β -catenin in Fällen in denen schwächere Tcf/Lef DNA-Interaktionen vorkommen, eine bedeutende Rolle für eine effiziente Transkription von Genen spielt.

In einer weiteren Studie wurde das Potential von chemisch modifizierten „Peptide Nucleic Acids“ (PNAs) für eine effiziente Herunterregulation von Genen, die mit dem Wnt-Signaltransduktionsweg interagieren, erfolgreich in Medakafischen gezeigt. PNAs stellen artifiziell synthetisierte Polymere, die aus N-(2-Aminoethyl)-Glycin Elementen bestehen, die über Peptidbindungen verknüpft und mit verschiedenen Purin- bzw. Pyrimidinbasen für eine RNA/DNA-Interaktion versehen sind, dar. Mit Injektionsexperimenten in Medaka Embryos konnte die Eigenschaften der PNAs mit Hilfe einer bestimmten Länge, eines chemisch veränderten Rückrats und variabler chemischer Endgruppen so weit verbessert werden, dass eine effiziente Herunterregulation von, während der Gastrulation im Neuralrohr exprimierten Gene, *Tcf3* und *Six3*, resultierte. Normalerweise wirken diese beide Gene entgegen dem aktiven Wnt-Signaltransduktionsweg, um einen von anterior nach posterior aufsteigenden Gradienten, der für die Entwicklung des Vorderhirns und Augen entscheidend ist, zu er-

zeugen. Die Hinunterregulationen von *Six3* and *Tcf3* führten zu starken Entwicklungsstörungen von Tel-, Diencephalon und der Augenanlagen, in Abhängigkeit der beeinträchtigten Genfunktionen. Diese Ergebnisse zeigten, dass diese modifizierten PNAs starke Werkzeuge zum Abschalten bestimmter Gene in der biologischen Forschung und für medizinische Anwendungen sein können.

3. List of abbreviations

293T	human embryonic kidney cell line
A	alanine or anterior
Ac	acetylation
AES	truncated human TLE isoform amino-enhancer-of-split
amp ^R	ampicilline resistance
APC	adenomatous polyposis coli
Arm repeats	armadillo repeats of β -catenin
ATG/AUG	translation start codon
ATP	adenosine-tri-phosphate
AtT20	human pituitary adenoma cell line
Bcl-9	B-cell CLL/lymphoma 9
BMP	bone morphogenetic proteins
Brg	brahma related gene, ATPase chromatin re-modelling factor
C or C-terminus	carboxyl end group of amino acid
C, T, A, G, U	cytosine, thymine, adenine, guanine, uracil
<i>C. elegans</i>	<i>Caenorhabditis elegans</i>
C57MG	mammary epithelial cell line
CaCl ₂	calcium chloride
CamKII	calmodulin-dependent kinase II
CAP	calf intestinal alkaline phosphatase
CAT	chloramphenicol transferase
CBP	cAMP response element-binding protein
cdc42	cell division control protein 42 homolog
cDNA	complementary DNA
cGMP, cAMP	cyclic guanosine or adenosine monophosphate
ChIP	chromatin immunoprecipitation
CMV	cytomegalovirus
c-myc	cellular myelocytomatosis oncogene
CNS	central nervous system
CO ₂	carbon dioxide
CRARF	conserved domain with amino acids cysteine, arginine, alanine, arginine, phenylalanine
CtBP	C-terminal binding protein
Cyclin D1	regulator protein of CDK kinases
Daam1	dishevelled-associated activator of morphogenesis 1
Dac	dachshund (<i>Drosophila</i>)
Dach1	dachshund1 (vertebrates)
DEPC	diethylpyrocarbonate
Dfz1/2	<i>Drosophila</i> frizzled1 and 2
dkk1	dickkopf
DMEM	Dulbecco's Modified Eagle's Medium
DNA	desoxyribonucleic acid
dnTcf	dominant negative form of Tcf
dNTPs	nucleoside triphosphates
Dpp	decapentaplegic
<i>Drosophila</i>	<i>Drosophila melanogaster</i>

Dsh	dishevelled (Wnt signaling pathway)
DTT	dithiothreitol
Dvl	dishevelled (Wg signaling pathway)
<i>E.coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediaminetetraacetic acid
EGR	early growth response
EMSA	electrophoretic mobility shift assay
Eng2	engrailed2
ERM	Eriksson medium
eya	eyes absent (<i>Drosophila</i>)
Eya1	eyes absent (vertebrates)
ey	eyeless
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
Fos	fos minimal promoter deriving from c-fos gene
Fz	frizzled
g	gram
GBX2	gastrulation brain homeobox 2 protein
GFP	green fluorescence protein
GSK3 β	glycogen synthase kinase 3 beta
H ₂ O	water
H3K4	histone 3 and lysine 4
HA	human influenza hemagglutinin
HCl	hydrochloric acid
HCT116	human colorectal carcinoma cells
HD	homeodomain
HDAC	histone deacetylase
HELA	human cervical cancer cell line
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer
HMG domain or box	high mobility group
hPa	hectopascal
ICAD	inhibitor of the caspase-activated nuclease
ICAT	inhibitor of β -catenin and Tcf
I-KB	inhibitory KB
JNK, JNK2	c-jun N-terminal kinases
K	lysine
K ₂ HPO ₄	potassium hydrogen phosphate
KCl	potassium chloride
kDa	kilodalton
KH ₂ PO ₄	potassium dihydrogen phosphate
l	liter
LB	Luria-Bertani medium
Lef	lymphoid enhancer-binding factor 1
Lgs	legless (<i>Drosophila</i>)
LiCl	lithium chloride
LIM domain	protein structural domains, discovered in Lin11, Isl-1 and Mec-3
LRP5/6	low density lipoprotein receptor-related protein
luc	firefly luciferase
Lys	lysine

Me	methylated
MgCl ₂	magnesium chloride
MgSO ₄	magnesium sulfate
miRNA	microRNA
ml	milliliter
MLL1/2	mixed-lineage leukemia proteins
mRNA	messengerRNA
N or N- terminus	amino end group of an amino acid
Na ₂ HPO ₄	sodium hydrogen phosphate
Na ₃ Citrate	sodium citrate
NaAc	sodium acid
NaCl	sodium chloride
NaHCO ₃	sodium hydrogen carbonate
NaOH	sodium hydroxide
NEMO	NF-KB essential modulator
NES	nuclear export signal
NF-AT	nuclear factor of activated T-cells
NF-κB	nuclear factor K-B
ng	nanogram
NHD	N-terminal homeodomain of pygopus
Nkd	naked cuticle
NLK	nemo-like kinase
NLS	nuclear localization signal
nm	nanometer
nmol	nanomolar
Nr-CAM	neuronal cell adhesion molecule
O ₂	oxygen
Oct-1	octamer-motif-binding protein
ODC	ornithine decarboxylase
Optix	homologous gene of vertebratal six3 for eye development (<i>Drosophila</i>)
Optx2	homobox gene of the six family for vertebratal retina development
Ori	origin of replication
Otx2	orthodenticle homeobox 2, target gene of the wnt signaling pathway for vertebratal anterior brain patterning
P	posterior
p	plasmid
p300	E1A binding protein
p50 or NF-κB1	subunit of nuclear factor KB
p65TA, p65, 65	transactivation domain of p65
PAF complex	RNA polymerase II associated factor complex
Pax	paired box gene family
PBS	phosphate buffered saline
PC12	rat pheochromocytoma 12 cells
PCP	the planar cell polarity Wnt signaling pathway
PCR	polymerase chain reaction
PDE	cyclic nucleotide phosphodiesterases
PDX-1	pancreatic and duodenal homeobox 1
PEG	polyethylene glycol
PEI	polyetherimide

pH	pondus Hydrogenii
PHD	plant homology domain of pygopus
<i>Photinus</i>	<i>Photinus photinus</i>
PIC	transcriptional preinitiation complex
PKC	protein kinase C
pm	picomolar
PKG	protein kinase G
Pla	Plakoglobin
PLC	phospholipase C
PML	promyelocytic leukemia
PNA	peptide nucleic acid
POP-1	posterior pharynx defect, homolog of Tcf/Lef
PP2A, PP1	protein phosphatases
PPi	diphosphate
puro	puromycin
Pygo	pygopus
Rac	rac-protein kinase
Ran BP3	ran-binding protein 3
RhoA	ras homolog gene family, member A
RK3	rat kidney cells
RLU	relative light unit
Rluc	<i>Renilla</i> luciferase
RNA	ribonucleic acid
RNAse	ribonuclease
ROCK	Rho-associated coiled-coil-forming protein kinase
rpm	revolutions per minute
Rx3	retinal homeobox gene 3
SB buffer	sodium borate buffer
SD	six domain
SDS	sodium dodecylsulfate
sec	second
Ser	serine
Six	sine oculis-related homeobox homolog gene (vertebrates)
So	sine oculis gene (<i>Drosophila</i>)
SOB	super optimal broth (medium)
SOC	derivate of SOB medium
SWI/SNF	switch/sucrose nonfermentable
TA	transactivation domain
TAK1	TGF-beta activated kinase 1
Taq	<i>Thermus aquaticus</i> (Taq DNA polymerase)
TB	tris borate buffer
TBP	TATA binding protein
Tcf	T-cell factor
tet ^R	tetracycline resistance
TFIIB	transcription factor IIB
Thr	threonine
TIP60/TIP49a	Tat interactive proteins
Tlc	protein for telencephalon development
TLE	transducin-like enhancer of split protein (corepressor in humans)

Toy	twin of eyeless gene (<i>Drosophila</i>), homologous to Pax6
Tris	THAM, tris(hydroxymethyl)aminomethane
Trp	trypsin
TRRAP	transcription domain associated protein
uPNAs	peptide nucleic acids with a specific backbone of ugi chem
VP16	transactivation domain of VP16
w/v	weight-volume percentage
Wg	wingless
Wnt	wnt genes, named from wingless and int-1
Wnt/Ca²⁺	wnt signaling calcium pathway
WRE	wnt response elements
X	any amino acid
X or <i>Xenopus</i>	<i>Xenopus laevis</i>
ZF or ZFHD-1	zinc finger homeoprotein, artificially combined of zinc finger structures, a polypeptide linker and the homeodomain of Oct-1
β-cat	β-catenin
β-Trcp	β-transducin repeat-containing protein
μg	microgram
μl	microliter

4. Introduction

4.1 The Wnt signaling pathway

The Wnt signaling pathway is based on the expression of small secreted glycolipoproteins and their binding to special receptors, leading to activation of particular target genes through a regulated signal transduction process. This event controls elementary processes in cells, including proliferation, polarity, survival, apoptosis, and motility and during embryonic development cell fate determination, tissue homeostasis and axis specification in all metazoan organisms (Logan and Nusse, 2004). In mammals the Wnt signaling pathway is involved in development and formation of the central nervous system, body axis, lots of organs, axial specification in limb development, ossification processes and in the regulation of different adult stem cells of brain, skin, blood and intestine (Cadigan and Nusse, 1997; Miller and Moon, 1996; Radtke and Clevers, 2005; Reya and Clevers, 2005). The specified and complex effects are performed by the known chemically different 19 Wnt ligands, which are distinctly segregated in time and place, their binding to the available receptors and the activation of target genes (Chen et al., 2008). These mammalian Wnt ligands can be subdivided into 12 conserved Wnt subfamilies (Prud'homme et al., 2002; Nusse, 2001). Due to its prominent function, some mutations, concerning its components, can often be connected to human birth defects, aging, cancer and other considerable diseases, including adiposis, osteoporosis, diabetes and cardiopathy (MacDonald et al., 2009; Klaus and Birchmeier, 2008).

The name of the Wnt genes is set up by the murine proto-onco gene *Int-1* and its homolog fly form *Wingless (Wg)* (Cabrera et al., 1987, Nusse et al., 1991). The first one was detected by Nusse and Varmus in 1982 and its corresponding gene *Wg* by Nüsslein-Volhard and Wieschaus in 1980. The mammalian form was identified by induced breast tumors when it is mutated. Its expression product was identified as a secreted protein with 23 conserved cysteine residues (Logan and Nusse, 2004). The *Wg* gene plays a prominent role in the development of the wings, which bases on the establishment of epidermal segment polarity in larval development (Nüsslein-Vollhard and Wieschaus, 1980; Clevers, 2006). Generally, all Wnt proteins are comprised by 350- 400 amino acids in length. In *Drosophila* the mutations of the oncogenes or tu-

more suppressor genes *porcupine*, *dishevelled*, *armadillo* and *pangolin* resulted in similar phenotypes to *wingless* (overdevelopment of cuticles). Therefore, they could be classified as important parts of the Wg signaling pathway (Kadowaki et al., 1996, Noordermeer et al, 1994; Peifer et al, 1994). In embryos of *Xenopus*, the injection of mRNA of the homolog vertebrate forms *dishevelled*, β -*catenin* or *Lef1* into the ventral side induced embryos with a duplicated body axis, including two heads, indicating their positive roles in the Wnt signaling pathway (Klaus and Birchmeier, 2008). All these findings, together with the identification of their interactions, led to the realization of one cohesive signal transduction pathway, the canonical Wnt signaling pathway. It is connected to highly conserved upregulating factors like Wingless/Wnt, Dishevelled/Dsh, Armadillo/ β -catenin and Pangolin/Tcf, Lef and downregulating factors like Axin, Adenomatous Polyposis Coli (APC) and Glycogen Synthase Kinase 3 β (GSK3 β) too. The importance of the Wnt signaling pathway in organismal patterning can be very well understood, when the incidence of different numbers of Wnt subfamilies in variable organisms is analyzed (Clevers, 2006). In the evolutionally lower animal groups of *Drosophila* and *Caenorhabditis elegans* only 5, respectively 7 Wnts could be identified, representing a low number in comparison to the 19 proteins in mice and humans (Cadigan, 2008; Clevers, 2006).

The key proteins for the inhibition of the Wnt signaling pathway in cytoplasm represent Axin and APC. In absence of Wnt binding to its coreceptors LRP5/6, both proteins form a degradation complex together with the kinases GSK3 β (Yost et al., 1998) and CK1 α (Yanagawa et al., 2002) and the phosphatase PP2A (Hsu et al., 1999). Through the establishment of this complex the binding affinity for β -catenin is strongly elevated, hindering its free accumulation in the cytoplasm. The more efficient β -catenin binding results through phosphorylation processes on APC as well on Axin, leading to stronger interactions between all three proteins (Huang and He, 2008). For an efficient depletion of β -catenin, it is phosphorylated at two conserved sites by CK1 α and GSK3 β , enabling the interaction with the E3 ubiquitin ligase subunit β -Trcp. Finally this process leads to ubiquitination and degradation of β -catenin (Aberle et al, 1997; Latres et al., 1999). In general, the degradation mechanism also accounts for a quick turnover of β -catenin in case of Wnt activation (Henderson, 2000). Some phosphorylation sites of β -catenin, APC and Axin could be mutated, leading to some types of cancer, especially in colorectal areas (Korinek et al., 1997; Kinzler et

al., 1991; Oates et al., 2006). In course of the whole β -catenin degradation process, the scaffold protein Axin fulfills the main controlling function over all performed phosphorylations., because it possesses different domains for interactions with GSK3 β , - α , CK1 α - δ , - ϵ , APC and β -catenin (Spink et al., 2000; Price, 2006).

The other protein which is mainly involved in the establishment of the degradation complex is the protein Adenomatous Polyposis Coli (APC), leading to Wnt downregulation (Polakis, 2000). Whether and how strong APC binds to Axin is regulated by phosphorylation through CK1 α and GSK3 β , similarly to Axin itself. Therefore, in the Wnt signaling pathway it assumes an upregulating as well as a downregulating role, depending on the context. But, its contribution for reaching Wnt induction is constricted to its ability to regulate the levels of Axin and thus the amount of β -catenin which gets degraded (Ha et al., 2004). The ability of APC for Wnt upregulation seems to be located in its amino terminal region, leading to the elimination of Axin (Takacs et al., 2008). Thus, in sum an upregulating effect of APC can get reached in ectopic and physiological Wg/Wnt signaling in *Drosophila*, where this phenomenon could be observed with both existing APC homologs (Lee et al., 2003). A further possible Wnt activating role of APC could also be identified by doing overexpression of APC in *Xenopus* (Vleminckx et al., 1997). The base for this effect was again the controlled degradation of Axin, which fulfills a clear negative role in the regulation of the Wnt signaling. However, the most important and negative function fulfills APC as a component of the Axin complex. In this context, when it is phosphorylated, probably it has a clear role in removing already phosphorylated β -catenin from Axin to make way for further unphosphorylated β -catenins (Kimelman and Xu, 2006). But, the most effective contribution of APC to β -catenin phosphorylation and degradation represents its property to hinder the phosphatase PPA2 to dephosphorylate β -catenin (Su et al, 2008). In other studies could be shown that in the nucleus APC can also work as a suppressor for Wnt target genes (Willert and Jones, 2006). This function seems to be chromatin dependent (Hamada and Bienz, 2004). Besides, it is probably involved in the nucleus export of β -catenin (Bienz, 2002).

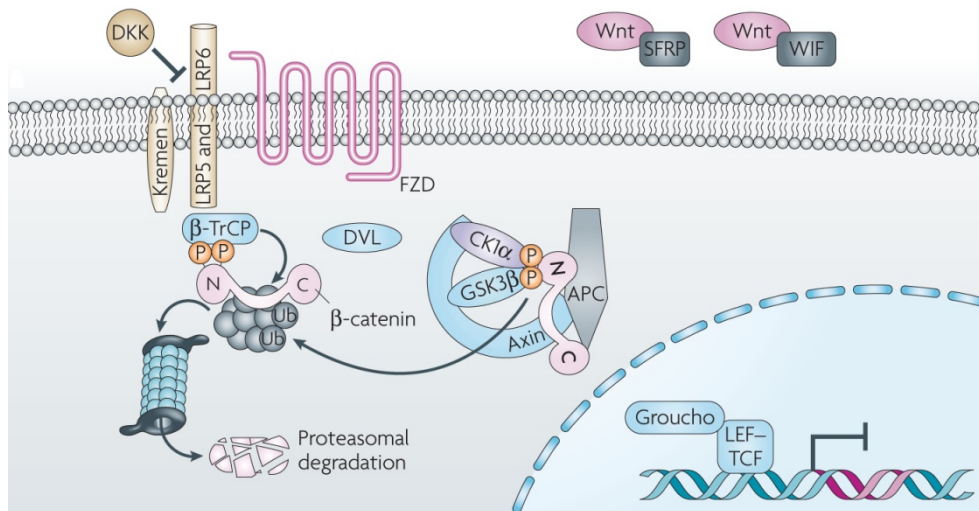


Figure 4.1.1. Illustration of the Wnt signaling pathway inhibiting mechanisms (see text for details; from Klaus and Birchmeier, 2008).

The activation of Wnt signaling is carried out by binding of Wnt ligands to the Fz seven-pass transmembrane receptors over their N-terminus (Dann et al, 2001) and to the coreceptors LRP5/6 (Pinson et al, 2000; Tamai et al, 2000) to form a Wnt- Fz- LRP5/6 complex (Wu and Nusse, 2002). In *Drosophila*, Dfz2 and Dfz1 work with Arrow as a LRP5/6 homolog for Wg reception (Wherli et al, 2000, Bhanot et al., 1996). The receptors Frizzled belong to the protein family of serpentine, in comparison to LRP5/6 which are part of the low-density-lipoproteins (He et al., 2004). In mammals 10 different Fz genes are known with different potentials for Wnt activation (Binners et al., 2007; MacDonald et al., 2009). An important point displays the ability of Wnt proteins to bind to the different Frizzled receptors and also in other direction (Dann et al., 1996). This fact shows the diversity of possibilities of Wnt induction.

After forming the Wnt-Fz-LRP5/6 complex, phosphorylation of the intercellular PPPSPxS sites of LRP6 is performed by GSK3β (probably bound to Axin) and CK1α (Davidson et al., 2005), so that binding of the Axin complex can take place (Tamai et al., 2004; Mao et al., 2001). In addition, the phosphorylation of LRP6 seems to be essential for the binding of GSK3β. As already discussed before, an interesting point is that the phosphorylation processes, carried out by CK1α and GSK3β, can lead to Wnt upregulation in combination with β-catenin degradation. Thus, these events must be very precisely controlled. However, a fact is that LRP6 phosphorylation is supported by interaction of Fz with Dsh/Dvl over the Fz C-terminal Lys-Thr-X-X-X-Trp motif, which is caused by a structural change of the receptor (Wong et al., 2003;

Umbhauer et al., 2000). Dvl itself represents a cytoplasmic Wnt component, which binds Axin-GSK3 β -CK1-Par1-CK2 upon Wnt activation, keeping them at the plasma membrane (Cadigan, 2002; Bienz and Clevers, 2003). It is proposed that Dvl can polymerize through its DIX domain at the plasma membrane to take up more and more Axin-GSK3 β complexes, forming large higher-order complexes, called signalosomes (Bilic et al., 2007).

For the Wnt signaling pathway activation, after binding of Wnt ligands to their receptors and the capture of the Axin-GSK3 β complex at the plasma membrane, the phosphorylation of the signaling protein β -catenin must be prohibited and its disassembly from the Axin complex must be achieved. These steps can be performed either by the activated LRP6 or by Dsh/Dvl or in combination of both. Looking at the underlying mechanism concerning the involvement of Dvl, it can directly effect Axin-GSK3 β - β -catenin disassembly (Logan and Nusse, 2004). In this context the phosphatase PP1 dephosphorylates Axin, followed by the process of dephosphorylation of β -catenin by the other phosphatase PP2A (Price et al., 2006; Su et al, 2008). The other ways would be that phosphorylated LRP6 directly inhibits the phosphorylation of β -catenin (Wu et al, 2009) or the Axin complex activity is hindered by phosphorylated Wnt receptors or high amounts of Dvl (Chen et al., 2008; Mao et al., 2001).

The high accumulation of stabilized β -catenin in the cytoplasm provides a basis for the import into the nucleus. Important is to mention that the pool of cytoplasmic β -catenin must be functionally differentiated by an amount of β -catenin, which fulfills its task as a component of adherens junctions. This could be shown in studies concerning *C. elegans* (Korswagen et al., 2000) and Zebrafish (Mo et al., 2009), in which two different forms of β -catenin with divergent functions could be identified. In this context, in case of Wnt activation increased levels of β -catenin import could be achieved by the phosphorylation of the β -catenin sites Ser191 and Ser605 by the Jun N-terminal kinase 2 (JNK2) (Wu et al., 2008). Thus, in mice the existing form of β -catenin must unify the functions of the both β -catenins of Zebrafish and *C. elegans*. However, the factors Axin and APC in cytoplasm (Cong and Varmus, 2004; Henderson and Fagotto, 2002; Willert and Jones, 2006; Neufeld et al., 2000) and the nucleic β -catenin cofactors BCL9 and Pygopus (Townesley et al., 2004) keep the corresponding β -catenin levels at the places they work. For the nucleus export β -catenin pos-

sesses a nuclear export sequence (NES). Thus, it is performed by binding of APC (Rosin-Arbesfeld et al., 2000), Axin (Bienz, 2002) and Ran binding protein 3 (RanBP3) (Hendriksen et al., 2005). Maybe more proteins like α -catenin (Giannini et al., 2000), Dsh (Habas and Dawid, 2005), ICAT (Tago et al., 2000), CK1, CK2 or GSK3 β (Willert and Jones, 2006) are also somehow involved in this process.

After translocation of β -catenin into the nucleus, it interacts with Tcf/Lef which are bound to their binding sites to activate special Wnt target genes. In the Wnt switch off situation, lots of inhibitors like CtBP, APC, HDAC and Groucho/TLE are associated with Tcf/Lef to hinder transcription activation (Clevers et al., 2006; Willert and Jones, 2006; Cavallo et al., 1998). Thus, all these proteins lead to a situation that more amounts of β -catenin are needed for Wnt upregulation. Subsequently, the nucleic β -catenin physically displaces Groucho and the other repression factors from Tcf/Lef, resulting in a conformation change and a stronger binding of Tcf/Lef to their recognition sites, following by efficient transcriptional activation (Daniels and Weis, 2005). A further transcription upregulation is reached by β -catenin itself through its C-terminal transactivation domain, on which several coactivators like BCL9, Pygopus (Thompson, 2004; Hoffmans et al., 2005), Brg (Barker et al, 2001), and CBP (Cadigan, 2002; Bienz and Clevers, 2003) bind to influence the promoter region of the target genes (for further detailed information about Tcf/Lef, β -catenin and their interaction see Chapters 4.2 – 4.4).

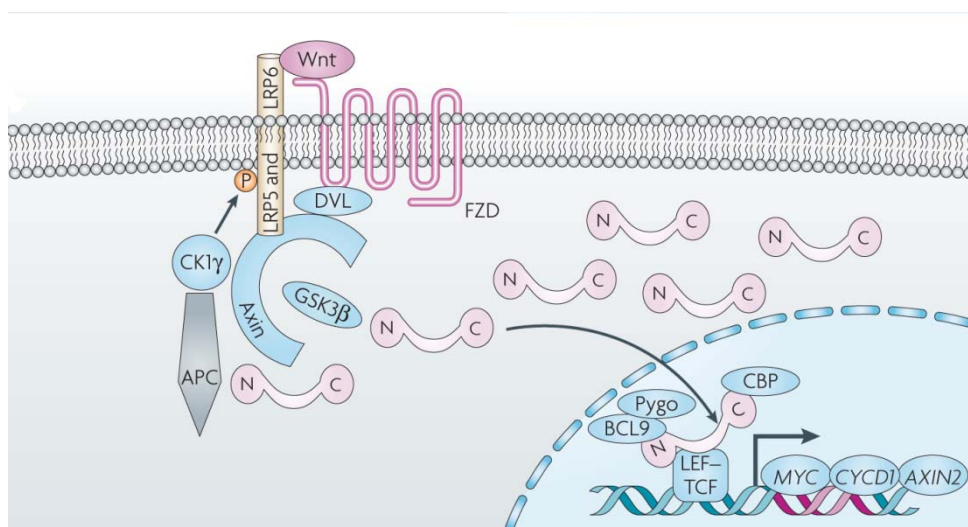


Figure 4.1.2. Schematic demonstration of the activating steps in the Wnt signaling pathway (see text for details; from Klaus and Birchmeier, 2008).

Nearby the most studied canonical Wnt pathways, the planar cell polarity (PCP) and the Wnt-calcium (Wnt/Ca²⁺) pathways are quite less described. Both signaling pathways are activated through different Wnt ligands (e.g. Wnt5, Wnt11) (Heisenberg et al., 2000; Kilian et al., 2003) and show interferences, especially concerning the regulation of intracellular calcium release. Furthermore, in both cases, the Wnt proteins signal through the components Frizzled (Strutt, 2003) and Dishevelled (Heisenberg et al., 2000). The first time the PCP was identified in *Drosophila* by evoking perturbation of hairs and bristles on the wings and thorax in Fz mutants (Vinson et al., 1989; Strutt, 2003). In the process of signaling, in addition to Wnt, Fz and Dvl, other components, such as the atypical cadherin Flamingo/Starry night, the Fz coreceptor Strabismus, the Formin-homology protein Daam1, the cytoplasmic protein with LIM domain, Prickle, the ankyrin-repeat protein Diego, the activated small G proteins RhoA and Rac and their respective effectors ROCK (Rock kinase) and JNK (Jun kinase) are involved (Wallingford, 2004). In course of this signaling pathway, Prickle and Strabismus associate with Dishevelled to regulate the cell asymmetry through their distribution and in some cases through calcium release (overlapping function with the Ca pathway) (Adler, 2002; Veeman et al, 2003). In this context a feed back loop is initiated through Daam1 (Habas et al., 2001). The functions of the other downstream components depend on the different operation areas (e.g. establishment of cytoskeleton, mitotic spindle orientation), in which they get activated. In *Drosophila* it is known that this pathway controls the epithelial planar polarity within the thorax, wing and eye and especially the neurogenesis (Mlodzik, 1999; Adler and Lee, 2001). Controversly, in vertebrates it is responsible for the patterning of the fur and the sensory hairs in the inner ear, epidermis formation and for the regulation of cell movement and convergent extension, during gastrulation and neurulation (Guo et al, 2004; Dabdoub and Kelley, 2005; Solnica-Krezel, 2005).

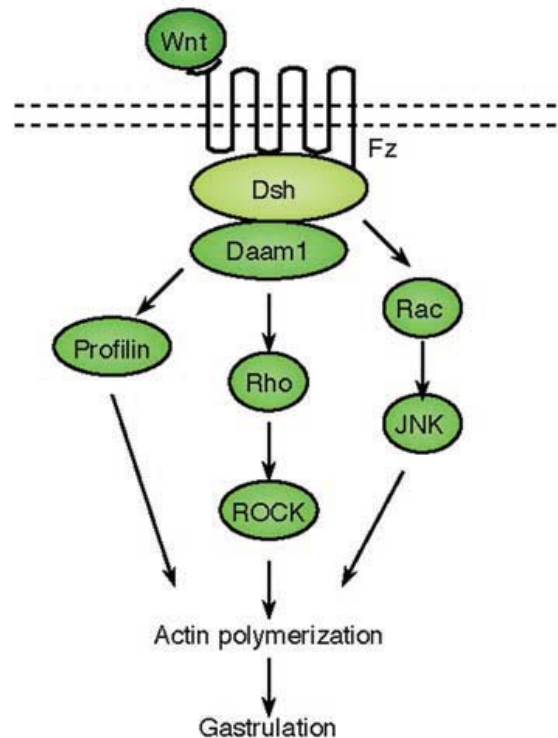


Figure 4.1.3. Representation of the planar cell polarity Wnt pathway, including the most important components (see text for details; from Komiya and Habas, 2008).

In contrast, the Wnt/Ca²⁺ pathway is more involved in regulation of cytoskeletal dynamics and cell adhesion processes through the control of intracellular calcium levels. The basic mechanism represents the influence of heterotrimeric G-proteins on the activation of phospholipase C (PLC) (Slusarski and Pelegri, 2007), releasing intercellular calcium, working as a second messenger (Slusarski et al., 1997; Westfall et al., 2003). This results then in the activation of protein kinase C (PKC), calcineurin and calcium/calmodulin-dependent kinase II (CamKII) (Kühl et al., 2000; Pandur et al., 2002). All three factors respond on calcium change. Each of them can be found in all metazoans, but only in vertebrates they act together in a related pathway. Often the calcium-sensitive transcription factor NF-AT is a target of the Wnt/Ca²⁺ pathway, which promotes ventral cell fate in *Xenopus* embryos (Saneyoshi et al., 2002). In development the Ca pathway can unfold its function as an antagonist of the canonical pathway by inhibiting dorsal axis specification (Slusarski and Pelegri, 2007). Furthermore, it is involved in slow muscle fiber formation in adult muscles (Anakwe et al., 2003). However, in *Drosophila* the existence of a Wingless-PKC pathway could be demonstrated, which probably displays a modified form of this pathway (Cook et al., 1996). Both noncanonical pathways must be part of extended studies to be able to

find out more about their functions, especially looking at their collective tasks and their connection to the canonical Wnt signaling pathway. In my experiments these pathways were not important.

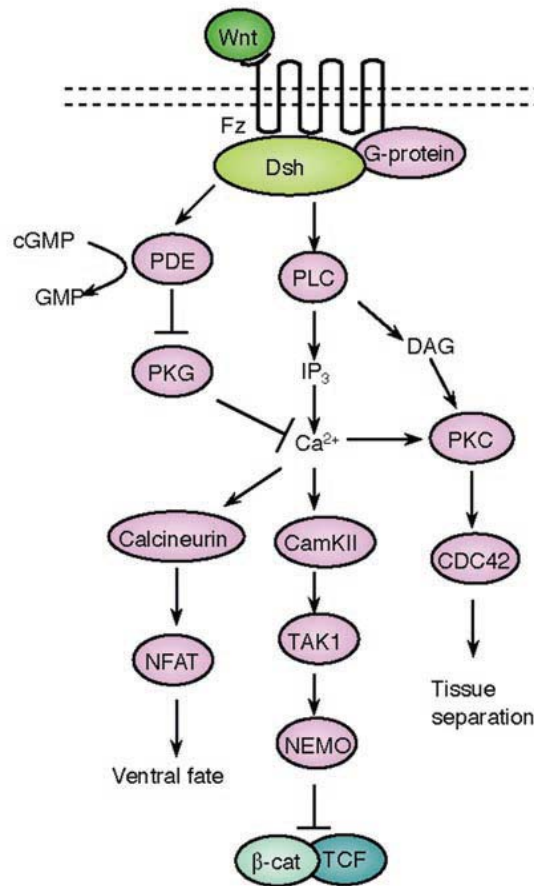


Figure 4.1.4. A schematical picture of the Wnt/Ca pathway, from its upregulation at the membrane and the intracellular calcium release to the activation of the kinases (see text for details; from Komiya and Habas, 2008).

4.2 The signaling proteins β -catenin and Plakoglobin

First, the orthologue form of β -catenin, Armadillo could be identified in *Drosophila*, playing an essential role for the establishment of the segment polarity (Sanson et al., 1996). In this context it fulfills its function downstream of Wingless, as the most important Wg/Wnt signaling component for upregulation. Subsequently, in diverse studies the multitudinous functions of β -catenin/Armadillo during development of invertebrates and vertebrates were discovered (Miller and Moon, 1996; Korswagen et al., 2000; Haegel et al., 1995; Kelly et al., 2000). In addition, in the field of stem cell re-

search, which is getting more and more important, β -catenin can manage cell self-renewal in several adult tissues in combination with other signaling pathways (e.g. Reya et al., 2003).

The large protein β -catenin fulfills important tasks in both adhesion and signaling functions. In the first function, β -catenin regulates the actin interactions and cell-cell contacts through its binding to cadherins, which represent transmembrane adhesion proteins. The interaction maps to the cytoplasmic tail of the cadherins (Ozawa et al., 1989). The coordination of changes in cell adhesion, followed by some relocation processes in the cytoskeleton and the upregulation of gene expression is a complicated mechanism with many factors and interactions involved and is not fully understood until now. However, only β -catenin unifies the pathways as being a component of both, of cell-cell adherens junctions and of a signaling pathway (Nelson and Nusse, 2004). Therefore, this protein is produced in all cells for the regulation of cell differentiation, cell proliferation, cell shape and cell death.

In approximately 3500 types of cancer β -catenin is overexpressed, therefrom in 90% of colorectal cancer (Klaus and Birchmeier, 2008). The overexpression of β -catenin is primarily caused by mutations in APC, which decrease the number of APC- β -catenin interactions and a lower part of β -catenin degradation (Kinzler et al., 1991; Rubinfeld et al., 1996). In APC the binding and regulation sites of β -catenin and the three Axin interaction sites can be mutated by truncations (Polakis, 1999). This fact could be demonstrated by studies in mice with overexpressed truncated APC forms (lacking one Axin binding site: Kartheuser et al., 1999; lacking two Axin binding sites: Walon et al., 1997), leading to colorectal polyposis. Concerning mutations in β -catenin regulation sites, an increased severity of polyposis could be shown in humans (Polakis, 2000). In addition, in knock-in mice, containing a stop codon in the *apc* gene, an augmented growth of adenomas could be mediated (Su et al., 1992). In β -catenin some point mutations could be identified on its N-terminal part, especially concerning specific serines or threonines, impairing phosphorylation, ubiquitination and degradation (Sparks et al., 1998; Miyaki et al., 1999). In many cases, the resulting strong Wnt activation leads to an increased proliferation of crypt progenitor cells due to the β -catenin/Tcf4 complex (van de Wetering et al., 2002). In this context, the Wnt target genes *cycline D1* and *c-myc* can be involved in this process. Usually, all sequence

abberations start in one allele and can be followed by modifications in the second allele, which finally result in development of malignant tumors. In addition, frameshift mutations in Axin1 and Axin2 can also lead to colorectal cancer (Liu et al., 2000).

The functions of β -catenin can be spatialized to its several regions. The N-terminal section, which is set up by 130 amino acids, can be phosphorylated by GSK3 β , representing the signal for degradation in the case of Wnt downregulation (Miller and Moon, 1996; Aberle et al., 1997). But, in the course of Wnt activation by binding of the Wnt ligand to its receptors, leads via some additional steps to dephosphorylation of this area and high amounts of the activated protein β -catenin in the cytoplasm and nucleus (Henderson and Fagotto, 2002; Stadel et al., 2006) (further information in Chapter 4.1).

The middle region of β -catenin, containing the Armadillo (Arm) repeats, definitely represents the most essential part for Wnt upregulation, because it contacts the transcription factors Tcf/Lef in the nucleus, physically connecting the transactivation capacity of β -catenin with the promoter regions of the Wnt target genes. This largest part of the protein encloses 550 amino acids, which are subdivided into 12 incomplete sequence repeats of each 42 amino acids. The Armadillo repeats are arranged as a superhelix of helices (each repeat region consists of 3 helices), forming a long positively charged groove to interact as a scaffold protein with the components of the destruction complex, Tcf/Lef, E-cadherin and several other corepressors and coactivators (Huber et al., 1997; Miller et al., 1999; Arce et al., 2006). One important coactivator for transcriptional initiation represents Legless/BCL9, which binds N-terminally to the first Armadillo repeats (Townesley et al., 2004). In *Drosophila*, in misexpression experiments, leading to Wingless similar phenotypes (Thompson et al., 2002), it could be shown that Legless and its interaction partner Pygopus (Pygo) are responsible for a pure β -catenin-dependent transcription (Mosimann et al., 2009). In addition, both proteins fulfill functions in transcription regulation, because Pygo is able, in connection with BCL9, to bind to dimethylated H3K4 components of chromatin, with or without Tcf (de la Roche and Bienz, 2007; Fiedler et al., 2008). In this context, the most essential point is that Pygo and Legless recruit β -catenin near the Wnt target genes (Townesley et al., 2004) and seem to control its interaction with Tcf/Lef. In mammals Pygo as well as BCL9 do not show such a prominent part in Wnt regulation

processes and their importance is restricted to defined cell tissues, for instance the kidney (Schwab et al., 2007).

The C-terminus, including 100 amino acids, harbors several binding sites for interactions with multiple coactivators for the transactivation of Wnt target genes (Hoffmans et al., 2005; van der Wetering et al., 1997). A collective negative charge is established at this region, due to several negatively charged amino acids (Huber et al., 1997). This is the reason, why the cofactors can interact more easily with the C-terminus. In a recent study it could be shown that the flexible negatively charged C-terminal area can contact the positively charged region of Armadillo repeats, hindering an interaction with Axin to reach stability in the cytoplasm (Mo et al., 2009). Looking at the interaction partners, the coactivators p300/CBP (cAMP response element-binding protein), (Sun et al., 2000), TRRAP/TIP60 (transcription domain associated protein/Tat interactive protein) (Feng et al., 2003), MLL1/2 (mixed-lineage leukemia proteins) (Sierra et al., 2006), SWI/SNF (switch/sucrose nonfermentable) (Barker et al., 2001), TIP49a/ Pontin52 (TATA interacting protein) (Hecht et al., 1999), and the PAF complex (RNA polymerase II associated factor) (Mosiman et al., 2006) bind to the C-terminal part of β -catenin for transcriptional upregulation. The proteins p300/CBP and TRRAP/TIP60 represent histone acetyltransferases, MLL1/2 methyltransferases and SWI/SNF ATPases for chromatin remodeling, TIP49a ATP-dependent helicases for regulation of DNA structures and the PAF complex plays important roles for the maintenance of the transcription and histone modifications (Mosiman et al., 2009; Willert and Jones, 2006). The application of all these factors mostly leads to histone acetylation and trimethylation (at H3K4) (Parker et al., 2008, Sierra et al., 2006) and as a consequence to highly active genes and accessible chromatin structures in vivo. But these processes need time for their realization and cannot happen within few minutes, which effect the activation of Wnt target genes. In new models, based on several studies, it is reported that the assembly and disassembly of coactivators can occur in short cyclic periods (Sierra et al., 2006; Wöhrle et al., 2007). In this context, APC seems to play a bifunctional role, which is known from cytoplasmic repressing functions. Through its recruitment to chromatin, it can mediate the exchange between corepressors and coactivators (Sierra et al., 2006). In addition, during transcription, ubiquitination and methylation of the coregulators it can participate in their distribution on the chromatin. These biochemical processes are performed by the protein chape-

rone 19S subcomplex with its ATPases in rapidly inducible genes (Ezhkova and Tansey, 2004).

A repressive effect on target gene activation can be achieved by disassembly of the coactivators or by interactions with corepressors, which can happen in combination (Hamada and Bienz, 2004; Fang et al., 2006; Sierra et al., 2006). Primarily, this antagonizing factors are represented by GSK3 β , β -Trcp, Groucho/TLE or C-terminal-binding protein (CtBP). Until now the first two proteins have only been renowned as compounds with repressive characterizes in the cytoplasm. Groucho/TLE itself interacts with hypo-acetylated histone H3 for maintaining the chromatin in a tranquilized state (Willert and Jones, 2006). CtBP works with Polycomb corepressors, depending on HDAC and other inhibiting factors to mediate chromatin repression (Chinnadurei, 2002). In addition, an interaction of CtBP with APC could be demonstrated, resulting in a more regulated repression of Wnt target genes (Willert and Jones, 2006).

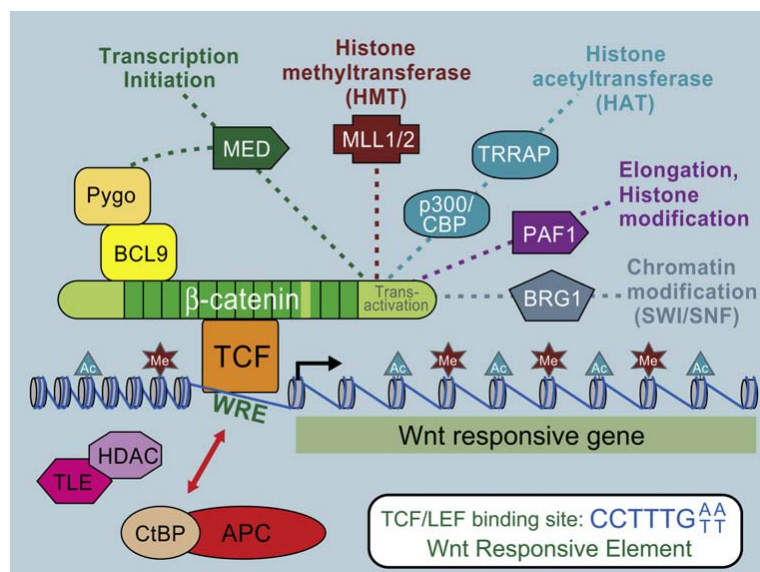


Figure 4.2.1. Schematic illustration of activation or repression of Wnt target genes by β -catenin, Tcf/Lef and numerous coactivators and corepressors, in combination with chromatin modifications (see text for more details; from MacDonald et al., 2009).

Through interaction events with other signaling pathways the β -catenin/Tcf complex itself can be involved in Wnt downregulation. In mammals the repressing effect of β -catenin/Lef1 could be identified by inhibiting the DNA binding of another transcription factor (Kahler and Westendorf, 2003). In *Drosophila*, a similar competitive repression, performed by β -catenin/Pangolin was observed by replacing certain coactivators

(Piepenburg et al., 2000). In combination with upregulating processes, the interaction of Groucho/TLE and histone deacetylases with DNA can be favored by β -catenin (Olson et al., 2006). Finally, it is also documented that some untypical Wnt corepressors can bind next to Wnt responsive elements (WREs) and work together with β -catenin-Tcf/Lef, resulting in downregulation of Wnt signaling on target gene level (Jamora et al., 2003; Theisen et al. 2007).

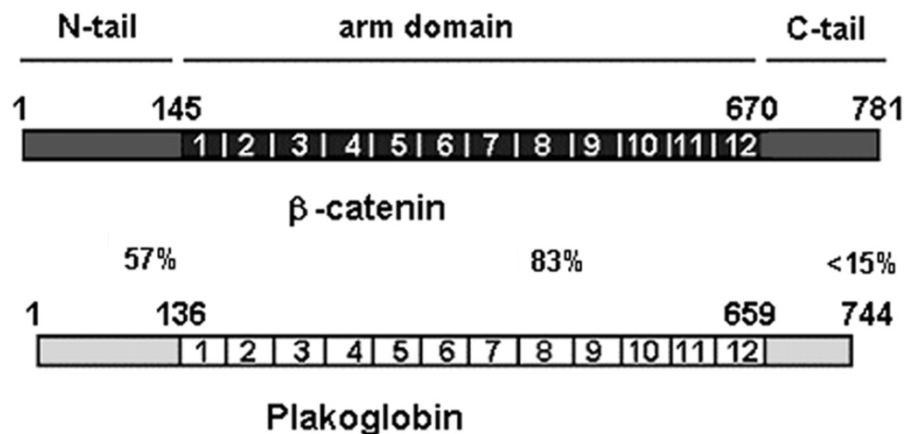


Figure 4.2.2. Structural comparison between β - and γ -catenin, including the sequence homology of the N- terminus, Armadillo repeat region and the C-tail (more information in the text; from Solanas et al., 2004).

The protein Plakoglobin represents a substitute of the group of catenins in vertebrates, including α -, β - and γ -catenin, which fulfill functions in cell adhesion processes (Cowin and Burke, 1996). Its membership was identified by Ozawa et al. in 1989, who classified it as γ -catenin. Although Plakoglobin possesses a 68% sequence homology in comparison to β -catenin (Butz et al, 1992), its tasks are quite different (Zhurinsky et al., 2000b). In detail, comparable with β -catenin, it mediates a contact between the actin cytoskeleton and E-cadherin adhesion receptors at the plasma membrane (Knudsen et al., 1995). On one hand this occurs by an interaction with the cytoplasmic part of E-cadherin and on the other hand by binding to α -catenin (Nagafuchi et al., 1994). But, in difference to β -catenin, Plakoglobin is responsible for the connection of the desmosomal cadherins desmocollin and desmoglein with intermediate filaments (Trojanovsky et al., 1994a, b; Kowalczyk et al., 1997). In the first case it binds to desmoplakin and in the latter to plakophilin. The stronger structural involvement of Plakoglobin in functions of cell adhesion, thus underlines probably the origin of both prominent catenins, resulting in major tasks of β -catenin in cell signaling during evolution.

Considering the structural formation of Plakoglobin, it resembles that of β -catenin, including the middle 12 Armadillo repeats and its C- and N-terminal regions (Hatzfeld, 1999). The N-terminus (57% sequence homology) also harbors target sites for degradation, although its analogous phosphorylation sites do not seem as essential as in β -catenin, following a lower rate of reduction (Williams et al., 2000). Nevertheless, in the context of Wnt downregulation interactions with APC, Axin, GSK3 β and β -Trcp could be demonstrated (Kodama et al., 1999; Sadot et al., 2000; Polakis, 1999). Unlike β -catenin, the N-terminal region does not possess any transactivation capacity. The C-terminal region (15% sequence homology) includes also a transactivation function (Hecht et al., 1999; Simcha et al., 1998), but in comparison to β -catenin it only enfolds a weak effect. In some studies the inhibiting capacity of the N- and C-termini of Plakoglobin when it binds to Tcf/Lef were detected (Huber et al., 1996; Hecht et al., 1999; Simcha et al., 1998; Zhurinsky et al., 2000a). This represents a big difference to β -catenin, whose termini do not affect these functions. The three-dimensional structure of the Armadillo repeats (83% sequence homology) resembles very strongly the conformation of the structure of β -catenin, resulting in similar binding partners and related functions (Zhurinsky et al., 2000a, Huber et al., 1997). Especially, the interactions with Lef1 (Behrens et al., 1996; Huber et al., 1996) and Tcf4 (Simcha et al., 1998; Zhurinsky et al., 2000a; Miravet et al., 2002), depending on cell types and context could be verified. The very similar sequence of the Armadillo repeats of β -catenin and Plakoglobin can point out a similar process for entering the nucleus (Cowin and Burke, 1996). But, the association of Plakoglobin with Tcf4 is carried out upon different sites of Tcf4 (amino acids 51-80 versus 1-51 in β -catenin) (Miravet et al., 2002). Additionally, only in few special cell types like rat PC12 pheochromocytoma (Bradley et al., 1993) and RK3 cells (Kolligs et al., 2000), murine AtT20 pituitary tumor (Papkoff et al., 1996; Hinck et al., 1994) and C57MG epithelial cells (Papkoff et al., 1996) and human HCT116 colon cancer cells (Shtutman et al., 2002; Pan et al., 2007), an accumulation of Plakoglobin in cytoplasm, caused by Wnt overexpression, could be observed.

In developmental processes Plakoglobin's participation in the differentiation of heart and skin could be shown in mice (Bierkamp et al., 1996; Williamson et al., 2006), Zebrafish (Martin et al., 2009) and humans (Gandarillas and Watt, 1997). In mice, both

proteins seem to play a fundamental role for survival, because in knock-out mice, concerning Plakoglobin or β -catenin, all embryos die (Haegel et al., 1995; Bierkamp et al., 1996). An explanation for this is the failed function in cell adhesion processes. In the development of *Xenopus*, the influence of Plakoglobin seems to be essential, because the formation of a second body axis (hallmark of increased Wnt activity) could be achieved by its overexpression (Karnovsky and Klymkovsky, 1995). But, Plakoglobin is not able to neutralize a deficit of β -catenin in mice, resulting in severe epithelial defects (Haegel et al., 1995). Furthermore, in *Drosophila* as well as in *Xenopus* it could be shown that an overexpression of Plakoglobin cannot balance the loss of β -catenin (White et al., 1998; Kofron et al., 1997). In lots of organs during tumorigenesis, in most cases a striking increase of β -catenin can be observed, often based on point mutations, concerning the β -catenin phosphorylation site and the components of the degradation complex, leading to high amounts of endogenous stabilized β -catenin and a deregulation of cell growth and proliferation (Polakis, 1999; Huang et al., 1999). In opposite, in several tumors Plakoglobin is downregulated, but an overexpression can elicit an inhibition of tumor growth (Simcha et al., 1996). Summarizing up these observations, β -catenin and Plakoglobin have essential functions during developmental processes and cancer formation, but these are dependent on context and position.

The transactivation potential of Plakoglobin is part of several discussions, based on different binding partners and the shorter C-terminus, although some target genes of the Wnt signaling pathway can be efficiently switched on by this protein. For instance, the prominent proto-oncogene *c-myc* (Kolligs et al., 2000; Williamson et al., 2006), the gene *PTTG* (Pan et al., 2007), expressing the protein securin, which is important for sister-chromatin separation and the *Nr-CAM* gene (Conacci-Sorell et al., 2002), an important adhesion molecule, could be identified as factors under the control of Plakoglobin. In addition, the binding capacity of Plakoglobin with TBP, and p300/CBP, which play a prominent role in transcriptional activation, could be demonstrated (Hecht et al., 1999; Zhurinsky et al., 2000b). But, further support for the enigmatic role of Plakoglobin is the fact, that it can also indirectly upregulate some Wnt target genes like *PML*, via a cooperation with β -catenin (probably, by hindering a strong degradation of β -catenin in the cytoplasm through its similar Axin-APC-GSK3 β complex binding potential) (Shtutman et al., 2002). In contrary, an overexpression of

Plakoglobin can effect a higher rate of β -catenin incorporation into desmosomes, following a decrease of transcriptional processes in mice (Simcha et al., 1998). Moreover, in the nucleus predominantly the interaction of Plakoglobin with Lef1 leads to a transcriptional downregulation and the association with Tcf4 results in an induction of the transcription of specific Wnt target genes (Kolligs et al, 2002; Williamson et al, 2006).

4.3 The transcription factors of the Tcf/Lef family

The HMG-box containing superfamily of transcription factors includes the proteins T cell factor/Lymphoid enhancer factor (Tcf/Lef). They represent specific DNA-bound transcription factors, which interact with activated nucleic β -catenin to regulate, together with other cofactors, the transcription of target genes (Arce et al., 2006; Brunner et al., 1997). In the switch off state of the Wnt signaling pathway the corepressors Groucho/TLE (Cavallo et al., 1998), HDAC (Billin et al., 2000) or CtBP (Brannon et al., 1999) inherit the most important roles. They mediate repression through binding to Tcf/Lef in connection with other antagonizing factors. The first two ones contribute to histone deacetylation and the clustering of chromatin too. Before transcriptional activation, the binding of β -catenin results in the direct replacement of Groucho (Daniels and Weis, 2005) and also in the disassembly of HDAC1 and CtBP (Willert and Jones, 2006). In course of this, further interactions with other cofactors play a role. In *Drosophila* and *Caenorhabditis elegans*, just one member of this gene family is known dTcf/pangolin and POP-1, respectively. In contrast, in vertebrates Tcf1, Tcf3, Tcf4 and Lef1 could be identified. The two members, which could be firstly found in the mammalian immune system, represent Tcf1 and Lef1. The protein Tcf1 is prominently expressed in T lymphocytes to enable the maintenance of early thymocyte progenitors in adult mice (Oosterwegel et al., 1993). An essential role plays Tcf4 in the development of the CNS, together with Lef1 during neurulation and proximated processes. Meanwhile, its function in the epithelium of the developing intestine, in connection with β -catenin and Plakoglobin, is well examined (Korinek et al., 1998; Shtutman et al, 2002). Generally, Lef1 seems to be the most important member of this protein family during developmental processes, because it appears in limb buds, thymus, tooth germs, whisker follicles, mammary buds, tail vertebrae and tel-, di- and mesencephalon and is hence involved in their establishment (Oosterwegel et al.,

1993; Zhou et al., 1995). Finally, Tcf3 fulfills essential tasks in the development of hair follicles, stomach epithelium and keratinocytes of the skin (Korinek et al., 1998; Merrill et al., 2001). It represents the gene, which is expressed earliest in embryogenesis. Tcf1 and Tcf4 can display their functions as activators or repressors, in comparison to Tcf3, which works in most cases as a repressor (in connection with CtBP, see also Chapter 3.5), but also in few situations as an activator and to Lef1, representing a typical Wnt activator (Hoppler and Kavanagh, 2007; Arce et al., 2006; MacDonald et al., 2009). Both, *Lef1* and *Tcf1* are themselves direct target genes of the Wnt signaling pathway in humans, being part of the feedback control mechanism (Hovanes et al., 2001).

In addition, the number of active Tcf/Lef proteins can be further increased by the production of some alternative splicing variants (Hoppler and Kavanagh, 2007; Arce et al., 2006). In this context, the only exclusion displays the transcription factor Tcf3. For instance, some forms of Tcf/Lef can be synthesized without the N-terminal β -catenin binding site, resulting in Wnt downregulation (Hovanes et al., 2001). This fact could be demonstrated with the dominant negative form of Tcf1 (dnTcf), which results in a suppression of tumor growth and in an inhibition of Tcf4 in stem cell renewal (Najdi et al., 2009; Hoppler and Kavanagh, 2007; MacDonald et al., 2009). An interesting detail are the isoforms Tcf1e and Tcf4e, which represents a very similar structure to the invertebrate form, displaying maybe a collective ancestral Tcf form (Atcha et al., 2003). This theory is further supported by the discovery of the existence of an additional DNA interaction region, called the C-clamp, interacting with a GC sequence, residing downstream of the intrinsic binding site (Atcha et al., 2007). In comparison to this, in *Drosophila*, the most variants contain a helper-site, which contributes to an additional DNA interaction with seven base pairs (Chang et al., 2008). This function can be functionally compared to the C-clamp in mammalian Tcfs.

The main task of Tcf/Lef is the physical mediation between the signal, arising from Wnt activation and nucleic β -catenin and the specific binding sites on the DNA of Wnt target genes. The highly conserved protein structure of Tcf/Lef contains the N-terminal β -catenin binding domain, a central region, a high mobility group region for DNA interaction with a nuclear localization signal (NLS) and a long C-terminus. Most importantly, the binding of these transcription factors is effected by their high- mobility

group domain (HMG domain), to Wnt responsive elements, representing the consensus sequence CCTTTGA/TA/T (Zhurinsky et al., 1996). This specified sequence is contacted by Tcf/Lef proteins via the DNA minor groove. Therefore, the DNA is bended (up to 130° away from the protein, towards the DNA major groove), leading to a change of the local chromatin structure (Giese et al., 1992; Love et al., 1995; Murphy et al., 1999; Bewley et al., 1998). Recent studies could show that Tcf/Lef can bind better to chromatin structures if the N-terminus is missing, indicating a positive reciprocal reinforcement between both, being important for transcriptional upregulation (Willert and Jones, 2006). The DNA recognition site itself compasses 88 amino-acids, which is set up by the 68-amino-acid HMG box and 9-amino-acid nuclear localization site (the remaining amino acids display some variations). The NLS supports and reinforces the DNA binding through the unspecific interaction of its positively charged side chains with the negatively charged phosphate backbone (Love et al., 1995). During the process of DNA binding the HMG box undergoes a conformational change from a low folded structure to a highly-ordered state with a well-folded figure (Love et al., 2004). In addition, in one study it could be shown that Tcf/Lef binds with higher affinity to linear duplex DNA than to single stranded one. Furthermore, their ability for DNA unwinding could be demonstrated (Giese et al., 1997). However, the transcriptional regulation through Tcf/Lef binding to the WREs can be performed over quite long distances downstream as well upstream from the transcription starting points. Primarily in cancer cells, especially in colon cancer cells this mechanism of transcription seems to be standard, also in connection with the involvement of binding sites within constricted areas (Hatzis et al., 2008). Summarizing up all properties of Tcf/Lefs, together with their different or concerted expression patterns in time and space, the regulation of the Wnt target genes can be controlled very precisely.

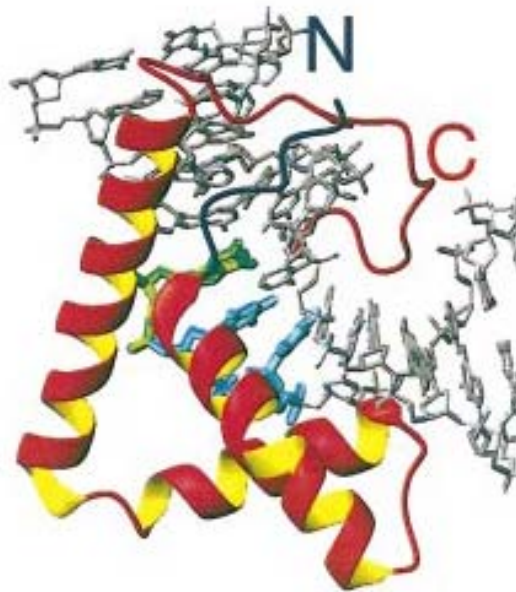


Figure 4.3.1. A schematic presentation of the three- dimensional interaction of the HMG domain of Lef1 with its target site on the DNA (see details in the text; from Allain et al., 1999).

Generally, some processes concerning special interactions, modifications and changing expression levels contribute to an altered behavior of Tcf/Lef in the context of regulation of the expression of Wnt target genes. For instance, in *Drosophila* the miRNA-315 could be identified, creating a Wnt activating effect by inhibiting the Wnt downregulating factors of Axin and Notum (Silver et al, 2007). In comparison to this, in a recent study a negative effect of the conserved miRNA-8 on the stability of Tcf/Lef could be demonstrated. According to this observation an influence on acetylation, sumoylation or phosphorylation was suggested (Kennell et al., 2008). Acetylating processes done by CBP usually lead to transcription antagonizing effects in flies and nematodes, by hindering or diminishing the complex binding to DNA (Phillips and Kimble, 2009; Hoppler and Kavanagh, 2007). In addition, it could be demonstrated that sumoylation, which resembles the mechanism of ubiquitination, can induce both, activation and repression, depending on the types of Tcf/Lef, which have different sumoylation sites. In connection with Tcf4 it supports the transcription (Yamamoto et al., 2003). In contrast, in Lef1 this biochemical modification leads to downregulation, because it is translocated in high amounts into nuclear bodies (Sachdev et al., 2001). The phosphorylation of Lef1 by CK2 ϵ and of Tcf3 by CK1 ϵ disturb the interaction with Groucho/TLE, leading to a transcriptional upregulation. Unlike to this process, a phosphorylation of Tcf4 and Lef1 by Nemo-like kinase (NLK) results in a weaker inte-

reaction of β -catenin/Tcf/Lef with DNA and in depletion of these two factors (Ishitani et al., 1999). Finally, there are efficient repressors, which can cut off the transcriptional active β -catenin-Tcf/Lef complex. Especially the inhibitors Chibby and ICAT bind to the Tcf/Lef binding domain of β -catenin, blocking further Tcf/Lef binding (Li et al, 2008; Daniels and Weis, 2005). Furthermore, in some Wnt target genes, Tcf can directly antagonize transcription by binding to the sequence AGAA/TAA/T, next to the WREs in *Drosophila* (Blauwkamp et al., 2008).

4.4 The Tcf/ β -catenin interaction

The formation of a Tcf/Lef- β -catenin complex and the stronger binding of Tcf/Lef to their DNA binding sites through Wnt signaling upregulation is key event for the activation of its target genes. Both Tcf/Lef and β -catenin need each other for an efficient upregulation of the Wnt target genes (van de Wetering et al., 1997). β -catenin possesses at the N-terminus as well as the C-terminus one potent transactivation domain, but no DNA interaction region and Tcf/Lef has the ability to bind to WREs through their HMG domain, but must associate with coactivators to effect transcription activation (Behrens et al., 1996; van de Wetering et al, 1997; Molenaar et al., 1996).

Generally, all four members of the vertebrate Tcf/Lef family are able to interact primarily with β -catenin via their highly conserved extended N-terminal β -catenin binding domain (located at the first 65-69 amino acids). This interaction domain can be divided in an extended part, consisting of residues 13-25 and another area, forming a helix, containing residues 40-50 (Poy et al, 2001). In Tcf3 a third interaction part exists reinforce the binding efficiency. The first one binds β -catenin at the Arm repeats 4-9 and the second one at the Arm repeats 3-5 (Poy et al., 2001). Probably, the extended part binds first and then the helix interaction follows. The process of Tcf/Lef- β -catenin interaction evokes changes in the β -catenin binding site (Love et al, 2004). On the one hand, this is caused by the central armadillo repeats 3-10 of β -catenin, forming an interaction groove, based on a shape of a superhelix with charged residues. On the other hand, Tcf/Lef establishes a negatively charged alpha helix in the area of the β -catenin binding domain, being able to interact via salt bridges with the positively charged parts of the superhelix of β -catenin (Graham et al., 2000; Hurl-

stone and Clevers, 2002). In addition to this, some electrostatic contacts between Tcf/Lef and β -catenin could be identified, supporting the conformational change of the N-terminal region. It is important to notice that during the binding process the conformation of β -catenin is not changed (Gooding et al, 2004). Usually, in solution Tcf/Lef proteins are representing largely unfolded proteins and only permissible interactions lead to folded structures, which could be identified in circular dichroism spectra, protease digestion and crystallographic analysis (Daniels and Weis, 2005; Poy et al, 2001).

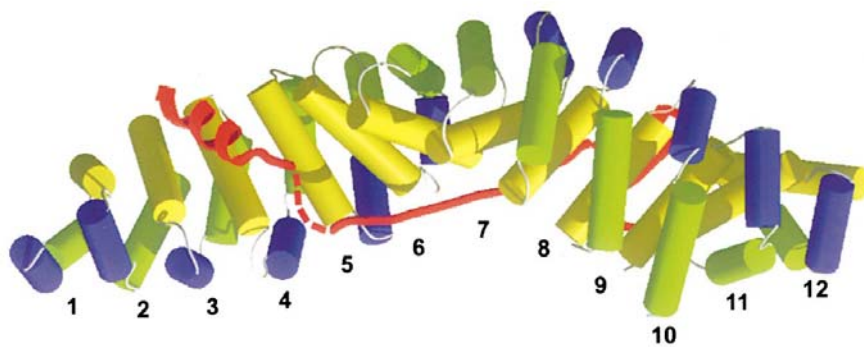


Figure 4.4.1. Three-dimensional model of the XTcf3-CBD/ β -catenin Armadillo complex. On the left hand side the N-terminal side of Tcf3 and on the right hand side the C- terminal side of the β -catenin binding site is shown. (see the text for details; from Graham et al., 2000).

4.5 Wnt signaling inhibition in brain development exemplified by Tcf3 and Six3

Tcf3 and Six3 represent both prominent transcription factors, which accomplish their functions during embryonic development in vertebrates, especially in neuronal tube patterning processes they inherit essential tasks in the anterior- posterior axis formation. In this context both proteins work in connection with the canonical Wnt signaling pathway, including the ligands, Wnt1, Wnt3, Wnt3A and Wnt8B (Buckles et al., 2004; Lagutin et al., 2003; MacMahon and Moon, 1989).

Generally, in gastrulation ectodermal cells take over neuronal characteristics to form a neural tube, followed by anterior versus posterior specification of neural tissues (Nieuwkoop, 1973; Yamaguchi, 2001; Kiecker and Niehrs, 2001). The first anterior-posterior axis determination is achieved by the anterior marker *Otx2* (orthodenticle homologue 2) and the posterior marker *Gbx2* (gastrulation brain homeobox 2), which

establish a functional boundary, lying upon the border between mid- and hindbrain (Ye et al., 2001; Raible and Brand, 2004). In course of this procedure, some further signal proteins as Wnts, Nodal, BMPs (bone morphogenetic proteins), FGFs (fibroblast growth factors) and retinoic acid contribute to the posterization of the neural tube, which could be shown in *Xenopus* (Gamse and Sive, 2000). The involved Wnt proteins are expressed throughout the whole neural tube, but are restricted by the antagonists Dkk1 (Hashimoto et al., 2000) and Cerberus (Leyns et al., 1997) at the front head side, forming a gradient from anterior to posterior (Kiecker and Niehrs, 2001). In *Xenopus*, the first hint for the posteriorizing effect of the Wnts could be shown by mediating a depletion of β -catenin, leading to ventralized embryos (Molenaar et al., 1996). In addition, an overexpression of *Wnt8* resulted in the suppression of anterior parts and the induction of posterior structures (Hamilton et al., 2001; Darken and Wilson, 2001; Wurst and Bailey-Cuif, 2001). In the same context, an increased expression of *Dkk1* causes the enlargement of anterior brain parts, representing the “Dickkopf” phenotype and defects at the posterior side (Glinka et al., 1998; Mukhopadhyay et al., 2001).

In this first influencing period of Wnt proteins on the posterization of the brain, the essential inhibiting function of Tcf3 could be identified (Houston et al., 2002; Kim et al., 2000). In *Xenopus*, overexpression of Tcf3 led to repression of anterior structures and to more prominent of posterior neural markers (Hamilton et al., 2001). In addition, Zebrafish *headless* mutants (point mutation in Tcf3) resulted in loss of eyes and forebrain and the frontal part of the midbrain (Kim et al., 2000). In exchange, the posterior area of the midbrain was enlarged. Furthermore, the enlarged expression patterns of genes defining the mid-hindbrain boundary could be shown. Therefore, Tcf3 does play a very important role as an intracellular inhibitor, enabling the formation of anterior and limiting caudal structures during early brain development. For instance, the negative control on some Wnt target genes through Tcf3 like *Pax2*, *Eng2* and *Dkk1* could be observed (Kim et al., 2000). In this context, in Zebrafish a second related gene *Tcf3b* could be identified, which strongly cooperates with *Tcf3* in early development. Actually, it has the ability of overtaking the function of *Tcf3* (Dorsky et al., 2003). This additional gene represents a further example for a gene duplication followed by mutations, leading to two different genes with variant functions (Ohta, 2000; Taylor et al., 2001). However, following the expression levels of Tcf3/Tcf3b dur-

ing gastrulation, *Tcf3* plays a much more prominent role in hindering the embryonic caudalization. To complete the functions of *Tcf3* during neural plate development, it is also common that it fulfills essential roles in the establishment of the rhombomers, indicating a Wnt antagonizing effect in the caudal region too (lowering the Wnt gradient in this specific areas) (Kim et al., 2000, Dorsky et al., 2003).

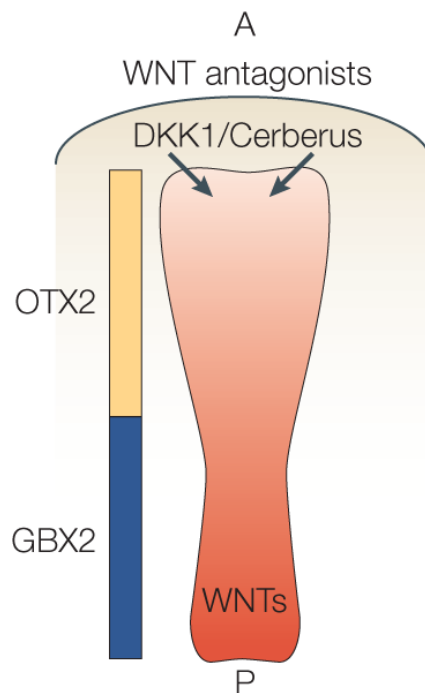


Figure 4.5.1. Schematic illustration of the vertebrate neural tube from the dorsal side during gastrulation. A broad Wnt gradient is established with is increased from anterior to posterior. The Wnt repressors Dkk1, Cerberus and Tcf3 play the most important role in this case. (from Ciani and Salinas, 2005).

In late gastrulation, an additional local gradient of Wnt proteins is formed in the forebrain, ranging over the di- and mesencephalon to the mid-hindbrain boundary. The neural tube is further divided into specific functional brain regions, representing the telencephalon, diencephalon, mesencephalon, and subsequently into metencephalon and myelencephalon. Striking structures of the forebrain display the lateral eye fields and the ventral hypothalamus (Wilson and Houart, 2004). The establishment of the additional Wnt gradient has been well documented by overexpression of *Wnt8* in *Xenopus*, resulting in loss of head and notochord and in bigger somatic muscles (Christian and Moon, 1993) and vice versa by its inhibition, leading to contrary phenotypes (Hoppler et al., 1996). Finally, the definite temporal relevance for the second Wnt gradient could be identified by Heisenberg and colleagues (Heisenberg et al., 2001)

and van de Water and colleagues (van de Water et al., 2001) in Zebrafish. In course of these experiments, based on the creation of the mutant *masterblind*, which represents a point mutation in Axin and therefore leading to the inhibition of GSK3 β binding, the fishes showed a strongly minimized eye field and telencephalon and larger posterior regions. In comparison to the earlier Wnt gradient, the later one is restricted to a smaller area, but with similar phenotypes. In addition, the extracellular inhibitor Tlc is involved in the establishment of this gradient. This could be demonstrated by overexpression experiments, resulting in expanded forebrain regions and diminished caudal areas (Houart et al., 2002). The opposite phenotype was reached by doing a knockdown of *Tlc* with morpholinos (Houart et al., 2002).

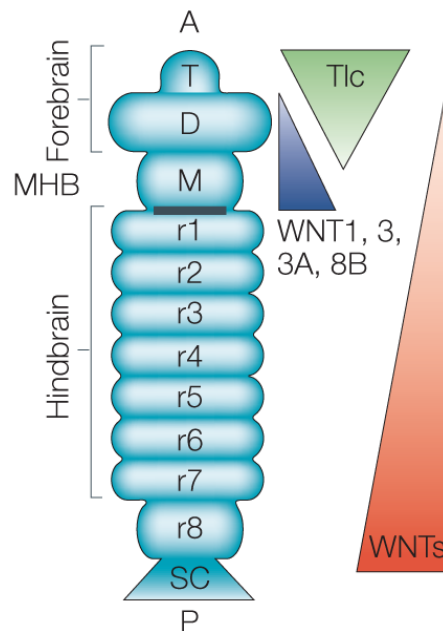


Figure 4.5.2. Dorsal view of the vertebrate neural tube during late gastrulation. An additional local Wnt gradient is arranged which is anteriorly restricted by Tlc and Six3. (from Ciani and Salinas, 2005).

In addition to the repressive effect of Tlc, the transcription factor Six3 is also expressed in most anterior regions and contributes to an efficient Wnt signaling inhibition there and to the constitution of the anterior-posterior Wnt gradient (Lagutin et al., 2003). *Six3* is part of the *Six* gene family, which can be subdivided into three subclasses *Six1/2*, *Six3/6* and *Six4/5*, characterized by the length of their C-termini (Kawakami et al., 2000; Seo et al., 1999). The members of this family could be found across the animal kingdom (fly: Serikaku and O'Tousa, 1994, Zebrafish: Kobayashi et al., 1998, mouse: Jean et al., 1999, human: Boucher et al., 1995). The most promi-

nent and conserved regions represent the homeodomain (HD), near the N-terminus (including approximately 60 amino acids) and the Six domain (SD), consisting of 110 to 115 amino acids (Kawakami et al., 2000). In difference to other known HD proteins, which contact the TAAT core sequence of specific DNA sites, the Six transcription factors bind to other DNA regions due to their missing amino acids arginine and glutamine at position 5, respectively 12 in the helix 1 (Kawakami et al., 2000; Serikaku and O'Tousa, 1994). An exception represents Six3/Six6, which binds to its target DNA site, including the sequence ATTA, being similar to the recognition sites of homeoproteins (Hu et al., 2008). Generally, all *Six* genes are involved in the development of skeletal muscles, neural crest derivatives, limb buds, ears, noses, kidneys and eye development in vertebrates.

In *Drosophila* imagines and larvae, *Six3*, respectively *Optix* (see below) and other homologous *Six* gene members are expressed in the eye imaginal disc, the optic lobe and the procephalic lobe, fulfilling their functions in the development and maintenance of the visual system, the ocelli and the larval photoreceptors (Cheyette et al., 1994; Seimiya and Gehring, 2000). Their effects on the eye formation and enclosed areas could be shown in several studies by using mutants, leading to lack of complete eyes, ocelli and optic lobe ganglions (Cheyette et al., 1994). In vertebrates, *Six3* and *Six6* are expressed in developing areas of the lens, neural retina, retinal pigmented epithelium, nasal placodes, optic chiasm and forebrain, taking over important tasks in forming the rostral brain and the eye, especially the retina and the lens (Jean et al., 1995; Lopez-Rios et al., 1999). The ability for regulation of the proximodistal axis patterning could be shown in Medaka *Six3* morphants, in which the expression levels of *Vax1*, being responsible for ventral forebrain and proximal eye structures and *Rx3*, playing a role in optic vesicle development, were strongly decreased. In addition, the evidence that *Six3* controls its own expression levels in the retinal precursor cells could be provided in Medaka too. This autoregulation process of *Six3* can also be blocked by Groucho interactions (Carl et al., 2002; Lopez-Rios et al., 2002). In *Six3* knock-out mice the structures of the telencephalon, optic vesicles and olfactory placodes are severely truncated (Lagutin et al., 2003). These observations indicate that *Six3* is the most important member of this gene family.

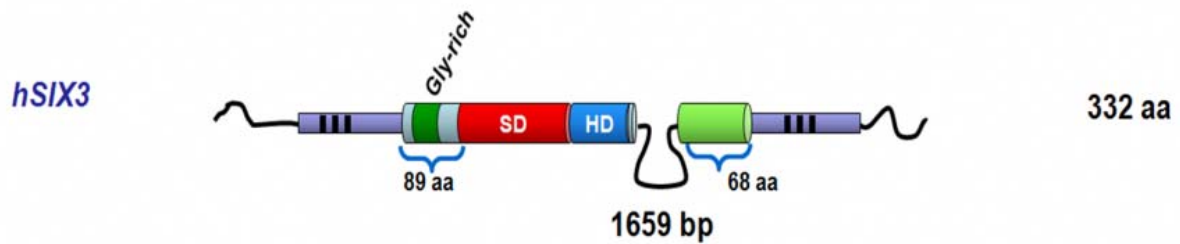


Figure 4.5.3. Schematic representation of the human Six3 structure. All coding areas are shown as cylinders, including the six domain (SD) and the homeodomain (HD). At the 5' end, within the structure and at the 3' end thin lines are depicted. These display the untranslated regions and the intron (from Rodriguez de Cordoba et al., 2001).

The genetic regulation of the vertebrate eye formation is still part of numerous studies, being in order to understand the process in detail. Basically, eye development starts with the development of the eye vesicle, arising from the diencephalon, followed by the induction of the lens placodes. Subsequently, by invagination the lens vesicle is developed for the establishment of the later adult lens. Meanwhile, the optic cup arises from the optic vesicle and grows around the lens vesicle. Then the adult retina is established as an inner part of the optic cup (Chow and Lang, 2001; Wawersik and Maas, 2000). The genetic pathways of eye formation are very similar in insects and vertebrates, although they have formed very different types of eyes in evolution through monophyletic processes (Gehring, 2005). This fact could be confirmed by the ectopic expression of *Pax6* in *Drosophila*, the key protein of eye development in vertebrates. This misexpression led to the formation of a normal functional compound eye in different body structures (Halder et al., 1995). The gene *Pax6* takes over the first prominent role during eye formation, being expressed in head ectoderm and afterwards in the lens placode, lens vesicle and optic vesicle and is directly involved in their establishments (Grindley et al., 1995). The function of *Pax6* is splitted into the homologous genes *twin of eyeless* (*toy*) and *eyeless* (*ey*) in *Drosophila*, which fulfill analogous tasks during eye formation (Czerny et al., 1999). In general, the expression of *Pax6* is responsible for the activation of the transcription factors Six3/Optx2, Eyes absent (*Eya1*) and Dachshund (*Dach1*) (Ohto et al., 1999), corresponding functionally to the fly genes *sine oculis* (*so*), *eya* and *dac* (Shen and Mardon, 1997). At this point it has to be mentioned that in fact the *Drosophila* gene *Optix* is the orthologue of the vertebrate *Six3* gene and that the factor *sine oculis* corresponds to the six subclass of *Six1/2* in higher organisms (Seimiya and Gehring, 2000). In

flies, this bases on different expression patterns, the independent function of *optix* in opposition to *eya* and the ability of *optix* to form ectopic eyes, what so cannot do (Seimiya and Gehring, 2000). In addition, the gene *Optx2* is also a member of the *Six3/6* group, performing together with *Six3* essential roles during the eye establishment. But, in this context, *Six3* is the more important factor, because *Optx2* only functions in retina development (Zuber et al., 1999). Comparing the different tasks of the subgroups of *Six3* and *Six1*, it seems that during evolution in vertebrates the gene *Six3* has inherited the functions of *so* and *optix*. A further difference between the eye development in flies and vertebrates is that in *Drosophila* exist reciprocal pathways among almost all of these genes which are involved in the network of eye formation (Pignoni et al., 1997). At least, a very well described mutual upregulation is known between *Six3* and *Eya* in vertebrates (Heanue et al., 1999). All these genes together are involved in the formation of optic vesicle, lens, retina and optic stalk and work as key regulators by controlling the expression levels of more subordinated genes. In this context, also the signaling pathways of Bmp or Dpp (decapentaplegic) play important roles (Chen et al., 1999).

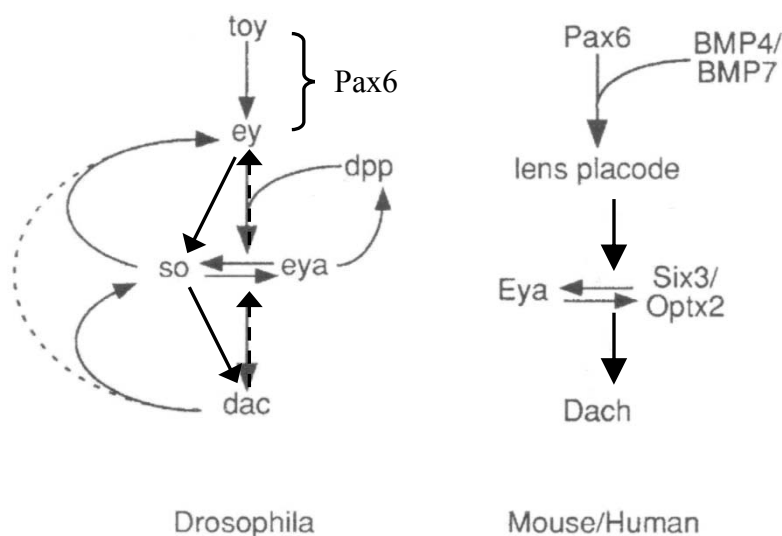


Figure 4.5.4. A comparison of the genetic pathways, which control the eye development in *Drosophila* and vertebrates (see more details in text; adapted from Wawersik and Maas, 2000)

The results of the *Six3* knock-out experiments in mice (lack of telencephalon, optic vesicles and olfactory placodes), indicate a repressing function of *Six3* on the Wnt signaling pathway in the anterior brain. In addition to the missing structures, the di- and mesencephalon show enlarged characteristics (Lagutin et al., 2003). Further-

more, an expansion of the *Wnt1* expression pattern in the diencephalon can be observed (Lagutin et al., 2003). Together with the last discovery and the verification that Six3 binds to characteristic regulatory sites within the *Wnt1* enhancer, it could be definitely shown that Six3 is responsible for *Wnt1* repression in the forebrain (Lagutin et al., 2003). The base for the repressor function of Six3 displays its ability for a stable interaction with proteins which are related to the corepressor Groucho (Lopez-Rios et al., 2002). In this context the binding of Groucho/TLE additionally leads to chromatin modifications (Courey and Jia, 2001). Further evidences for the repressor function of Six3 are the formation of an enlarged forebrain when it is overexpressed and its ability to rescue the *headless* phenotype in Zebrafish (Lagutin et al., 2003). It is also known that in humans the phenomenon of holoprosencephaly based on a heterozygous mutation in the Six3 homeodomain or Six domain (Pasquier et al., 2000). However, the repressing activity of Six3 can be abolished by an efficient interaction with AES, a dominant negative form of TLE (Lopez-Rios et al., 2002).

4.6 The transactivation domains of VP16 and p65

The herpes simplex virion protein VP16 represents an activator, which function of efficient viral replication was identified by Cress and Triezenberg in 1991 (Cress and Triezenberg, 1991). This protein contains two potent transcription activation domains (TAs), which independently contact the important human cofactor PC4 (Kretzschmar et al., 1994) and the well known transcription factor TFIIB (Lin et al., 1991). In course of this process, a conformation change takes place (Jonker et al., 2005). Through binding of VP16, the assemblies of the preinitiation complex (PIC) and of chromatin remodeling enzymes are supported (Kingston et al., 1996) in eukaryotes. In many experiments this VP16 transactivator domain was fused to transcription factors for upregulating specific target genes. For example the fusion with PDX-1, a homeodomain-containing transcription factor led to increased insulin biosynthesis (Kaneto et al., 2005) or in connection with Gal4, it can induce specific reporters (Hagmann et al., 1997). In the latter case, it could be shown that the function of VP16 is only limited to proximal and singular promoters, making it to an efficient tool for transcription activation.

In difference to VP16, the transactivator domain of p65 displays a subunit of the human transcription factor NF- κ B, which plays important roles in cell proliferation, cell death and immune response. The activation of this transcription factor is regulated by its detachment of the inhibitor I κ B, resulting in its nuclear transport (Baeuerle and Baltimore, 1988). The binding of this antagonist is directly mediated by p65 (Ruben et al., 1992). After the arrival of NF- κ B in the nucleus, it binds to its DNA target sites by another subunit, called p50. P65 itself could be shown to work as a strong transactivator (Ballard et al., 1992), without having the ability for DNA interaction (Urban and Baeuerle, 1990). In a study, performed by Ballard and colleagues, the transactivator function of p65 was detected (Ballard et al., 1992). For this purpose, among others, the C-terminally transcriptional active region of p65 was fused to the Gal4 protein for reaching a CAT reporter upregulation. The design of this protein was done in a way, that p65 was added C-terminally. As a result, an induction of about 200 fold could be observed, 4 times higher than with VP16. Thus, this study showed that the fusion of p65 to any transcription factor functions in a strong upregulation.

5. Material and Methods

5.1 DNA methods and cloning

5.1.1 Cultivation conditions of bacteria

The bacterial stocks of *Escherichia coli* bacteria “TOP10F” (for usual cloning procedures) and “stable2” (for cloning processes with repeats) were cryopreserved in 1x glycerol buffer at -80°C. For cultivation the standard Luria-Bertani (LB) medium at 37°C was used. The liquid cultures were incubated over night on a shaker at 220 rpm. An efficient selection was reached through usage of an Ampicillin resistance gene (amp^R) on the transformed plasmid in combination with an addition of Ampicillin to the medium at a concentration of 100 µg/ml. The sterilisation of all media was carried out by autoclaving for 120 minutes at 130°C.

LB composition (per litre):

bacto tryptone	10g
yeast extract	5g
NaCl	5g
NaOH 1M	1ml
final pH 7.2- 7.5	

for agar plates: 15g agar per litre added

10x glycerol buffer: 36 mM K₂HPO₄, 13 mM KH₂PO₄, 20 mM Na₃Citrate, 10 mM MgSO₄, 50% Glycerol in H₂O dest., sterilization by filtration.

5.1.2 Cloning procedures

For cloning all buffers and restriction enzymes derived from Fermentas/MBI and Gibco. Usually 5-10 µg DNA for vector or insert was digested with 2 µl enzyme (~20 units) for 2 hours at the recommended temperature (mostly at 37°C). In addition, the onset included 10x buffers which were introduced in a ratio 1:10 and in consideration

of the used enzymes. Gel purification with 0.5-2% agarose gels led to an isolation of the needed DNA.

On demand for the synthesis of blunt ends the digested plasmids were “filled in” by usage of 10 units T4 DNA polymerase (Fermentas) per 5 or 10 µg plasmid DNA and 0.5 µl of 10 mM dNTPs (Fermentas). This reaction was started by incubation for 5 minutes at room-temperature, following a phase of inactivation at 75°C for 10 minutes.

In most cases 1 µl of the enzyme fast calf intestinal alkaline phosphatase (CAP) from Fermentas was used for dephosphorylation of vector DNA. For this purpose the CAP was added to digested plasmid DNA and exposed at 37°C for 10 minutes and then arrested by the application of 5mM EDTA in combination with an incubation phase at 80°C for 20 minutes.

The purification of the vector and insert DNA was directly performed by the Invisorb Spin DNA Extraction Kit. Before this step the second one was separated and cut out of an agarose gel. The ligation was carried out by the usage of 50 ng vector fragment and 1-3 fold molar excess of the insert in connection with the Fermentas enzyme T4 DNA ligase. The application of the amount of the enzyme was dependent on blunt or sticky ends. In the first case 2 units and in the second case 1 unit for the ligation was used. After an over night incubation, the inactivation of the ligase at 65°C for 10 minutes followed.

5.1.3 Transformation into *E. coli*

The transformation in TOP10F⁺ was started with the application of 100 µl competent bacteria (in combination with 10 µl of the product of the ligation reaction) on ice for 10 minutes. Subsequently a heat shock at 42°C for 1minutes 30 sec. was performed and an incubation on ice for 10 minutes followed. 1 ml LB medium was then added and the whole reaction batch incubated for 30 minutes at 37°C with shaking. The removal of the supernatant followed the centrifugation for 4 minutes at 5000 rpm. The resulting pellet was resuspended in approximately 100 µl of the remaining LB and finally plated out on LB + Ampicillin Petri dishes and incubated at 37°C over night. In

comparison to the transformation in TOP10F⁺ cells, the procedure was changed in some points in the stbl2 (Invitrogen) line. The first ice incubation step was performed for 30 minutes and the subsequent heat shock at 42°C for only 25 seconds. Then the ice incubation was carried out for 2 minutes. Instead of the LB medium 1 ml SOC (derivate of super optimal broth = SOB, high rich medium) medium was added and shaken at 225 rpm at 30°C for 90 minutes. Finally, the distribution was done on agar plates composed of SOB medium.

SOB composition (per litre):

bacto tryptone	20g
yeast extract	5g
NaCl	0.5g
KCl	2.5ml
ddH ₂ O to	1l

final pH 6.8-7.0, adjusted with 10N NaOH
after sterilization, add sterile 10ml 1M MgCl₂ and 10ml 1M MgSO₄

for agar plates: 15g agar per litre added

SOC composition (per litre):

SOB medium plus 20 mM Glucose
final pH 6.8-7.0

5.1.4 Mini-preparation of plasmids

Ampicillin selected colonies were taken off the Petri dish and transferred into 10ml tubes with 2 ml LB + Ampicillin for suspension and incubated at 37°C over night. In case of performing cloning with stbl2 bacteria, the bacteria colonies were incubated in 2 ml TB medium at 30°C for at least 20 hours.

TB composition (per litre):

bacto tryptone	12g
yeast extract	24g
glycerol	4ml
ddH ₂ O to	1l

after sterilization, add 0.17M KH₂PO₄ and 0.72M K₂HPO₄

Then this bacteria suspension was splitted in one small amount stored at 4°C for Midi-prep inoculation and one remaining part for further working. After a centrifugation step for 2 minutes at 6000 rpm the supernatant was discharged. For reaching homogeneity 100 µl P1 solution (50 mM Tris, 10 mM EDTA, HCl ad pH 8.0 plus RNase) was added to the pellet and mixed. In the next step 200 µl P2 solution (200 mM NaOH, 1% SDS v/w) for cell lysis was added, 5 times inverted carefully and incubated at room temperature for at least 3 minutes, but not more than 5 minutes. Before next centrifugation (20 minutes at 14000 rpm) for further DNA extraction 200 µl P3 solution (3.1 M potassium acetate and acetic acid ad pH 5.5) for neutralization was filled in and again immediately mixed by inverting the tubes 5 times. For DNA precipitation the supernatant was given into a new Eppendorf tube and 500 µl sterile filtered PEG 24% was added. The mixing step includes incubation for 15 minutes at 37°C in combination with shaking. A further centrifugation for 15 minutes at 14000 rpm at 4°C was done, resulting in the removal of the supernatant. For the purpose of DNA washing 500 µl 70% ethanol was carefully added and a centrifugation for 5 minutes at 14000 rpm was performed. The ethanol was carefully drawn off so that the DNA pellets could be dried for 10 minutes. Finally they were dissolved in 20 µl H₂O.

5.1.5 Midi-preparation for getting higher amounts of plasmid DNA

On the same day as the Mini prep synthesis 10 µl of the stored liquid culture from Mini Prep culture was transferred in an Erlenmeyer flask with 100 ml LB + Ampicillin for over night incubation at 37°C. Doing the Midi preparation in stbI2 cells 10 µl were preincubated in 10 ml LB medium for at least 4 hours and then transferred in 100 ml LB medium with Ampicillin (same amounts like before). As a first step the cells were separated from the medium by centrifugation at 4000 rpm for 5 minutes. The addition of 4 ml of the first reagent of the used JETstar kit (Genomed GmbH), solution E1 (RNase included) caused cell homogeneity. Cell lysis was carried out by 4 ml solu-

tion E2 in combination with 5 times inversion and incubation for 5 minutes at room temperature. For neutralization, 4 ml solution E3 was added and gently mixed by inverting the tube 5 times again, followed by a centrifugation at 4200 rpm for 20 minutes. During the centrifugation step, the columns for DNA extraction were preequilibrated with absolute ethanol. Subsequently, the real equilibration was performed by adding solution E4 (600 mM NaCl, 100 mM sodium acetate and 0.15% Triton X-100, acetic acid pH 5.0). The supernatant resulting from centrifugation was transferred into the equilibrated columns and twice washed with 10 ml solution E5 (800 mM NaCl, 100 mM sodium acetate, acetic acid p 5.0). The plasmid elution was achieved by addition of 5 ml solution E6 (1250 mM NaCl, 100 mM Tris pH 8.5). For plasmid DNA precipitation 3.5 ml isopropanol was added, followed by strong mixing and a centrifugation step at 4200 rpm at room temperature (4°C) for 30 minutes. Finally the plasmids were washed with 70% ethanol, air dried for 10 minutes and resuspended in 100 µl of H₂O. For a better DNA purification and subsequent dissolving the resulting solution was gently shaken at 65°C for one hour and then centrifuged for 5 minutes with 10000 rpm (this is not recommended for highly sensitive plasmids).

The concentration of plasmids was quantified with the Christian- Warburg method by measuring with a spectrometer at 260 nm and then on an agarose gel. For this purpose 5 µl DNA dilution (contained 50 ng plasmid) was loaded on a 1% or 2% agarose gel with 30 µg/ml ethidium bromide and SB buffer. For visualizing, the DNA samples were mixed with DNA loading buffer before loading on the gel. A mixture, called λ Mix, of two Fermentas DNA ladders (Gene Ruler 100bp DNA ladder and λ DNA/Eco91I (BstEII)) was used for DNA fragment length identification and semi-quantitative analysis. The visualization was done on the Alphamager (Alpha Innotech). Usually, the DNA quantification was combined with a specific digest for the prepared DNA plasmid. This was done by using the appropriated restriction enzymes, leading to the corresponding DNA band patterns on the agarose gel.

Composition of used materials:

Agarose gel:	20x SB-buffer (per 1000ml):	
	NaOH 5M	40 ml
	pH adjusted to 8.5 with boric acid	

5x loading buffer (per 100 ml):

Ficoll 400	20 g
EDTA 0.5M pH8.0	20 ml
SDS 20%	5 ml
Bromphenol blue	0.1 g

λ- Mix (per 500 µl):	100 bp ladder (0.5 µg/ µl)	43 µl
	λ BstEII (0.5 µg/ µl)	42 µl
	5x loading buffer	100 µl
	H ₂ O to 500 µl	

5.1.6 PCR and sequencing reactions used in context of cloning

The PCR batch consisted of 100 ng template DNA, 50 pmol of each primer (Gibco), 10 nmol dNTPs, 2.5 units Taq polymerase (Fermentas), 5 µl 10x standard SB buffer with Tween (Fermentas) and 3 µl 25 mM MgCl₂ in a volume of 50 µl. All PCR reactions were performed in the PCR machine MJ Research PTC-200 Peltier Thermal Cycler and following program was used:

94°C	5 min	
94°C	30 sec	} 25-30 cycles
55-65°C	30 sec	
72°C	30 sec	
72°C	5 min	

The control of cloned plasmids was done by sequencing. The sequencing batch in a PCR tube contained 250 ng plasmid DNA, 5 pmol primer and 2 µl Sequence Pre mix in final volume of 10 µl. For the cycling reaction was used the same PCR machine as described above, with following program:

95°C	20 sec	} 25 cycles
50°C	15 sec	
60°C	60 sec	

15°C forever

After cycling reactions the precipitation of sequencing products was done with 12 μ l 500 mM Sodium Acetate (diluted from NaAc 3 M pH 4.6) and 58 μ l 96% ethanol. Subsequently a centrifugation at 14000 rpm at 4°C for 30 minutes was performed and the pellet was twice washed with 200 μ l cold 70% ethanol. For the drying step the tubes were transferred into a speed vacuum machine for 5 minutes. Finally the pellet was dissolved in 20 μ l 70% formamide. The sequencing itself was performed in an ABI PRISM 377 machine (Applied Biosystems).

5.1.7 Cloning strategies and design of DNA constructs

For almost all transient reporter assays the expression vectors pKC (polylinker modification of pKW) or pMC, containing CMV promoters were used and the constructs which were cloned, derived from murine, human or *Xenopus* cDNA.

In this study the possible existence of a second interaction domain of Tcf/Lef with β -catenin was examined. This domain is part of the HMG boxes of this protein family. For design, the DNA binding region of the murine protein Lef1 was gained by performing a targeted PCR. In course of this, the forward and reverse primers were exactly placed at start and end points, comprising the nucleotides for the expression of the amino acids 293 to 397 (HMG box contains the amino acids 293 to 380). Subsequently, the resulting cDNA for Lef Δ 293 expression was cloned into the pGemT (Promega) and then into a pMC vector including a FK binding domain, resulting in the construct pMCLef Δ 293FK. The final construct pKCLef Δ 293 was generated by an exchange of Lef Δ (240-397) with Lef Δ 293. For this process the vector pKCLef Δ (240-397) as well the insert pKCNLSLef Δ 293 were digested by SmaI. Parallel to the construct which expresses the Lef1 deletion mutant Lef Δ 293, two constructs with further shortened Lef1 deletion variants were cloned: pMCLef Δ (240-397) and pKCLef Δ (78-397). The construct pMCLef Δ (240-397) (HMG box plus a short N-terminal region of Lef1) was generated by inserting Lef Δ (240-397), deriving from pMCLef Δ (240-397)FK into a pMC vector (insert und vector were cut by ClaI and XhoI). The coding sequences of Lef Δ (240-397) and Lef Δ (78-397) were isolated by targeted PCRs, the second one was cloned directly into the pKC vector (ClaI, HindIII digestions). The

constructs pKCLef1 and pKCTcf3 for expressing the full length proteins of Lef1 or Tcf3 were cloned from the cDNAs of murine Lef1 and *Xenopus* Tcf3 and the pKC vector. Another Tcf3 expressing variant was cloned into a pRL plasmid (Promega), which includes an HA tag. This was done by cutting the insert and the vector with BamHI and treated them with T7 polymerase for generating blunt ends. Finally, the original construct pSC2Tc1e originates from Andreas Hecht (Wöhrle et al., 2007) (see Figure 5.1.7.1 for cloning steps).

vector	restriction enzymes	insert	restriction enzymes	construct
pKC	Clal HindIII	pGemTLef1	Clal HindIII	pKCLef1
pKC	Clal HindIII	pGemTLefΔ(78-397)	Clal HindIII	pKCLefΔ(78-397)
pMC	Clal XhoI	pMCLefΔ(240-397)FK	Clal XhoI	pMCLefΔ(240-397)
pKCLefΔ(240-397)	SmaI	pMCLefΔ293FK	SmaI	pKCLefΔ293
pKC	Clal HindIII	pGemTTcf3	Clal HindIII	pKCTcf3
pRL-CMV	BamHI	pGemTXTcf3	BamHI T7	pHAXTcf3

Figure 5.1.7.1. Illustration of the cloning steps for isolating the full proteins and the HMG boxes of Lef1, Tcf3 and Tcf1 and their insertion processes into the vectors pKC, pMC and pRL.

The influence of β -catenin on the binding efficiency of Lef(Δ293) on the special Tcf/Lef binding sites was tested by using some reporter constructs expressing firefly luciferase. In all cases oligos with variable Tcf/Lef binding sites were inserted upstream of a Fos minimal promoter for luciferase expression. In one case the plasmid plucF, which originates from the pGL3 reporter (Promega) and in another case a modified version pMlucF, including a backbone from the pMC vector, was used. Firstly, the plucF6Top reporter with six artificial high affinity Tcf/Lef binding sites was used to examine the effect of β -catenin on the DNA binding of LefΔ293. The Tcf/Lef binding sites are derived from the TopFlash reporter (Korinek et al. 1997), which is known from several studies concerning the Wnt signaling pathway. In addition, the reporter pLefluc, containing seven Lef1 consensus binding sites (differ somewhat in sequence) and developed by Grosschedl et al. (Hsu et al. 1998) was applied for further analysis. At that time this reporter was designed for expressing chloramphenicol acetyltransferase. In the same context, I created reporters with three or six Tcf/Lef binding sites of the same consensus sequences, named plucF3Lef or pLucF6lef.

One step further, the reporter pMlucF6Lefcons was generated, which includes six Tcf/Lef binding motifs from the TopFlash reporter again, but with another sequence in between (see Figure 5.1.7.4). By using this construct, a possible binding effect of β -catenin on Lef Δ 293 DNA binding could be evaluated, by comparing the expressing levels with other reporters with natural Tcf/Lef binding sites in combination with the same insertional sequence. Nevertheless, examining possible binding differences, in series, firefly luciferase expressing reporters with various non-artificial natural motifs were created. In a first case, the Tcf/Lef binding motifs Otxa and Otxb, derived from the Medaka Otx2 midbrain enhancer (Kurokawa et al., 2004) were used (reporters plucF4LefOtxab, plucF6LefOtxb, plucF8LefOtxab, pMlucF24LefOtxa/b, pMlucF24LefOtxab). In ChIP analysis it could be shown that Wg/Wnt activation, considering special clusters, respectively low affine Tcf/Lef binding sites of Axin2 (Wöhrle et al., 2007), *c-myc* (Yochum et al., 2008; Williamson et al., 2006) and *naked cuticle* (*nkd*) (Parker et al., 2007), causes an increased amount of Tcf/Lef and β -catenin binding. In addition, in these areas some DNA Tcf/Lef binding elements seem to have similar aberrances in their sequences in comparison to the binding sites in the Otx enhancer. This supports the idea, that in some situations through Wnt upregulation, Tcf/Lef proteins bind to characterized binding elements with the help of β -catenin. Therefore, individual Tcf/Lef binding sequences of the *c-myc* (human) and the *nkd* (*Drosophila*) enhancers were used for the design of further reporters (pMlucF24Lefmyc, pMlucF24Lefnkd). To exclude an effect, which may derive from the close distance between the Lef/Tcf binding sites, a reporter with binding sites for the zinc finger homeodomain protein ZFHD-1 (ZF) in between the Otxb motifs, was generated (plucF24ZFLefOtxb). The ZFHD-1 protein consists of Zif268 zinc finger structures, the Oct-1 homeodomain (HD) and of a short polypeptide linker in between (Pomerantz et al., 1995). The same binding system was used for creating a pSGFluc (twelve ZF binding motifs) and a plucZF24 (24 ZF binding motifs) reporter. These both reporters were used to distinguish between a Wnt target gene induction, arising from Tcf/Lef binding and a luciferase upregulation, just caused by the binding of the zinc finger protein. The exact sequence of the Otx2 enhancer was used for the reporter pMlucF16OtxFMcore. In case of the reporters pMyc5'3'luc and plucAxin2 the whole promoters/enhancers were used for luciferase expression. The myc reporter was generated by S. G. Yochum (Yochum et al., 2008) and the reporter plucAxin2 by

A.Hecht (Wöhrle et al., 2007). The design of all reporters, inclusively the different Tcf/Lef binding sites are shown in Figures 5.1.7.2, 5.1.7.3 and 5.1.7.4).

vector	restriction enzymes	insert	restriction enzymes	construct
plucF	BglII	Oligos TC813/814		plucF3LefOtxb
plucF3LefOtxb	BglII	Oligos TC813/814		plucF6LefOtxb
plucF	BglII	Oligos TC815/816		plucFLefOtxab
plucLefOtxab	BglII	Oligos TC815/816		plucF2LefOtxab
plucF2LefOtxab	BglII	Oligos TC815/816		plucF3LefOtxab
plucF3LefOtxab	BglII	Oligos TC815/816		plucF4LefOtxab
plucF4LefOtxab	BglII	plucF4LefOtxab	BglII	plucF8LefOtxab
pKC	BglII BamHI	Oligos TC696/697		pKCOtxFMcore
pKCOtxFMcore	Sall BglII	pKCOtxFMcore	Sall BamHI	pKC2OtxFMcore
pKC2OtxFMcore	Sall BglII	pKC2OtxFMcore	Sall BamHI	pKC4OtxFMcore
pKC4OtxFMcore	Sall BglII	pKC4OtxFMcore	Sall BamHI	pKC8OtxFMcore
pKC8OtxFMcore	Sall BglII	pKC8OtxFMcore	Sall BamHI	pKC16OtxFMcore
pMlucF	BglII	pKC16OtxFMcore	BglII BamHI	pMlucF16OtxFMcore
plucF	BglII	Oligos TC807/808		plucF3Lef
plucF3Lef	BglII	Oligos TC807/808		plucF6Lef
plucF	BglII	Oligos TC809/810		plucF3Top
plucF3Top	BglII	Oligos TC809/810		plucF6Top
pKC	BglII Xho	Oligos TC986/987		pKC3Lefcons
pKC3Lefcons	BglII	pKC3Lefcons	BglII BamHI	pKC6Lefcons
pMlucF	Sal XhoI	pKC6Lefcons	Sal XhoI	pMlucF6Lefcons
pKC	BglII BamHI	Oligos TC811/812 Oligos TC813/814		pKC3LefOtxa/b
pKC3 LefOtxa/b	BamHI Sfi	pKC3 LefOtxa/b	BglII Sfi	pKC6LefOtxa/b
pKC6LefOtxa/b	BamHI Acc65	pKC6LefOtxa/b	BglII Acc65	pKC12LefOtxa/b
pKC12LefOtxa/b	BamHI Acc65	pKC12LefOtxa/b	BglII Acc65	pKC24LefOtxa/b
pMlucF	Xho BamHI	pKC24LefOtxa/b	BglII Xho	pMlucF24LefOtxa/b

Figure 5.1.7.2. Presentation of the different cloning strategies for the reporters plucF6LefOtxb, plucF4LefOtxab, plucF8LefOtxab, pMlucF16OtxFMcore, plucF3Lef, plucF6Lef, plucF6Top, pMlucF6Lefcons, pMluc24LefOtxa and pMluc24LefOtxb, in combination with the numbers of the used oligos. The reporters by which the experiments were finally performed are shown in bold.

vector	restriction enzymes	insert	restriction enzymes	construct
pKC	BglII BamHI	Oligos TC815/816		pKCLefOtxab
pKCLefOtxab	BamHI Sfi	pKCLefOtxab	BglII Sfi	pKC2LefOtxab
pKC2LefOtxab	BamHI Sfi	pKC2LefOtxab	BglII Sfi	pKC4LefOtxab
pKC4LefOtxab	BamHI Acc65	pKC4LefOtxab	BglII Acc65	pKC8LefOtxab
pKC8LefOtxab	BamHI Acc65	pKC4LefOtxab	BglII Acc65	pKC12LefOtxab
pKC12LefOtxab	BamHI Acc65	pKC12LefOtxab	BglII Acc65	pKC24LefOtxab
pMlucF	XhoI BamHI	pKC12LefOtxab	BglII XhoI	pMlucF12LefOtxab
pMlucF	XhoI BamHI	pKC24LefOtxab	BglII XhoI	pMlucF24LefOtxab
pKC	BamHI BglII	Oligos TC1088/1089 Oligos TC 1086/1087		pKC3Lefnkd/myc
pKC3Lefnkd/myc	BamHI Acc65	pKC3Lefnkd/myc	BglII Acc65	pKC6Lefnkd/myc
pKC6Lefnkd/myc	BamHI Acc65	pKC6Lefnkd/myc	BglII Acc65	pKC12Lefnkd/myc
pKC12Lefnkd/myc	BamHI Acc65	pKC12Lefnkd/myc	BglII Acc65	pKC24Lefnkd/myc
pMlucF	XhoI BamHI	pKC24Lefnkd/myc	BglII XhoI	pMlucF24Lefnkd/myc
pSceBS	Sall NotI	pGFluc	Sall NotI	pSGFluc/plucF12ZF
plucF 12ZF	SacI T4 Acc65	plucF 12ZF	SacI T4 Acc65	plucZF24
pKC	BamHI	Oligos TC883/884		pKC3ZFLeFotxb
pKC3ZFLeFotxb	BamHI	pKC3ZFLeFotxb	BglII BamHI	pKC6ZFLeFotxb
pKC6ZFLeFotxb	Sall BglII	pKC6ZFLeFotxb	Sall BamHI	pKC12ZFLeFotxb
pKC12ZFLeFotxb	Sall BglII	pKC12ZFLeFotxb	Sall BamHI	pKC24ZFLeFotxb
pMlucF	BglII	pKC24ZFLeFotxb	BamHI BglII	plucF24ZFLeFotxb

Figure 5.1.7.3. Presentation of the different cloning strategies for the reporters pMluc12LefOtxab, pMluc24LefOtxab, pMlucF24Lefnkd, pMlucF24Lefmyc, pSGFluc, plucZF24 and plucF24ZFLeFotxb, in combination with the numbers of the used oligos. The reporters by which the experiments were finally performed are shown in bold.

oligo	reporter	Tcf/Lef binding sites
813	EOtxFM Tcf2	GATCCCAC GCTTTGTT CCTCGCAC GCTTTGTT CCTCGCAC GCTTTGTT CCTCA
814	EOtxFM Tcf2	GATCTGAGGAACAAAGCGTGCGAGGAACAAAGCGTGCGAGGAACAAAGCGTGG
815	EOtxFM Tcf1+Tcf2	GATCCCACGTTCAAAGGCTTTGCTGG GCTTTGTT CCTCA
816	EOtxFM Tcf1+Tcf2	GATCTGAGGAACAAAGCCCAGCAAAG CCTTTGAAC GTGG
696	OtxFMcore	AGATCTGTTCAAAGGCTTCGCTGG GCTTTGTT CCACTGTGTGGGGATTAGGCCTGGATCC
697	OtxFMcore	TCTAGACA AAGTTTCC GAAGCGACCCGAAACAAGGTGACACACCCCTAATCCGGACCTAGG
807	Lefluc	GATCCCAC CCTTTGAAG CTCGCAC CCTTTGAAG CTCGCAC CCTTTGAAG CTCA
808	Lefluc	GATCTGAGCTTCAAAGGGTGCGAGCTTCAAAGGGTGCGAGCTTCAAAGGGTGG
809	TopFlash	GATCCCAC CCTTTGATC CTCGCAC CCTTTGATC CTCGCAC CCTTTGATC CTCA
810	TopFlash	GATCTGAGGATCAAAGGGTGCGAGGATCAAAGGGTGCGAGGATCAAAGGGTGG
986	Lefcons	AGATCTTTT CCTTTGAT CTTCTTTT CCTTTGAT CTTCTTTT CCTTTGAT CTTTGGATCCTCGAG
987	Lefcons	TCTAGAAAAGGAAACTAGAAAGAAAGGAAACTAGAAAGAAAGGAAACTAGAAACCTAGGAGC TC
811	LefOtxa	GGATCCCAC CCTTTGAAC CTCGCAC CCTTTGAAC CTCGCAC CCTTTGAAC CTCAGATCT
812	LefOtxa	CCTAGGGTGGGAAACTTGAGCGTGGGAAACTTGAGCGTGGGAAACTTGAGTCTAGA
813	LefOtxb	GGATCCCAC GCTTTGTT CCTCGCAC GCTTTGTT CCTCGCAC GCTTTGTT CCTCAGATCT
814	LefOtxb	CCTAGGGTGCGAAACAAGGAGCGTGCGAAACAAGGAGCGTGCGAAACAAGGAGTCTAGA
1088	Lefnkd	GGATCCCAC GCTTTGTA ACTCGCAC GCTTTGTA ACTCGCAC GCTTTGTA ACTCAGATCT
1089	Lefnkd	CCTAGGGTGCGAAACATTGAGCGTGCGAAACATTGAGCGTGCGAAACATTGAGTCTAGA
1086	Lefmyc	GGATCCCAC CCTTTGAA ACTCGCAC CCTTTGAA ACTCGCAC CCTTTGAA ACTCAGATCT
1087	Lefmyc	CCTAGGGTGGGAAACTTTGAGCGTGGGAAACTTTGAGCGTGGGAAACTTTGAGTCTAGA
883	ZFLefOtxb	GGATCCGCT TAATGATGGGCGCGCTCG CAC CCTTTGTT CCTCAGATCT
884	ZFLefOtxb	CCTAGGCGATTACTACCCGCGCGAGCGTGGGAAACAAGGAGTCTAGA
	pSGFluc/ plucZF24	TAATGATGGGCGCGCTCG x12 or x 24

Figure 5.1.7.4. Schematic presentation of the oligo sequences, which were used for the creation of the reporters above. The Tcf/Lef binding sites are shown in bold and the letters in italic shape represent the binding site for the zinc finger homeodomain protein.

To normalize cell culture data for variations in transfection efficiency and protein expression, a fusion of the cDNA of the truncated Lef variant to Renilla luciferase (Rluc) cDNA was made. For example the construct pKCLefΔ293VP16Rluc was cloned by doing an insertion of Rluc (cutting with NruI and XbaI) at the C-terminus of pKCNLSLefΔ293VP16, which was digested by the enzymes NruI and XbaI. Furthermore, another construct pKCLefΔ293mVP16 was created by an insertion of a fragment containing 3 myc-linkers which was cut by HindIII and EcoRI. The fusion of the 3 myc-linkers should cause a physical connection between the Lef protein and the transactivation domain of VP16, which therefore could freely exhibit its biological effect of transactivation. The second step in this context was then the fusion of Rluc at the N-terminus by a ClaI/Spel digestion to get the construct pKCRlucLefΔ293mVP16. For the expression of Renilla luciferase alone as an internal reference the construct pCMVRluc was created, which originates from the insertion of the Rluc cDNA into the pRL vector (detailed cloning steps see Figure 5.1.7.5).

vector	restriction enzymes	insert	restriction enzymes	construct
pRL-CMV	BamHI	pGemTRLuc	BamHI T7	pCMVRLuc
pKCLefΔ293VP16	NruI XbaI	pRAPL	NruI XbaI	pKCLefΔ293VP16RLuc
pKCLefΔ293VP16	HindIII EcoRI	pGemTmycHindEco	HindIII EcoRI	pKCLefΔ293mVP16
pKCRLucNLS	Clal SpeI	pKCLefΔ293mVP16	Clal SpeI	pKCRLucLefΔ293mVP16

Figure 5.1.7.5. Presentation of the cloning steps for the expression of a fusion protein with a functional Renilla luciferase, in combination with LefΔ293, a myc linker and the transactivation domain of VP16.

Without the C-terminus of β -catenin, including a strong transcriptional transactivation domain, only a weak or a complete loss of transactivation could be observed. In this context, to be able to analyse the interaction between LefΔ293 and β -catenin other strong transactivators had to be used. As a first step the cDNA of the transactivation domain of VP16 (VP16), a component of the herpes simplex virus type 1 virion, important for transcriptional activation of immediate early genes (Cress and Triezenberg, 1991), was added. The constructs for expressing the Lef1 variants LefΔ78VP16 and LefΔ293VP16 were cloned by adding C-terminally VP16. For this in the first case the insert and vector were cut by XhoI and NotI and in the second case by XhoI and EcoRV. For the generation of pKCLefΔ240VP16 the pMC vector of pMCLEfΔ240VP16 was changed to pKC (BglII and EcoRV digestions). To further increase the transactivation, VP16 was replaced by the transactivation domain of p65 (p65TA; Ballard et al. 1992; derived from the NF- κ B a transcription factor). For the design of pKCLefΔ293m65 (expressing a fusion protein of LefΔ293 and p65) an exchange of VP16 (pKCLefΔ293mVP16) by p65TA was done (by cutting with XhoI and SpeI). Subsequently, the resulting construct was inserted into pKCRLucNLS by using Clal and SpeI to get pKCRLucLefΔ293m65. Further cloning of pKCRLucTcf3Δ(320-434)m65 and pKCRLucTcf1Δ(269-398)m65 were performed by using the construct pKCRLucLefΔ293m65 and doing an exchange of LefΔ293 with pGemTTcf3Δ(320-434) or pGemTTcf1Δ(269-398) (cut by Clal and HindIII). The cDNAs of the conserved HMG domains of Tcf3 (positions 320-434) and Tcf1 (positions 269-398) were gained by targeted PCR. In order to compare the binding functions, constructs for expressing fusion proteins, including the Lef1 HMG domain or the zinc finger homeo-domain protein were created. The last version represents a negative control.

The first truncated variant of β -catenin, used together with Lef Δ 293 in experiments, was a version which contains only the Armadillo repeats, called β -cat Δ (134-663) or β -cat Δ . Thus, the characteristics of this protein are the deletion of the N-terminus, being essential for degradation through phosphorylation, carried out by the kinase GSK3 β and of the C-terminus which is normally responsible for transactivation of target genes. The cloning process itself was done by inserting β -cat Δ (134-663) into a pKC vector using the restriction enzymes BamHI and XhoI. To combine the transactivation domain of p65 with β -cat Δ (134-663) to pKC β -cat Δ 65, the vector pKC β -cat Δ (134-663) and the insert pKCRlucLef Δ 293m65 were cut by XhoI and NotI, gaining p65, followed by an insertion into pKC β -cat Δ (134-663). The construct pKCDP β -cat for expression of the full length β -catenin protein with N-terminal point mutations for preventing its degradation (phosphorylations cannot take place) was created by exchanging the DNA of β -cat Δ with DP β -cat. In this context, the vector was digested by Bsp119I and EcoRV and the insert by XbaI and Bsp119I (blunt ends were generated by a Klenow fragment). All cloning steps are listed in Figure 5.1.7.6.

vector	restriction enzymes	insert	restriction enzymes	construct
pKCLef Δ (78-397)	XhoI NotI	pKCGal4-VP16	XhoI NotI	pKCLef Δ 78VP16
pKC	BglII EcoRV	pMCLef240VP16	BglII EcoRV	pKCLef Δ 240VP16
pKCNLSLef Δ 293	XhoI EcoRV	pKCgfpB-VP16	XhoI EcoRV	pKCLef Δ 293VP16
pKCLef Δ 293mVP16	XhoI SpeI	pMCP1TA65	XhoI SpeI	pKCLef Δ 293m65
pKCRlucNLS	Clal SpeI	pKCLef Δ 293m65	Clal SpeI	pKCRlucLef Δ 293m65
pKCLef Δ 293m65	Clal HindIII	pKCLef Δ HMGVP16	Clal HindIII	pKCRlucLef Δ HMGm65
pKCRlucLef Δ 293m65	Clal HindIII	pGemTTcf3 Δ (320-434)	Clal HindIII	pKCRlucTcf3 Δ (320-434)m65
pKCRlucLef Δ 293m65	Clal HindIII	pGemTTcf1 Δ (269-398)	Clal HindIII	pKCRlucTcf1 Δ (269-398)m65
pMC RlucZFb6m	XhoI SpeI	pKC RlucLef Δ 293m65	XhoI SpeI	pMCRlucZFb6m65
pKCATG	BamHI XhoI	pGemT β -cat Δ (134-663)	BamHI XhoI	pKC β -cat Δ (134-663)
pKC β cat Δ (134-663)	XhoI NotI	pKCRlucLef Δ 293m65	XhoI NotI	pKC β -cat Δ 65
pKCDP β -cat Δ	Bsp119I EcoRV	pCMVDP β -cat	XbaI Klen Bsp119I	pKCDP β -cat

Figure 5.1.7.6. Illustration of the cloning steps for the design of constructs, which contain the coding sequences of Renilla luciferase, the functional proteins or the transactivation domains of VP16 or p65 or combinations of them.

Since cotransfection experiments with pKCRlucLef Δ 293m65 together with pKC β -cat Δ did not effect any strong increase, which would have given a hint for an interaction between these domains, the fusion protein pKCRlucLef Δ 293m β cat Δ 65 was cloned. The three myc linkers between these two domains should work as a covalent link and should guarantee free movements of Lef Δ 293 and β -catenin Δ . Furthermore, Lef Δ 293, the myc linker and β -catenin Δ should together simulate the primary high affinity interaction domain in the N-terminus of Lef1. For this purpose, β -cat Δ (134-663) cut by BamHI and XhoI was inserted into the construct pKCRlucLef Δ 293m65. The same strategy was applied to Lef Δ HMG, Tcf3 Δ (320-434) and Tcf1 Δ (269-398), for creating pKCRlucLef Δ HMGm β cat Δ 65, pKCRluc Δ Tcf3(320-434)m β cat Δ 65 and pKCRlucTcf1 Δ (269-398)m β cat Δ 65. Again the zinc finger homeodomain protein was added to β -catenin Δ and p65 for excluding a Tcf/Lef independent effect (cloning steps are listed in Figure 5.1.7.7).

vector	restriction enzymes	insert	restriction enzymes	construct
pKCRlucLef Δ 293m65	BamHI XhoI	pKC β -cat Δ (134-663)	BamHI XhoI	pKCRlucLef Δ 293m β cat Δ 65
pKCRlucLef Δ HMGm65	BamHI XhoI	pKC β -cat Δ (134-663)	BamHI XhoI	pKCRlucLef Δ HMGm β cat Δ 65
pKCRlucTcf3 Δ (320-434)m65	BamHI XhoI	pKC β -cat Δ (134-663)	BamHI XhoI	pKCRlucTcf3 Δ (320-434)m β cat Δ 65
pKCRlucTcf1 Δ (269-398)m65	Clal HindIII	pKC β -cat Δ (134-663)	Clal HindIII	pKCRlucTcf1 Δ (269-398)m β cat Δ 65
pMCRlucZFb6m	BamHI XhoI	pKCRlucLef Δ 293m β cat Δ 65	BamHI XhoI	pMCRlucZFb6m β cat Δ 65

Figure 5.1.7.7. Presentation of the cloning strategies for the synthesis of fusion proteins with Renilla luciferase, Lef Δ 293, Lef Δ HMG, Tcf3(320-434), Tcf1 Δ (269-398), β -catenin Δ and p65.

To further experiments the part of β -cat Δ , which is responsible for the interaction with the HMG box of Lef1, a row of deletion mutants were cloned and again fused to Lef Δ 293 via the myc linker and combined with the transactivation domain of p65. The Armadillo repeats from the C- and/or N-termini were removed. The mutant pKCRlucLef Δ 293m β cat Δ 309N65 was cloned by inserting β -cat Δ (309-663), cut by the restriction enzymes BamHI and XhoI, into pKCRlucLef Δ 293m65. The same was made for pKCRlucLef Δ 293m β cat Δ 518C65, pKCRlucLef Δ 293m β cat Δ 423N65, pKCRlucLef Δ 293m β cat Δ (423-622)65, pKCRlucLef Δ 293m β cat Δ 479N65, pKCRlucLef Δ 293m β cat Δ (423-518)65 and pKCRlucLef Δ 293m β cat Δ 521N65, inserting β -cat Δ (134-518), β -cat Δ (423-663), β -cat Δ (309-622), β -cat Δ (479-663), β -cat Δ (423-518) and β -cat Δ (521-663). After the successful mapping of the interaction between

Lef Δ 293 and the last three C-terminal repeats, additional constructs like pKCRLucLef Δ 293m β cat Δ 544N65, pKCRLucLef Δ 293m β cat Δ 566N65, pKCRLucLef Δ 293m β cat Δ 584N65 and pKCRLucLef Δ 293m β cat Δ 625N65 were designed. They also include the insertions of β -cat Δ (544-663), β -cat Δ (566-663), β -cat Δ (584-663) and β -cat Δ (625-663) into pKCRLucLef Δ 293m65. Also two other members of Tcf/Lef family, Tcf3 and Tcf1 were used, supporting the hypothesis that β -catenin can generally reinforce the DNA binding efficiency of Tcf/Lef. Constructs for expression of fusion proteins consisting of the HMG box and the last C-terminal part of Tcf3 and Tcf1, together with β -cat Δ 65 were designed and generated by inserting β -cat Δ , cut by BamHI and XhoI, into the constructs pKCRLucTcf3 Δ (320-434)m65 or pKCRLucTcf1 Δ (269-398)m65 see Figure 5.1.7.8).

vector	restriction enzymes	insert	restriction enzymes	construct
pKCRLucLef Δ 293m65	BamHI XhoI	pKC β -cat Δ (309-663)	BamHI XhoI	pKCRLucLef Δ 293m β cat Δ 309N65
pKCRLucLef Δ 293m65	BamHI XhoI	pKC β -cat Δ (134-518)	BamHI XhoI	pKCRLucLef Δ 293m β cat Δ 518C65
pKCRLucLef Δ 293m65	BamHI XhoI	pKC β -cat Δ (423-663)	BamHI XhoI	pKCRLucLef Δ 293m β cat Δ 423N65
pKCRLucLef Δ 293m65	BamHI XhoI	pKC β -cat Δ (423-622)	BamHI XhoI	pKCRLucLef Δ 293m β cat Δ (423-622)65
pKCRLucLef Δ 293m65	BamHI XhoI	pKC β -cat Δ (479-663)	BamHI XhoI	pKCRLucLef Δ 293m β cat Δ 479N65
pKCRLucLef Δ 293m65	BamHI XhoI	pKC β -cat Δ (423-518)	BamHI XhoI	pKCRLucLef Δ 293m β cat Δ (423-518)65
pKCRLucLef Δ 293m65	BamHI XhoI	pKC β -cat Δ (521-663)	BamHI XhoI	pKCRLucLef Δ 293m β cat Δ 521N65
pKCRLucLef Δ 293m65	BamHI XhoI	pGemT Δ (544-663)	BamHI XhoI	pKCRLucLef Δ 293m β cat Δ 544N65
pKCRLucLef Δ 293m65	BamHI XhoI	pKC β -cat Δ (566-663)	BamHI XhoI	pKCRLucLef Δ 293m β cat Δ 566N65
pKCRLucLef Δ 293m65	BamHI XhoI	pKC β -cat Δ (584-663)	BamHI XhoI	pKCRLucLef Δ 293m β cat Δ 584N65
pKCRLucLef Δ 293m65	BamHI XhoI	pKC β -cat Δ (625-663)	BamHI XhoI	pKCRLucLef Δ 293m β cat Δ 625N65

Figure 5.1.7.8. Illustration of the cloning strategies for expressing fusion proteins of Lef Δ 293 with the variable β -catenin deletion mutants.

After identifying the critical domains of Lef Δ 293, Tcf3 Δ (320-434) and Tcf1 Δ (269-398), which show an interaction with β -catenin, the same region of Plakoglobin was tested with the same three transcription factors. The sequence of the Armadillo repeats of Plakoglobin is highly conserved in comparison to β -catenin. Therefore Plakoglobin Δ (Pla Δ) and Plakoglobin Δ 473 (Pla Δ 473) were generated by targeted

PCR again. The first region corresponds to all Armadillo repeats and the second one to the Armadillo repeats 7 to 12. The cDNAs of both deletion mutants were again inserted into pKCRlucLefΔ293m65 by cutting with XhoI and EcoRV (all cloning steps listed in table 5.1.7.9).

vector	restriction enzymes	insert	restriction enzymes	construct
pKCRlucLefΔ293m65	EcoRI XhoI	pGemT PlaΔ	XhoI EcoRV	pKCRlucLefΔ293mPlaΔ65
pKCRlucLefΔ293m65	EcoRI XhoI	pGemT PlaΔ473	XhoI EcoRV	pKCRlucLefΔ293mPlaΔ473N65

Figure 5.1.7.9. Presentation of the cloning steps for achieving the expression of Plakoglobin, or a truncated form of Plakoglobin in combination with LefΔ293, Rluc and p65.

Consequently, several point mutations were introduced in the coding sequence of LefΔ293 to be able to determine which amino acid(s) are most important for an interaction between β -catenin and the HMG box of Lef1. The insertion of point mutations resulted in the expression of alanine instead of the original amino acids in exposed positions. Hence a hint for an involvement of several amino acids in the interaction would further support the hypothesis that β -catenin reinforces the DNA binding of Lef1. The point mutations were produced by site-directed mutagenesis. All gained cDNAs of the LefΔ293 mutants were first cloned into the pGemT vector and then inserted into the construct pKCRlucNLS. As a second step, for showing their influence on β -catenin binding, the cDNA of β -cateninΔ was inserted to them to create fusion proteins. All designed and created point mutations of LefΔ293 and also their combination to β -cateninΔ for expressing fusion constructs are displayed in Figure 5.1.7.10.

vector	restriction enzymes	insert	restriction enzymes	construct
pKCRlucNLS	ClaI SpeI	pGemTLefΔ293MIm65	ClaI SpeI	pKCRlucLefΔ293MIm65
pKCRlucLefΔ293MIm65	BamHI XhoI	pGemTβcatΔ(134-663)	BamHI XhoI	pKCRlucLefΔ293MImβcatΔ65
pKCRlucNLS	ClaI SpeI	pGemTLefΔ293MIIm65	ClaI SpeI	pKCRlucLefΔ293MIIm65
pKCRlucLefΔ293MIIm65	BamHI XhoI	pGemTβcatΔ(134-663)	BamHI XhoI	pKCRlucLefΔ293MIImβcatΔ65
pKCRlucNLS	ClaI SpeI	pGemTLefΔ293MIIM65	ClaI SpeI	pKCRlucLefΔ293MIIM65
pKCRlucLefΔ293MIIM65	BamHI XhoI	pGemTβcatΔ(134-663)	BamHI XhoI	pKCRlucLefΔ293MIIMβcatΔ65

pKCRlucNLS	ClaI SpeI	pGemTlucΔ293MIVm65	ClaI SpeI	pKCRlucLefΔ293MIV m65
pKCRlucLefΔ293 MIVm65	BamHI XhoI	pGemTβcatΔ(134-663)	BamHI XhoI	pKCRlucLefΔ293MIV mβcatΔ65
pKCRlucNLS	ClaI SpeI	pGemTlucΔ293MVm65	ClaI SpeI	pKCRlucLefΔ293MV m65
pKCRlucLefΔ293 MVm65	BamHI XhoI	pGemTβcatΔ(134-663)	BamHI XhoI	pKCRlucLefΔ293MV mβcatΔ65
pKCRlucNLS	ClaI SpeI	pGemTlucΔ293MVIm65	ClaI SpeI	pKCRlucLefΔ293MVI m65
pKCRlucLefΔ293 MVIm65	BamHI XhoI	pGemTβcatΔ(134-663)	BamHI XhoI	pKCRlucLefΔ293MVI mβcatΔ65
pKCRlucNLS	ClaI SpeI	pGemTlucΔ293MVIIIm65	ClaI SpeI	pKCRlucLefΔ293MVII m65
pKCRlucLefΔ293 MVIIIm65	BamHI XhoI	pGemTβcatΔ(134-663)	BamHI XhoI	pKCRlucLefΔ293MVII mβcatΔ65
pKCRlucNLS	ClaI SpeI	pGemTlucΔ293MVIIIIm65	ClaI SpeI	pKCRlucLefΔ293MVII Im65
pKCRlucLefΔ293 MVIIIIm65	BamHI XhoI	pGemTβcatΔ(134-663)	BamHI XhoI	pKCRlucLefΔ293MVII ImβcatΔ65
pKCRlucNLS	ClaI SpeI	pGemTlucΔ293MIXm65	ClaI SpeI	pKCRlu- cLef293MIXm65
pKCRlucLefΔ293 MIXm65	BamHI XhoI	pGemTβcatΔ(134-663)	BamHI XhoI	pKCRlucLefΔ293MIX mβcatΔ65
pKCRlucNLS	ClaI SpeI	pGemTlucΔ293MXm65	ClaI SpeI	pKCRlucLefΔ293MX m65
pKCRlucLefΔ293 MXm65	BamHI XhoI	pGemTβcatΔ(134-663)	BamHI XhoI	pKCRlucLefΔ293MX mβcatΔ65
pKCRlucNLS	ClaI SpeI	pGemTlucΔ293MXIm65	ClaI SpeI	pKCRlucLefΔ293MXI m65
pKCRlucLefΔ293 MXIm65	BamHI XhoI	pGemTβcatΔ(134-663)	BamHI XhoI	pKCRlucLefΔ293MXI mβcatΔ65
pKCRlucNLS	ClaI SpeI	pGemTlucΔ293MXIIIm65	ClaI SpeI	pKCRlucLefΔ293XIIIm 65
pKCRlucLefΔ293X IIIm65	BamHI XhoI	pGemTβcatΔ(134-663)	BamHI XhoI	pKCRlucLefΔ293MXII mβcatΔ65
pKCRlucNLS	ClaI SpeI	pGemTlucΔ293MXIIIIm65	ClaI SpeI	pKCRlucLefΔ293XIII m65
pKCRlucLefΔ293X IIIIm65	BamHI XhoI	pGemTβcatΔ(134-663)	BamHI XhoI	pKCRlucLefΔ293MXII ImβcatΔ65

Figure 5.1.7.10. Presentation of the cloning strategies for achieving expression of Lef293 point mutants in combination with β-cateninΔ, Rluc and p65.

For the PNA study, the cloning of pS(GUAS)CgfpLucdNpuro for expressing GFP and luc under the control of a CMV promoter was started by inserting pMCgfpdegCN6 into pS(GUAS)TATA, resulting in pMClucdecN (digestions were done by HindIII and NotI). Meanwhile, pS(GUAS)Cgfpdegluc was generated with the following cloning steps. First pS(GUAS)C was generated by inserting pCS2 into pS(GUAS) (cloning was performed by SalI/T4/BglII, respectively NheI/T4/BglII). Subsequently, pS(GUAS)Cgfpdegluc was constructed by pSHgfpdeg (GFP with ornithine decarboxylase degradation signal for faster degradation) and pMAgfpdegluc (expressing luciferase protein). Before the last cloning step by bringing in the puromycin resistance

(pGemT β -catpuro), the first produced construct pMClucdecN was inserted into pS(GUAS)Cgfpdegluc, resulting in ps(GUAS)CgfpdecN (all involved steps and enzymes are listed in the Figure 5.1.7.11). For more details see Chapter 5.2.1.

vector	restriction enzymes	insert	restriction enzymes	construct
pS(GUAS)TATA	HindIII NotI	pMCgfpdegCN6663	HindIII NotI	pMClucdecN
pS(GUAS)	NheI T4 BamHI	pCS2	Sall T4 BglII	pS(GUAS)C
pS(GUAS)C	EcoRI NotI	pSHgfpdeg	EcoRI NotI	pS(GUAS)Cgfpdeg
pS(GUAS)Cgfpdeg	Eco72I NotI	pMAgfpdegluc	Eco72I NotI	pS(GUAS)Cgfpdegluc
pS(GUAS)Cgfpdegluc	Bsp119I NotI	pMClucdecN	Bsp119I NotI	pS(GUAS)CgfpplucdecN
pS(GUAS)CgfpplucdecN	SacI NotI	pGemT β -catpuro	SacI NotI	pS(GUAS)CgfpplucdNpuro

Figure 5.1.7.11. Presentation of the cloning strategies for expressing pS(GUAS)CgfpdNpuro, which was used for the establishment of a stable 293T cell line.

5.2 Cell culture methods

5.2.1 Cell lines, cell culture reagents and cell transfection assays

In an incubator from Salvis (model Biocenter 2001) under standard conditions at 37°C and 5% CO₂ Human HELA cervix carcinoma cells were kept.

Cell culture reagents:

Cell medium: DMEM high glucose with L-Glutamine and Sodium Pyruvate (PAA, No.E15-843) used with 100x Penicillin/Streptavidin (PAA, No.P11-010) and 10% fetal bovine serum gold (EU-approved; PAA, No.A15-151).

10x PBS: 1.37 M NaCl, 27 mM KCl, 43 mM Na₂HPO₄, 14 M KH₂PO₄, pH 7.4

10xTrypsine (PAA, No.L11-001)

The transfections were performed with PEI (Polyethylenimin) or the highly efficient liposome reaction reagents TransFast (Invitrogen) or Turbofect (Fermentas). Firstly

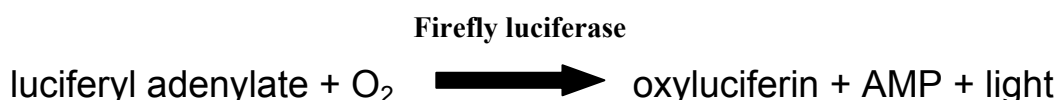
the cells, being confluent up to maximum 80%, in a tissue culture flask (PAA) with filter, were trypsinised and the cell number was determined with a Neubauer counting chamber. Performing experiments in 24 well-plates, together with 0.5 ml medium 0.25×10^5 cells of HELA cells were sowed in each well and subsequently, they were over night incubated to achieve a stable attachment and at least one division per cell, representing normal growth conditions. In the first step the transfection reaction itself was carried out with the addition of 1.6 μ l PEI, 0.8 μ l TransFast or 0.8 μ l Turbofect to 100 μ l serum free medium for each well. Then, the same amount of serum free medium was mixed with the prepared DNA (containing in every batch 400ng). Immediately after this both reaction batches were mixed and incubated for 10 minutes at room temperature. After the complete aspiration of the medium from the cells, the reaction solution was transferred onto the cells as fast as possible. The transfection reaction was stopped after 2 hours with 1 ml medium with serum per well. During the transfection reaction the cells were incubated at 37°C and 5% CO₂ in the incubator. Doing experiments with 96 well format, as a first step, the plates were coated for better adhesion with 30 μ l 2.5 μ g/ml PEI (mw: 750000, pH 7.4 adjusted by 5mM HEPES) per well and incubated for 30 minutes at 37°C. Then the liquid PEI was taken off, each well was washed with 30 μ l PBS and every coated plate was stored coated at 4°C. Subsequently, 0.5×10^4 cells of HELA were sowed in 100 μ l medium and incubated over night. Directly for transfection for each well 0.14 μ l Turbofect in 20 μ l incomplete medium was prepared. In parallel, 20 μ l serum free medium was added to the pipetted 90 ng DNA. Both solutions were mixed and incubated for 30 minutes. After removing the medium, 40 μ l of the DNA-liposome mixture were transferred onto the cells in each well and the transfection reaction was started. This procedure was stopped after 2 hours with 100 μ l medium with serum per well. In both methods a meaningful analysis was possible 24 hours after the starting point of the transfection procedure.

For the establishment of the stable cell line, including pS(GUAS)CgfpLucdNpuro, in 293T cells, 2.1×10^6 were sowed and the 11 μ g of the construct in 3.2ml incomplete medium were transferred onto the cells on the next day, in combination with 25.6 μ l Turbofect in 3.2ml serum free medium. After an incubation time of two hours the reaction was stopped by 10ml. The selection itself was done by adding puromycin in a concentration of 1:1000 and by transferring the grown single clones with the help of

cloning discs from Sigma-Aldrich. As a control group for the GFP and luciferase degradation, the translation blocker 100µl/ml Cycloheximide was added. The knock-down of *gfp* was performed with up to 200µM of the Peptide Nucleic Acids (PNAs) (see also chapter 6.4).

5.2.2 Luciferase reporter assay

Generally reporter gene assays are applied for studies of gene expression, to find out which factors control and how they regulate the expression of a gene of interest e.g. DNA/RNA sequences, transcription factors, RNA-binding proteins, signal transduction pathways and specific stimuli (Recchia et al. 2008). Especially they are used for identification of a gene reporter region or specific elements within a promoter, like transcription factor binding sites or regulatory elements. Well known and commercially available reporter gene assays are chloramphenicol transferase (CAT), β galactosidase (β -gal), secreted alkaline phosphatase and luciferases or fluorescent proteins. The intercellular enzyme firefly luciferase, derived from *Photinus* a terrestrial Coleoptera, is capable of bioluminescence utilizing D-luciferin as substrate. The resulting light is produced by the oxidation of luciferin, involving Adenosintriphosphate (ATP). Firstly luciferyl adenylate is produced by ATP addition following oxidation to oxyluciferin catalyzed by luciferase and production of light flashes, which then can be measured by luminometers (Penzlin et al., 1996).



In this study to be able to measure quantitatively the activity of firefly luciferase in response to the activity of the canonical Wnt signaling pathway, following firefly luciferase reporters with different Tcf/Lef binding sites were used: pLefluc, plucF3Lef, plucF6Lef, plucF6Top, pMlucF6Lefcons, pMlucF16OtxFMcore, plucF6LefOtxb,

pMlucF24LefOtxb, pMlucF24LefOtxa, plucF4LefOtxab, plucF8LefOtxab, pMlucF12LefOtxab, pMlucF24LefOtxab, pMlucF24Lefnkd, pMyc5'3'luc, pMlucF24Lefmyc, plucF24ZFLefOtxb, plucAxin2 (design described in Chapter 4.1.7). For the cell lysis, done like the transfection in 24 well plates, medium was removed and in each well 100 μ l of lysis buffer was added. The incubation time for the lysis buffer was 10-15 minutes. Afterwards 50 μ l of the liquid solution of each well was quantitatively measured with the luminometer Lumat LB 9507 from EG&G Berthold with 100 μ l injection solution I and 100 μ l injection solution II. In the case of using the 96 well microplates for performing the experiments, in every well 10 μ l of the lysis buffer was given onto the cells. Subsequently, the plate was strongly shaken (3600 rpm) for 10 minutes. The actual measurements were done with the Luminoskan Ascent microplate luminometer from Thermo scientific. For detection 40 μ l of the ATP/luc reagent was injected in each well and after measuring the luciferase reaction was stopped by 100 μ l 0.33M HCl.

Firefly luciferase reporter assay materials:

24 well plate:

lysis buffer: 25mM Tris, 0.03% Triton X-100, 1mM DTT (freshly added)

injection solution I: (100 μ l for 1 reaction + 600 μ l for initial wash):

for 3 ml: 60 μ l 10 mM luciferin and 60 μ l 1 M Tris pH 7.5 in H₂O dest.

injection solution II: (100 μ l for 1 reaction + 600 μ l for initial wash):

for 3 ml: 75 μ l 0.2 M ATP pH 7.0; 75 μ l 1 M Tris pH 7.5; 45 μ l 1 M MgCl₂ in H₂O dest.

96 well plate:

lysis buffer: see 24 well format

injection solution (ATP/luc reagent): 50 μ l 10mM Luciferin, 62.5 μ l 0.2M ATP, 31.25 μ l 1M Tris pH 7.5, 46.88 μ l 1M MgCl₂, 4.8 ml H₂O

5.2.3 Renilla luciferase (Rluc) assay

The intracellularly synthesized enzyme Renilla luciferase (Rluc) is produced by the soft coral *Renilla* for light emission using the substrate Coelenterazine, which is converted to Coelenteramide (Matthews et al., 1977). Often Rluc is cotransfected on a separate plasmid or can also be fused to proteins having an interne control of transfection efficiency, cell number, cell viability and protein synthesis. That means that Renilla Luciferase activity is proportional to the amount of firefly luciferase-encoding plasmid taken up by the cells.

Detection of Renilla luciferase activity was always carried out directly after the measurement of the firefly luciferase activity (also with the same lysis buffer). For this purpose 10 µl of the cell lysate from the firefly luciferase reporter assays was measured with the luminometer in 24 well format as well as in 96 well format experiments by injecting 100 µl, respectively 40 µl Rluc reagent. But, in the second case the cell lysis was performed with 25 µl lysis buffer and then the cell solution for the Renilla luciferase measurement was transferred well per well into another empty 96 well plate. To avoid signal inhibition through medium with serum, before the transfer the medium was sucked off and every well was carefully washed with 30 µl PBS.

Renilla luciferase reporter assay materials:

lysis buffer: same as for firefly luciferase

injection solution (Rluc reagent): 3.125 µl Coelenterazine, 25 µl 0.5M EDTA, 31.25 µl 1M Tris pH 7.5, 4.9 ml H₂O

5.3 Peptide nucleic acid (PNA) application and preparation

In course of the establishment of specific PNAs being an effective knock down medium for certain genes in Medaka fish, the chemical properties of the PNAs must be changed to improve their abilities in membrane permeability, solubility and DNA binding. In order to do this, the length of the molecules was varied, ranging from 12 to 17meres, leading to better DNA hybridization. The DNA binding could be further im-

proved by synthesizing a mixed backbone, consisting of Nielsen PNA elements (N-(2-aminoethyl)-glycine units) in the middle parts and ugiPNA elements (Ubichem PNA elements) in border molecular areas. In addition, the diffusion properties could be advanced by these changes. The problem of low solubility could also be largely eliminated by adding a fourfold trimethyl-lysine group. The distribution of ugiPNAs in 293T cells and into intestine bordering cells of Medaka fish embryos could be demonstrated by FITC and TexasRed labelled PNAs (further details in Chapter 6.2).

5.3.1 PNA design for gene targeting in Medaka

Based on the chemical modifications, which are described above, several PNAs were designed and synthesized by our cooperation partner Ubichem GmbH, for testing their ability for an efficient knock-down of target genes in Medaka. Firstly, their anti-sense potential could be demonstrated by an efficient degradation of *gfp* mRNA. For this, as a first step, in consideration of the known knockdown competence of morpholinos, one molecule for GFP, comprising 25 units, was designed targeting the 5' region of mRNA. In course of this experiment, *gfp* mRNA was synthesized by in vitro transcription. Firstly, the GFP expressing plasmid was digested over night by *Apal* for linearization. Subsequently, the in vitro transcription was performed by the Ampli Cap Mix T7 High Yield Message Marker Kit (Ambion). The batch comprises 3 µl 500ng digested DNA, 4 µl Cap-d-NTP- Mix, 1 µl DTT, 1 µl T7 polymerase and 1 µl 10x T7-Buffer. After performing the procedure for 2 hours at 37°C, 1 µl DNase was added for removing the templates. Subsequently, the reaction was completely stopped, respectively RNA precipitated by 30 µl nuclease free water and 30 µl LiCl. Immediately the reaction batch was mixed and stored for approximately 30 minutes at -20°C, following centrifugation for 15 minutes at 4°C at maximum speed. After removing the supernatant, the pelleted DNA was washed with 1ml 70% ethanol by recentrifugation and then 50 µl ddH₂O was added and the entire batch freezed by -70°C. The extraction was started by adding 115 µl nuclease free water and 15 µl Ammonium Acetate. Subsequently, the real extraction was performed by an equal volume of phenol/chloroform solution and then by an equal volume of chloroform. The precipitation was done by adding 1 volume isopropanol and thorough mixing. Then, the mixture was stored at least 15 minutes at -20°C and centrifuged for 15 minutes at 4°C with maximum speed to pellet the RNA. Finally, the pellet was resuspended in 50 µl water

and stored at -20°C. The produced *gfp* mRNA was quantified on a 1% Agarose gel and immediately frozen at -20°C. For injection, it was carefully thawed and mixed with RNase inhibitor and DEPC water as a control group (for details see Chapter 5.3.2).

The preparation of the PNAs was carried out by dissolving them in nuclear free water, in combination with shaking and vortexing. These steps have to be repeated several times in order to get them completely in solution. A treatment with a sonicator is also recommended. Subsequently, the PNAs were frozen in aliquots of 100 µl with a concentration of 2mM at -80°C. Before injection, the PNAs were added to the Gfp mix and to DEPC water (plus RNase inhibitor, Fermentas) on ice. Generally, the supplement of RNase inhibitor should prevent a premature degradation of the *gfp* mRNA (for the exact procedure and the reaction batch, see Chapter 5.3.2).

Details for the quantification of PNA concentrations and the whole-mount insitu hybridizations in the embryos for the analysis of changed gene expression levels due to Six3 or Tcf3 knock downs are explained in the attached manuscript in the appendix.

5.3.2 Medaka injection

5.3.2.1 Preparations

All injections were done at the embryo's first or second cell stages directly in one Blastomere. The embryos derived from the Medaka Cab inbred strain. Therefore one day before injection male and female were separated to be able to get on the next day embryos at the 1-cell stage (30 minutes after mating).

To be able to immobilize the embryos an agarose coated Petri dish (1.5% agarose in 1x Yamamoto buffer) with furrows (addition of a mold to the liquid agarose forms the furrows) was casted. After cooling down the plate it was stored at 4°C and precooled 1x Yamamoto was added directly before injection. Then the tips of the borosilicate injection needles (GC100-10; Harvard No. 30-0016) were pulled with a Flaming/Brown Micropipette Puller (model P-97; Sutter instrument Co).

5.3.2.2 Fish mating

For mating 1 male fish was added to each fish tank containing up to 3 females (1 hour after feeding/breakfast is recommended). Then for the mating process itself the fish room was kept empty for 30 minutes. After this time the eggs could be collected from the females with the help of an inoculating loop. For this purpose the egg clusters were carefully pelt from the belly and transferred into a pre-cooled Petri dish containing pre-cooled 1x ERM (through this, the embryo's development is arrested/slowed down for 30-60 minutes).

5.3.2.3 Injection process

Firstly the eggs were separated through gently rolling them on a Whatman paper. The remaining traces of attachment filaments could be manually removed by using tweezers and a dissecting microscope. Subsequently the separated embryos were transferred into the prepared pre-cooled Petri dish containing agarose and the eggs were arranged into the furrows. After filling in the injection mix into the needle with the help of an Eppendorf microloader tip the needle tip was opened in the ERM buffer by touching with fine tweezers. Injection with the Microinjector Femtojet (Eppendorf) was done with an injection pressure of 150-600 hPa and a back-hold pressure of 50-150 hPa. Around 1/6 of the cell volume was injected into the cytoplasm of one blastomere of the early embryo. 1x Yamamoto buffer was replaced with pre-warmed 1xERM after injection and the embryos were incubated at 27°C (17°C for slower development).

Materials for injection:

Injection solution (Gfp mix) for *gfp* mRNA injection:

1.15 µl mRNA *gfp* (10-20 ng/µl)
0.4 µl RNase inhibitor (2 units/µl)
3.5 µl DEPC water

Injection solution for *gfp* mRNA- PNA injection:

1 µl mRNA Gfp mix

1 μ l PNA (50- 600 μ M endconc.)
3 μ l DEPC water + RNase inhibitor

Injection solution for PNA oligo injection:

1xYamamoto buffer
50-1200 μ M PNA
ad 5 μ l with ddH₂O

Injection solution for morpholino oligo injection:

0.5 μ l 10xYamamoto
10-1500 μ M morpholino
ad 5 μ l with autoclaved MilliQ water

Further solutions for oligo injections:

1xERM:

17 mM NaCl
0.4 mM KCl
1.0 mM CaCl₂ x H₂O
0.65 mM MgSO₄ x H₂O

10xYamamoto:

1.28 M NaCl
27 mM KCl
14 mM CaCl₂
2.4 mM NaHCO₃
adjust to pH 7.3
(diluted with water to
1xYamamoto)

6. Results

6.1 Indication for a second β -catenin Tcf/Lef interaction domain

6.1.1 Effects of β -catenin on Lef1 and truncated Lef1 forms

The high affinity interaction between β -catenin and Tcf/Lef involves the core region of β -catenin, consisting of Armadillo repeats 3-10 (Huber et al., 1997, Graham et al., 2000) and the first 65-69 amino acids of Tcf/Lef (Behrens et al., 1996, Van de Wetering et al., 1997, Molenaar et al., 1996). In detail, the third helices of each of the 12 Armadillo repeats (a 42-amino acid sequence motif fits one Armadillo repeat) forming a superhelix of helices, compose a long positively charged groove (Huber et al., 1997, Graham et al., 2000). During interaction along this groove the Tcf/Lef β -catenin binding domain is located in an antiparallel direction. Daniels and Weis (Daniels and Weis, 2005) could also provide experimental data indicating a second possible β -catenin interaction domain of Lef1, positioned at the C-terminus (amino acids 265-397, containing the HMG box), but it was not mapped exactly. The binding of this second interaction domain of β -catenin to Lef1 could only be shown by using high amounts of β -catenin, indicating that this interaction has a very low affinity. But in this study the authors could not further analyze this potential second interaction, because the C-terminal region of Lef1 could not be purified without DNA. However, in 2006 experiments done by Cornelia Dworak suggested that this interaction domain might be positioned at the C-terminal part of Lef1 (amino acids 293-397; Dworak, 2006). I repeated these experiments as a starting point for my thesis. I used deletion variants of Lef1, lacking the N-terminal β -catenin binding domain, in combination with the transactivation domain of VP16. One construct, named Lef Δ 78 contained the amino acids 78 to 397 of Lef1, including the interaction domain of the inhibitor of the caspase-activated nuclease (ICAD) and the HMG box, but not the β -catenin binding domain. Usually ICAD hinders the function of the caspase activated DNA nuclease, but lost its function in case of apoptosis. Another deletion variant Lef Δ 240 included the amino acids 240 to 397, which consisted of the HMG domain with a short additional N-terminal and the whole C-terminal part of Lef1. Finally, the shortest protein form Lef Δ 293 included the HMG domain and the remaining C-terminal part (all Lef deletion

versions are illustrated in Figure 6.1.1.1). The transactivation domain of the Herpes simplex virus type 1, VP16 protein was C-terminally fused to the different Lef deletion variants for achieving a higher basal activation, independent of β -catenin. This component mediates transcriptional activation of viral immediate early genes (Cress and Triezenberg, 1991). For detection of the transcriptional activities of the Lef-VP16 fusion proteins the reporter construct pLefluc, which consists of Tcf/Lef DNA binding sites, a minimal Fos promoter (Fisch et al., 1989) and a firefly luciferase gene, was used. The reporter contains seven high affinity Tcf/Lef consensus binding sites and was developed by Grosschedl and colleagues (Hsu et al., 1998). Generally, the firefly luciferase is often used for the detection of certain transcriptional levels. The luciferase itself displays an oxidative enzyme, which consists of only one 61kDa unit. In course of the biochemical process it interacts with the substrate D-luciferin for producing bioluminescence, which can be detected by luciferase analyzers. An essential reason for the usage of this reporter assay is the fact that the enzyme can properly work without any post-translational processing (deWet et al., 1985). In addition, with certainty any cross-reaction, respectively interference can be excluded.

Upon binding of Lef1, Lef1 Δ 78, Lef Δ 240 or Lef Δ 293 different luciferase levels were obtained (Figure 6.1.1.2). The experiments were done in human HELA cells by co-transfecting the expression vectors (driven by the strong human cytomegalovirus promoter = CMV promoter, being highly active in vertebrate cells; Foecking and Hofstetter, 1986) for the different Lef deletions in combination with the luciferase reporter construct pLefluc and the β -catenin expressing plasmid pKCDP β cat. The β -catenin cDNA of this vector includes N-terminal point mutations for avoiding its phosphorylation and degradation (Morin et al., 1997). After 24 hours the absolute firefly luciferase expression levels RLU (luc) were measured and the relative induction calculated in relation to the cells, which were only transfected with the reporter plasmid. In this experiment, the truncated protein Lef Δ 293 was still activated by β -catenin, showing a weak but clear luciferase induction with the luciferase reporter pLefluc.

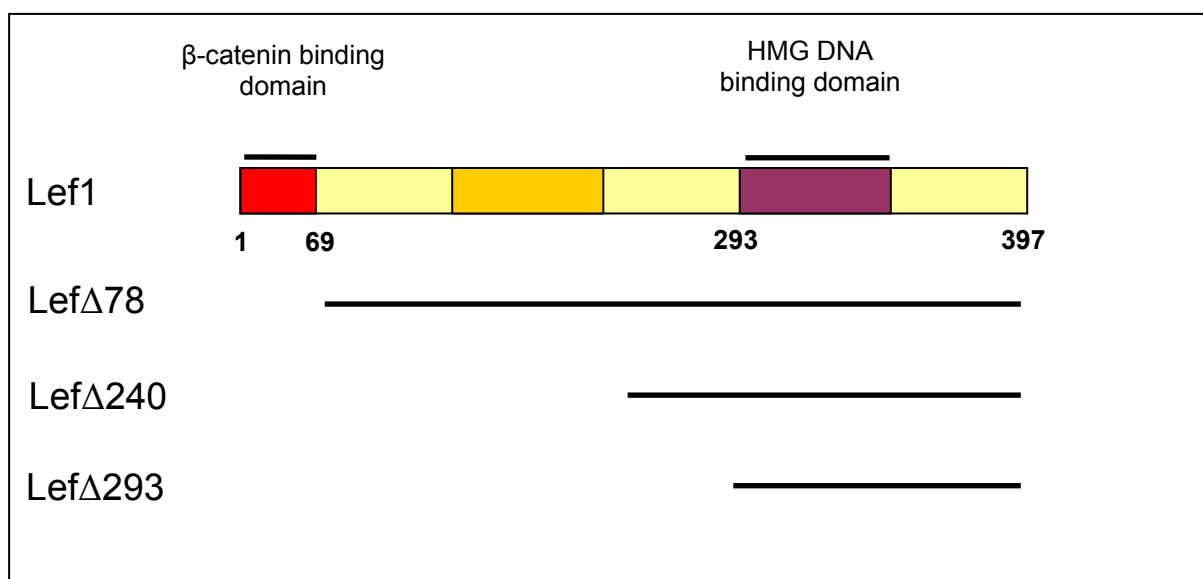


Figure 6.1.1.1. The different Lef protein variants, which include full length Lef1 or the proteins without the β-catenin binding domain (LefΔ78) and the interaction domain of the inhibitor of the caspase-activated nuclease (ICAD, yellow) (LefΔ240). The shortest version only encompasses the HMG DNA binding domain with the remaining C-terminus (LefΔ293).

Cell line: HELA

Format: 24 well

Reporter: 300ng pLefluc

Background (luc): 243 RLU

	0	1ng pKCLef1	1ng pKCLefΔ78VP16	1ng pKCLefΔ240VP16	1ng pKCLefΔ293VP16
RLU (luc)	5801	9227	1375308	71260	11227
RLU (luc) + 30ng DPβcat		425821	5168938	536221	176586
relative luc activity	1.0	46.1	3.8	7.5	15.7

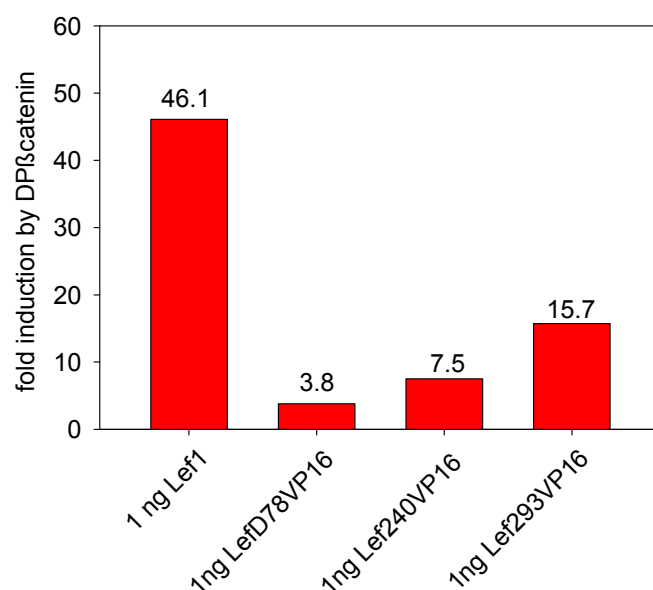


Figure 6.1.1.2. Indication for a second Lef1- β -catenin binding domain, located in the HMG box. In cotransfections 1ng of pKCLef Δ 78VP16 (lacking the β -catenin binding domain), pKCLef Δ 240VP16 (amino acids 240 to 397) and pKCLef Δ 293VP16 (amino acids 293 to 397), which are representing different deletion mutants fused to the transactivation domain of VP16, were combined with 300ng of the luciferase expressing reporter pLefluc (contains seven Tcf/Lef binding sites) and 30ng of pKCDP β cat (full length β -catenin) in HELA cells. The background (luc) data were calculated from four luciferase measurements of the lysis buffer. The values RLU (luc) represent an average of four luciferase measurements subtracted by the background (luc). Both calculation schemes were applied to all following experiments in the 24 well format. The luciferase induction caused by the expression of DP β -cat (relative luc activity) was calculated relative to cells only transfected with the reporter pLefluc (see text for more details). The names of the fusion proteins can include a "D" instead of " Δ ".

This finding indicates the existence of a potential interaction of β -catenin within the HMG domain of Lef1 (see experiment shown in Figure 6.1.1.2). The HMG domain is unstructured in solution but acquires its structure when bound to DNA (Love et al., 2004). Since β -catenin seems to bind to the HMG domain-DNA complex it might shift the equilibrium between unstructured and structured state to the structured one. This would result in an enhanced affinity for DNA. Therefore, the main part of my thesis was to identify and characterize the interaction domain of β -catenin, with the HMG box of Tcf/Lef factors.

6.1.2 The transactivation domain of VP16 improves, but another transactivation domain of p65 further improves the transactivation of Lef1

Lef1 can only work as a transcriptional transactivator of Wnt target genes through the cooperation and interaction with β -catenin, which binds several coactivators C-terminally, effecting transcriptional initiation and elongation. In order to study the influence of β -catenin on the DNA binding of Lef1 and the additional interaction between β -catenin and Lef1, I used the truncated Lef1 protein Lef Δ 293, which contains the HMG domain, but lacks the primary β -catenin binding site (see schematical illustration in Figure 6.1.1.1). Thus, the normal transactivation caused by β -catenin does not work. Therefore, this Lef1 deletion mutant lacking the β -catenin binding site had to be shifted into an activated state by adding a transactivation domain similar to the first experiments. To further increase the transactivation, the VP16 transactivation domain was replaced by the transactivation domain of p65 (p65TA), which is involved in cell proliferation, cell death and immune response (Ballard et al., 1992). A significantly higher potential for this transactivation domain could be shown in luciferase induction experiments (Rivera, 1996). The p65 transactivation domain is a part of the

subunit p65 of the human transcription factor NF- κ B, which leads to very strong upregulation processes and is often used to increase low transcription levels. The application of a transactivation domain with a stronger transactivation potential than that of β -catenin should lead to a stronger transcriptional response in the experiments. In this context, an increased binding efficiency of Lef1 through β -catenin interaction should become visible. In Figure 6.1.2.1 the interaction of β -catenin with Lef1 and the potential interaction between β -catenin and the Lef HMG box, detected by the transactivation domain p65 are schematically shown.

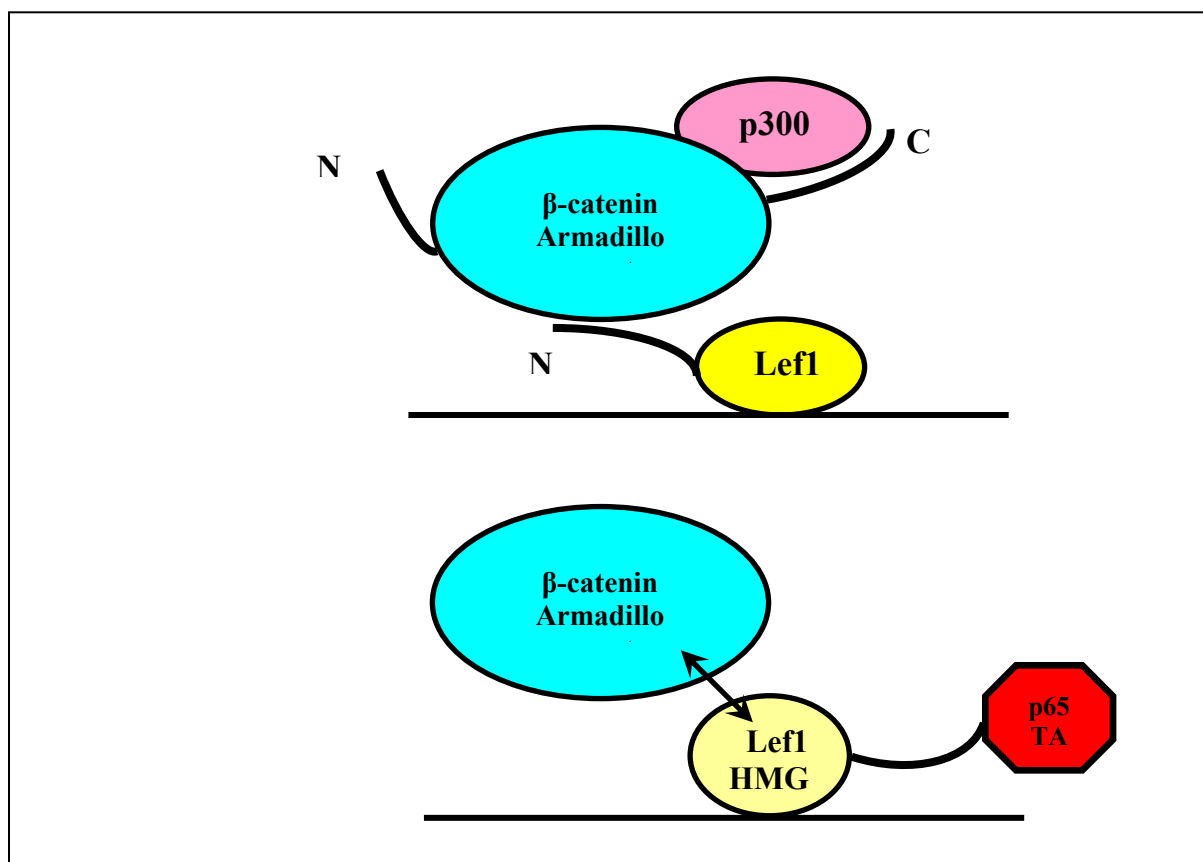


Figure 6.1.2.1. Schematic view of the interactions of the Armadillo repeats of β -catenin with Lef1 via its N-terminus and probably via its HMG box fused to p65. The Armadillo repeats of β -catenin are shown in blue and Lef1 in yellow. The N-termini (N) of β -catenin and Lef1 and the C-terminus of β -catenin (C) are indicated by lines. The pink colored cycle represent the protein p300, which interacts with the Armadillo repeats 10 to 12 and the C-terminus of β -catenin. It fulfills its function as a transcriptional coactivator by catalyzing the acetylation of histones. The transactivation domain of p65 (p65TA) is shown in red. In order to detect a possible β -catenin-Lef interaction, the p65TA is fused to the Lef1 HMG domain. The possible weak interaction between the Armadillo repeats of β -catenin and Lef HMG is represented by a double arrow.

In the first experiments the transactivation domain of VP16 was used. In order to improve transactivation, p65 was C-terminally fused to the Lef Δ 293 protein and cotransfected with the reporter pLefluc into HELA cells (see experiment shown in Figure

6.1.2.2) (the cloning strategies and transfection procedures are explained in Chapters 5.1.7 and 5.2.).

Cell line: HELA

Format: 24 well

Reporter: 300ng pLefluc

Background (luc): 200 RLU

	0	1ng pKCLefΔ293VP16
RLU (luc)	796	23685
relative luc activity	1.0	39.0

Cell line: HELA

Format: 24 well

Reporter: 300ng pLefluc

Background (luc): 406 RLU

	0	1ng pKCLefΔ293m65
RLU (luc)	951	282876
relative luc activity	1.0	519.0

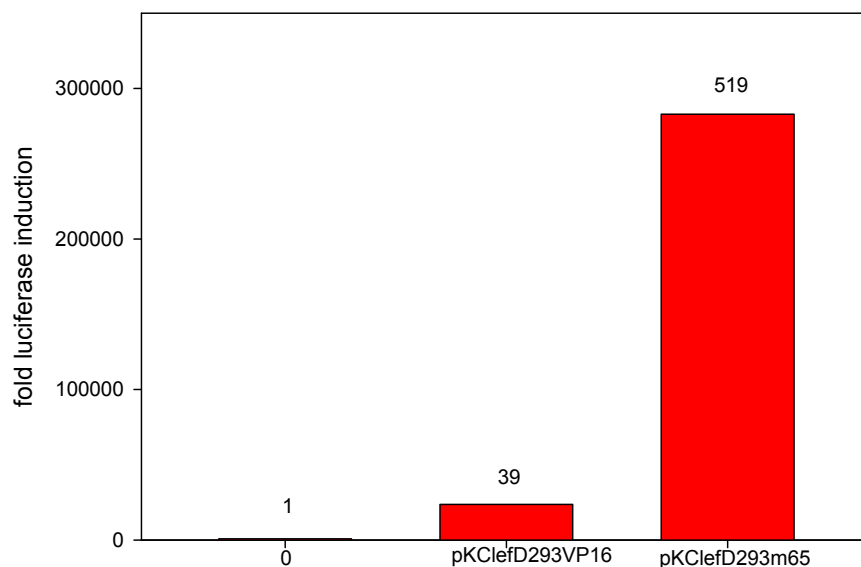


Figure 6.1.2.2. Comparison of the transactivation domains of VP16 and p65. The Lef deletion mutant LefΔ293 (amino acids 293 to 397, including HMG domain) fused to the transactivation domains of VP16 or p65, was overexpressed. The expression of both fusion proteins led to pLefluc luciferase induction levels which were analyzed relative to cells only transfected with the reporter. For transfections all DNA amounts were used as indicated. The names of the constructs can include a "D" instead of "Δ".

The application of the transactivation domain of p65 showed a 11.9 fold higher relative reporter activation compared to the activation of the VP16 transactivation domain (Figure 6.1.2.2). Therefore the transactivation domain of p65 was selected for further experiments.

6.1.3 Renilla luciferase fusion constructs for normalization and sensitive quantification of protein expression levels

In all experiments which were performed so far, a reliable conclusion about an improved DNA binding of specific constructs mediated by reporters could not be done. Parameters like transfection efficiency, expression levels and protein stability could not be regarded. For elimination of these effects, the cDNA of the enzyme *Renilla* luciferase (Rluc) was fused in frame to the Lef constructs. This should allow an exact quantification of the amount of the Lef protein within the cell (for further details see Chapters 5.1.7 and 5.2.3). The intracellularly expressed enzyme *Renilla* luciferase (Rluc) originates from the soft coral *Renilla* for light emission using the substrate Coelenterazine, which is converted to Coelenteramide (see Figure 6.1.3.1) (Matthews et al., 1977). The enzyme consists of just one molecule with a molecular weight of 36kDa. Like the firefly luciferase it displays its activity without post-translational processings. Often Rluc is cotransfected on a separate plasmid as an internal control for transfection efficiency, cell number, cell viability and protein synthesis. Here, a direct fusion with Lef1 was used. The ratio of the amount of the produced reporter protein (firefly luciferase activity) to the activity of the *Renilla* luciferase of the fusion proteins was used for normalization and accurate quantification of the actual protein expression levels.

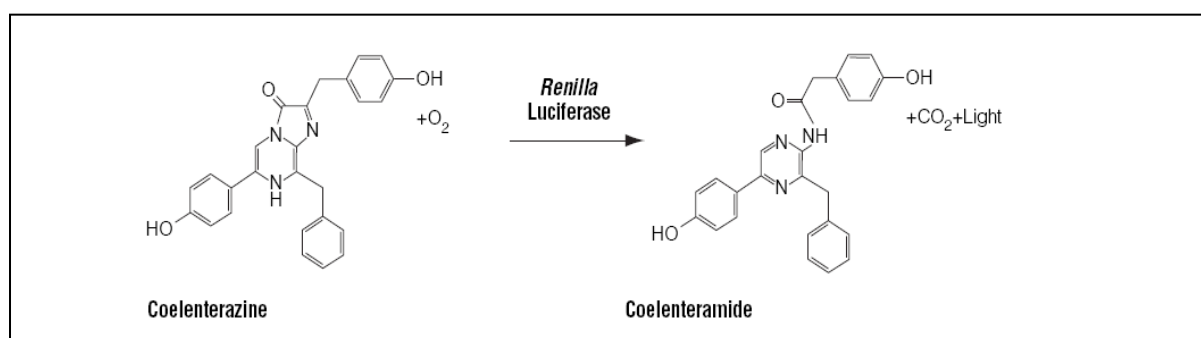


Figure 6.1.3.1. *Renilla* luciferase converts its substrate Coelenterazine into Coelenteramide, CO_2 and light (from Penzlin et al., 1996).

As a first step the Renilla luciferase cDNA was fused to the C-terminus of Lef Δ 293VP16 (for detail of the cloning procedures see Chapter 5.1.7). The constructs pKCLef Δ 293VP16 and pKCLef Δ 293VP16Rluc were used for cotransfections to analyze their transactivation potential and their expression levels in the following experiments (see Figures 6.1.3.2 and 6.1.3.3) within 293T or HELA cells.

Cell line: 293T			
Format: 24 well			
Reporter: 30ng pLefluc			
Background (luc): 260 RLU			
Background (Rluc): 541 RLU			
	0	10ng pKCLef Δ 293VP16	10ng pKCLef Δ 293VP16Rluc
RLU (luc)	41616	3856415	61779
relative luc activity	1.0	93.0	1.0
RLU (Rluc)		542	545

Cell line: HELA			
Format: 24 well			
Reporter: 300ng pLefluc			
Background (luc): 290 RLU			
Background (Rluc): 327 RLU			
	0	10ng pKCLef Δ 293VP16	10ng pKCLef Δ 293VP16Rluc
RLU (luc)	1715	19302	5599
relative luc activity	1.0	11.3	3.3
RLU (Rluc)		289	292

Figure 6.1.3.2. Impact of the expression of C-terminal Rluc fusion constructs on luciferase activity. The fusion proteins Lef Δ 293VP16 or Lef Δ 293VP16Rluc, consisting of Lef Δ 293, VP16 and selectively of the Renilla luciferase (C- terminally added) were overexpressed in 293T or HELA cells, respectively. For detection 30ng or 300ng of the reporter pLefluc were transfected. The transfected DNA amounts are declared in the tables. The background (luc) and (Rluc) data represent the mean of four measurements of the lysis buffer (luc) or the cell extract transfected only with the pLefluc reporter (Rluc). The values RLU (luc) and RLU (Rluc) represent an average of four firefly or Renilla luciferase measurements subtracted by background (luc) or (Rluc). This calculation schemes were applied to all following experiments in 24 well format. The luciferase inductions caused by the expression of both fusion proteins were calculated relative to cells only transfected with the reporter.

The construct with the truncated protein Lef Δ 293, C-terminally fused with VP16, was cotransfected with the reporter pLefluc. High reporter activations could be observed (93 fold with 293T cells and 11.3 fold with HELA cells; Figure 6.1.3.2). Using the fusion constructs, containing Renilla luciferase at the C-terminus, the reporter activity

did not exceed the basal level of the reporter in 293T cells and in HELA cells, indicating that the binding affinity was strongly diminished by a changed protein conformation. In both cases no Renilla luciferase activity could be measured. A possible explanation for this could also be a modification of the protein conformation, leading to a defective function of Renilla luciferase. Therefore the enzyme was next fused to the N-terminus of Lef Δ 293 instead of the C-terminus. For analysis of the transcriptional activity of the Lef proteins the firefly luciferase reporter plucF3Lef with three Tcf/Lef binding sites was used (all cloning procedures in Chapter 5.1.7).

Cell line: HELA				
Format: 24 well				
Reporter: 300ng plucF3Lef				
Background (luc): 260 RLU				
Background (Rluc): 311				
	0	10ng pKCRlucLef Δ 293VP16	10ng pKCLef Δ 293m65	10ng pKCRlucLef Δ 293m65
RLU (luc)	1573	9610	18925	29150
relative luc activity	1.0	7.1	14.2	22.0
RLU (Rluc)		35856	315	71885

Figure 6.1.3.3. Testing N-terminal Rluc fusion constructs. The deletion mutant Lef Δ 293 was optionally fused to the transactivation domains of VP16 or p65 and N-terminally to the Renilla luciferase, resulting in expression of the fusion proteins RlucLef Δ 293VP16 and RlucLef Δ 293m65. In cotransfections 10ng of the constructs were combined with 300ng of the reporter pLucF3Lef (contains three Tcf/Lef binding motifs). The luciferase/Renilla luciferase inductions caused by the expression of all three fusion proteins were analyzed relative to cells only transfected with the reporter (see text for more details).

In experiment 6.1.3.3 (Figure 6.1.3.3), the functionality of the Renilla luciferase as part of the fusion proteins RlucLef Δ 293VP16 and RlucLef Δ 293m65 could be demonstrated by the detection of high values of firefly luciferase, produced by the activated plucF3lef reporter. This means that the Lef protein was able to bind to and activate the reporter similarly compared to the construct lacking Renilla luciferase. The Renilla luciferase fusion worked for both the VP16 and the p65 fusion constructs. However, this experiment again showed higher activities for the transactivation domain of p65 in comparison to VP16. Putting all results together, a reliable quantification of the expression levels of Lef proteins was possible by using Lef constructs with a N-terminal fusion of Rluc.

6.1.4 An effect of the Armadillo repeats comprising part of β -catenin on Lef/Tcf activation

As mentioned before, β -catenin represents the most important transactivator protein of the canonical Wnt signaling pathway. After accumulated in the cytoplasm it is shuttled into the nucleus, where it interacts with Tcf/Lef to activate target genes (Arce et al., 2006; Brunner et al., 1997). The protein can be divided into an N-terminal part, which can be phosphorylated, resulting in degradation, a C-terminal region with binding sites for other proteins which are involved in transactivation and a middle region, consisting of 12 Armadillo repeats for the primary interaction with Tcf/Lef. However, in this study we were interested in the interaction between β -catenin and Lef1. Therefore a deletion protein β -cat Δ (pKC β -cat Δ) was designed, comprising only of the 12 Armadillo repeats of β -catenin (exact design in Chapter 5.1.7). The resulting protein is stabilized by the loss of the N-terminus. Additionally, any possible effect of C-terminal partners on the interaction between β -catenin and Lef Δ 293 could be excluded too. In the following experiments in addition to the interaction of β -cat Δ with Lef1, also interactions of β -cat Δ with Tcf3 and Tcf1 were examined (the linear structures of all three proteins are illustrated in Figure 6.1.4.1).

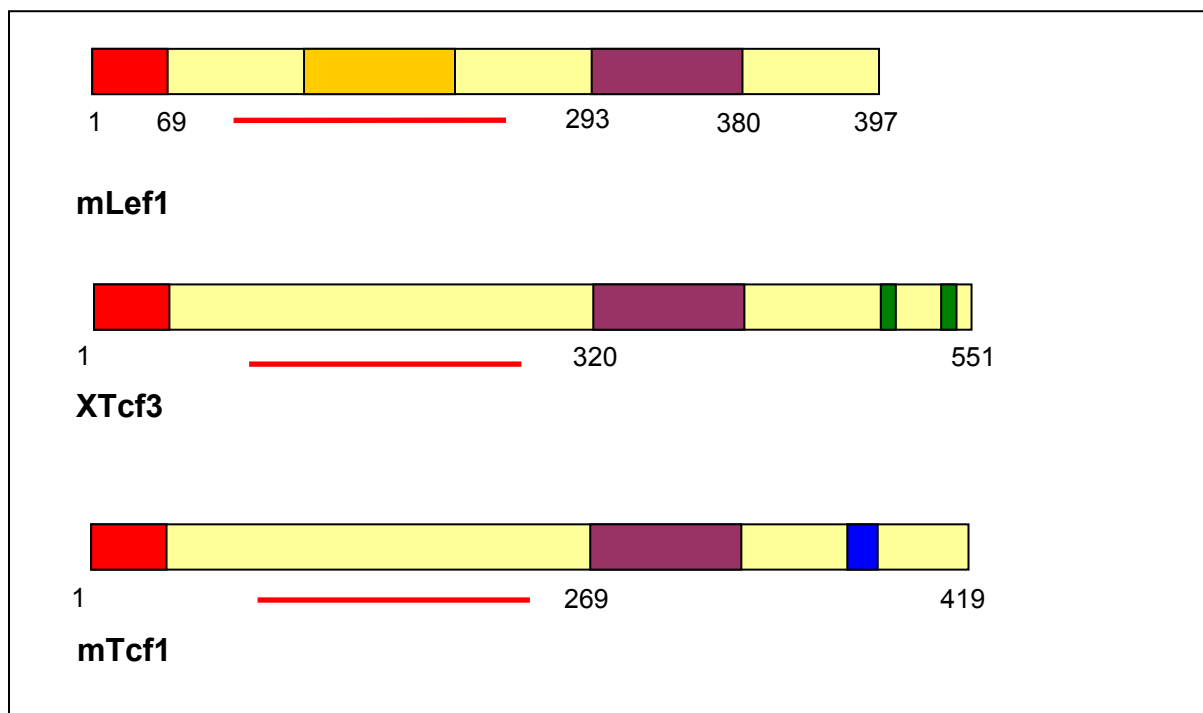


Figure 6.1.4.1. The murine proteins Lef1, Tcf1 and the *Xenopus* protein Tcf3 with their N-terminal highly conserved β -catenin binding sites (red), their also highly conserved HMG domains (violet), near

the C-terminus and their Groucho/TLE interaction sites (red lines). Groucho or TLE proteins represent typical corepressors, which fulfill several functions during developmental processes. In addition, the Lef1 protein includes a region for the interaction with the inhibitor of the caspase-activated nuclease (ICAD) (yellow), whose function gets lost in case of apoptosis. The binding domains for the corepressor CtBP (C-terminal binding protein) are shown in green in Tcf3 and in Tcf1 the conserved CRARF domain (blue), which is responsible for activation of the Lef1 promoter and additional DNA binding (it is also found in the Tcf4 protein).

Cell line: 293T

Format: 24 well

Reporter: 300ng plucF6Lef

Background (luc): 177 RLU

		0	10ng pKCDP β cat	10ng pKC β cat Δ	10ng pKC β cat Δ 65
1ng pKCLef1	RLU (luc)	1988	337699	54178	3463837
	relative luc activity	1.0	169.9	27.3	1742.4
10ng pHAXTcf3	RLU (luc)	3377	505536	17575	2114157
	relative luc activity	1.0	149.7	5.2	626.1

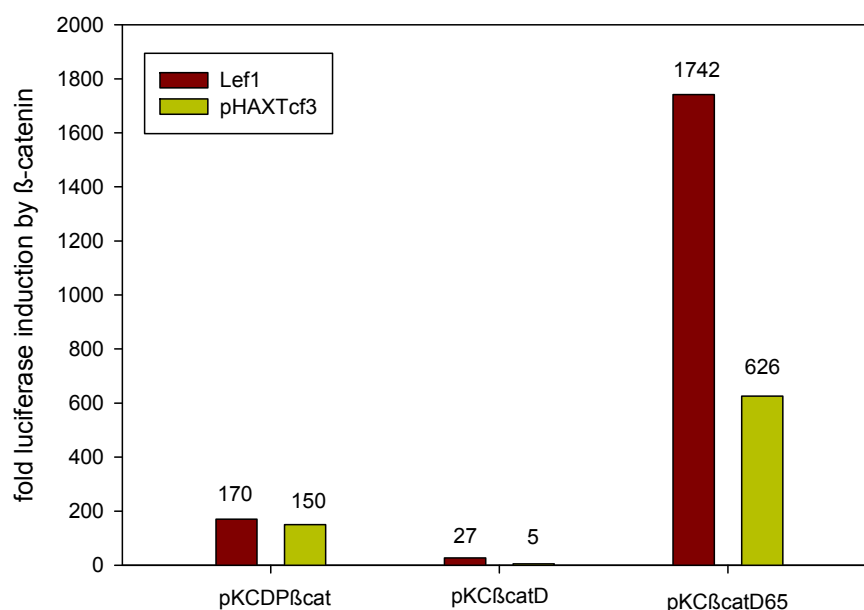


Figure 6.1.4.2. Comparison of the luciferase inductions of full β -catenin, the Armadillo repeats of β -catenin and the Armadillo repeats fused to p65 in 293T cells. 10ng pKCLef1 or 1ng pHAXTcf3 (full Lef1 or Tcf3 encoding constructs) were cotransfected with 10ng pKCDP β cat (activated full β -catenin), pKC β cat Δ (Armadillo repeats of β -catenin) or pKC β cat Δ 65 (Armadillo repeats fused to p65), and 300ng of the reporter pLucF6Lef (six Tcf/Lef binding sites). The luciferase inductions caused by the expression of DP β -cat, β -cat Δ or β -cat Δ 65 were calculated relative to cells which express only Lef1 or Tcf3 (see text for more details). The names of the constructs can include a "D" instead of " Δ ".

The transactivation potential of full length β -catenin was compared with that of a deletion version containing only the Armadillo repeats. The constructs pKCLef1 and pHAXTcf3, containing full length cDNAs, containing pKCDP β cat, the whole human β -catenin protein with several point mutations of the N-terminus preventing phosphorylation for degradation, pKC β cat Δ , containing all 12 Armadillo repeats and pKC β cat Δ 65, possessing in addition the transactivation domain of p65, were used. The reporter plucF6Lef consists of 6 Tcf/Lef binding sites for expression of the reporter gene luciferase (design of all constructs is described in Chapter 5.1.7).

In 293T cells high reporter activation could be observed upon application of the stabilized full length β -catenin protein together with Lef1 (approximately 170 fold activation) and with Tcf3 (approximately 150 fold) (see Figure 6.1.4.2). This strong upregulation of the Wnt signaling pathway by binding of Tcf/Lef and β -catenin was consistent with our expectations and the literature (e.g. Bachar-Dahan et al., 2006). On contrary to the experiments with the full length β -catenin protein, the cotransfection of Lef1/Tcf3 with β -cat Δ showed only low luciferase expression levels, using Lef1 27 times and Tcf3 5 times weaker. This reduction can be explained by the lack of the highly active C-terminal transactivation region of β -catenin. The strongest activations were reached by application of β -cat Δ 65 under the same conditions. Together with Lef1 the expression levels of the Armadillo repeats could be upregulated 1742 fold and 626 fold with Tcf3. Therefore the presence of the p65 transactivation domain upregulates the activity of the isolated Armadillo repeats (β -cat Δ) almost 100 fold.

Cell line: HELA

Format: 24 well

Reporter: 300ng plucF6Lef

Background (luc): 212 RLU

		0	10ng pKCDP β cat	10ng pKC β cat Δ	10ng pKC β cat Δ 65
1ng pKCLef1	RLU (luc)	250	26874	4425	105418
	relative luc activity	1.0	382.9	4.6	1208.2
10ng pKCTcf3	RLU (luc)	6	8169	97	25775
	relative luc activity	1.0	101.4	16.7	397.8

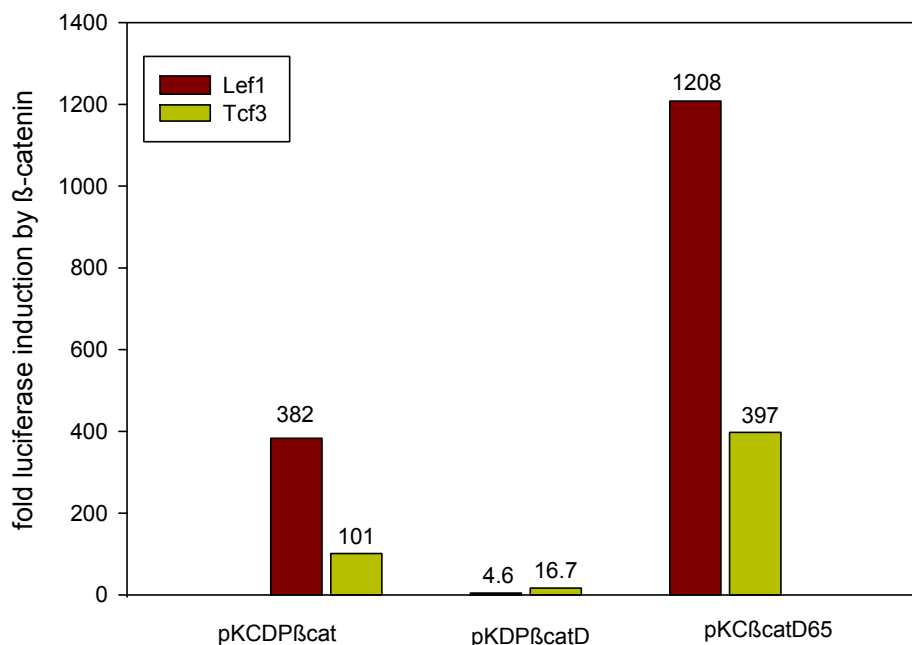


Figure 6.1.4.3. Comparison of the luciferase inductions of full β -catenin, the Armadillo repeats of β -catenin and the Armadillo repeats fused to p65 in HELA cells. The proteins DP β -cat, DP β -cat Δ or β -cat Δ 65 were expressed in combination with Lef1 or Tcf3. For luciferase detection the reporter pLucF6Lef was transfected. The β -catenin variants resulted in luciferase induction levels which were calculated relative to cells which expressed only Lef1 or Tcf3. All transfected DNA amounts are indicated in the table. The names of the constructs can include a "D" instead of " Δ ".

In almost the same manner like in the experiments with 293T cells, the addition of Lef1/Tcf3 together with DP β -cat led to a 383 or 101 fold activation in HELA cells (see experiment shown in Figure 6.1.4.3). Only weak upregulation of the luciferase reporter levels could be reached again by usage of β -cat Δ with Lef1 (83 fold) and Tcf3 (6 fold). Far stronger reporter gene activation could be observed again with the cotrans-

fection of β -cat Δ 65 with Lef1 and Tcf3. These activations with Lef1 were 3 times higher and with Tcf3 4 times higher than using DP β -cat together with Lef1 or Tcf3. In HELA cells the upregulation of the transactivation of the Armadillo repeats by the p65 domain was again roughly 100 fold. Comparing the results of the experiments with 293T and HELA cells, in both cases could be shown the essential influence of β -catenin's C-terminus, being responsible for transactivation and activation of the reporter gene firefly luciferase. The application of the fusion protein β -cat Δ 65 could dramatically improve the natural transactivation ability of β -catenin. Perhaps the lower background levels, in HELA cells in opposition to the background levels in 293T cells might give a hint for a slightly higher endogenous Wnt signaling activity in 293T cells than in HELA cells.

Cell line: HELA

Format: 24 well

Reporter: 300ng plucF8LefOtxab, 1ng pCMVRluc

Background (luc): 373 RLU

Background (Rluc): 1009 RLU

	10ng pSC2Tcf1e		
	0	100ng pKC	100ng pKC β cat Δ
RLU (luc)	3608	6054	107697
relative luc activity	1.0	1.7	29.8
RLU (Rluc)	45431	38178	67345
relative luc/Rluc activity	1.0	2.0	19.9

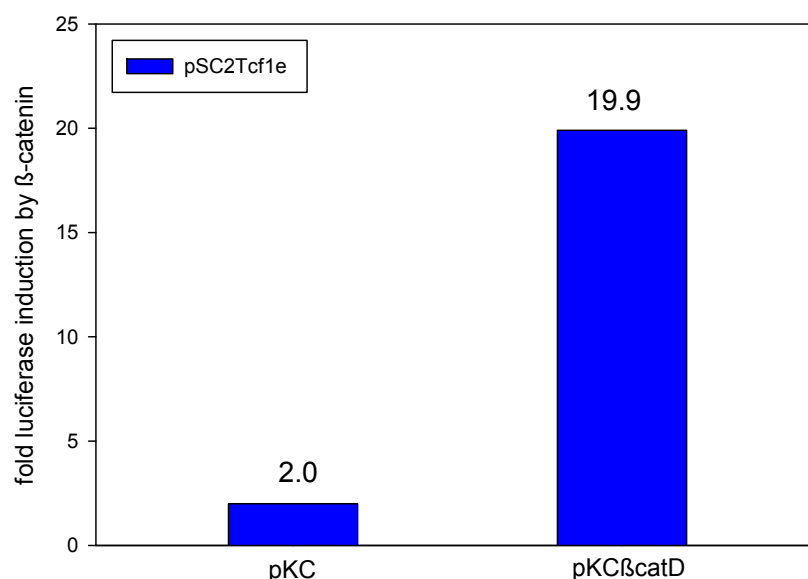


Figure 6.1.4.4. Luciferase induction of the β -catenin Armadillo repeats in combination with Tcf1e. pKC as well Tcf1e in combination with Renilla luciferase were overexpressed and for luciferase detection the reporter plucF8LefOtxab (eight Otxa and Otxb motifs; see text) was used. The different transfected plasmid amounts are shown in the table. The firefly luciferase/Renilla luciferase inductions caused by the expression of Tcf1 and β -cat Δ were calculated relative to cells which produce high levels of Tcf1 and the empty vector pKC. The relative luc/Rluc activity was calculated by dividing the luc values of Tcf1/pKC or Tcf1/ β -cat Δ by the Rluc values of pCMVRluc. The names of the constructs can include a "D" instead of " Δ ".

To show that the effect of the Armadillo repeats is not only limited to Lef1 or Tcf3, a Tcf1e expressing construct was cotransfected with pKC β cat Δ in HELA cells. The protein Tcf1e represents a specific splice variant of Tcf1, which includes the CRARF domain (see Figure 6.1.4.1). A clear induction of approximately 20 fold could be demonstrated (see experiment shown in Figure 6.1.4.4). For this experiment a luciferase reporter with 16 low affinity Tcf/Lef binding sites, derived from the *Otx2* midbrain enhancer was used. This highly conserved enhancer was isolated by Kurokawa and colleagues (Kurokawa et al., 2004) and contains 2 Tcf/Lef binding sites here referred to as Otxa and Otxb. These binding sites represent natural sequence motifs with a weaker binding character than the high affinity binding sequences of pLuc6Lef. The function of the *Otx2* enhancer for *Otx2* expression and brain development was determined in mouse mutants, in which the *Otx2* enhancer was deleted. The strongly impaired *Otx2* expression led to strongly reduced di- and mesencephalon (Kurokawa et al., 2004). In the same study the high relevance of the two Tcf/Lef binding sites for controlling the *Otx2* expression could be shown by introducing point mutations, leading to a complete loss of β -gal expression (driven by the *Otx2* enhancer) in the fore- and midbrain. The results of the experiment (Figure 6.1.4.4) show that β -cat Δ based activation works for Tcf1, in addition to Lef1 and Tcf3.

6.1.5 Tcf/Lef activation through cotransfection of Lef Δ 293 and β -catenin Δ

Our goal was to analyze potential effects of the second weak interaction between Lef Δ 293 (containing the highly conserved DNA binding HMG box) and β -catenin. Especially, we were interested in the influence of β -catenin on the DNA binding affinity of Lef1. For further experiments, the construct pKCRlucLef Δ 293m65 was designed, which lacks the primary β -catenin binding region of Lef1 and contains N-terminally the Renilla luciferase for quantification and C-terminally the transactivation domain of

p65, for strong transactivation (for cloning procedures see Chapter 5.2). Addition of pKC β cat Δ , expressing a β -catenin protein containing only the 12 Armadillo repeats (amino acids 134-664), should show a positive effect on reporter gene activity by an improvement of Lef1 HMG box-DNA binding (in Figure 6.1.5.1 the interaction between β -catenin and Lef1 and the indicated interaction between β -catenin and the Lef1 HMG box in combination with Rluc and p65 are shown). However, the β -cat Δ protein should not be able to improve the activity of the strong transactivation domain of p65 (for comparison see experiments 6.1.4.2 and 6.1.4.3).

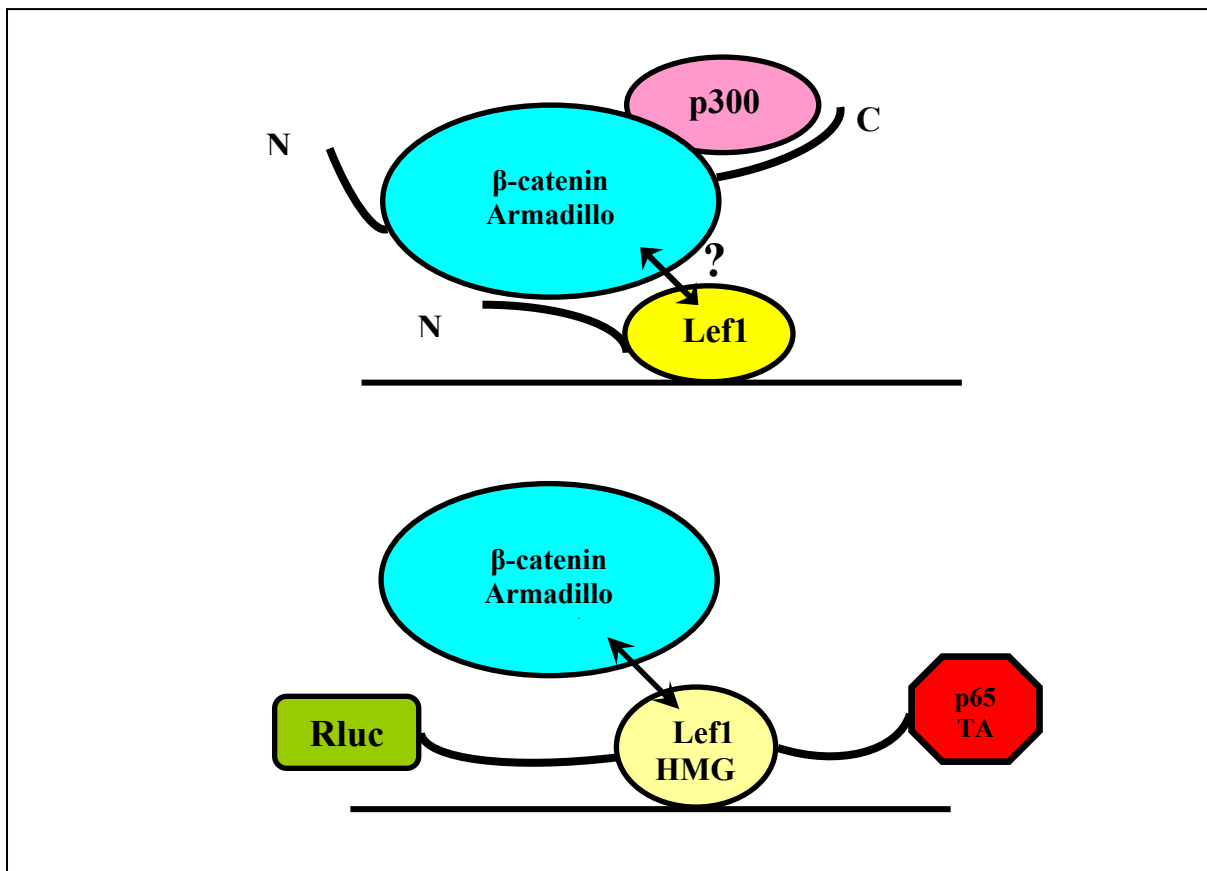


Figure 6.1.5.1. Schematic representation of the interaction of the Armadillo repeats of β -catenin with Lef1 and the potential interaction between the Armadillo repeats of β -catenin and the Lef1 HMG box, in combination with Rluc and p65. The proteins Lef1, β -catenin and p300 are represented in yellow, blue and in pink. The HMG box of Lef1 is indicated in light yellow. The potential interaction of the Armadillo repeats with Lef1 marked by a question mark. In the lower part the HMG box of Lef1 is shown as a fusion protein in combination with Renilla luciferase (Rluc) and the transactivation domain of p65 (p65TA). The N-termini (N) of β -catenin and Lef1 and the C-terminus (C) of β -catenin are indicated by lines. The double arrow between β -catenin and Lef1 HMG represents the possible weak interaction between them.

Cell line: 293T

Format: 24 well

Reporter: 10ng plucF6lefOtxb

Background (luc): 347 RLU

Background (Rluc): 455 RLU

		pKCRlucLefΔ293m65			
		0	1ng	4ng	8ng
30ng pKC	RLU (luc)	574	725	1928	12232
	relative luc activity	1.0	3.6	19.3	155.3
	RLU (Rluc)	347	28124	85761	376440
	relative luc/Rluc activity		0.05	0.08	0.25
30ng pKCβcatΔ	RLU (luc)	1260	1118	5002	21193
	relative luc activity	1.0	8.7	59.5	271.1
	RLU (Rluc)	455	35013	182225	589755
	relative luc/Rluc activity		0.09	0.1	0.19

Figure 6.1.5.2. Wnt pathway upregulation caused by the combined expression of β -cat Δ and Lef Δ 293 in 293T cells. The HMG-box containing protein Lef Δ 293 fused to Rluc and p65 was expressed in combination with pKC or β -cat Δ . For luciferase detection the six Otxb sites containing reporter plucF6LefOtxb was used. All amounts of plasmids were used as indicated. The resulting firefly luciferase/Renilla luciferase induction levels of the fusion protein with or without β -cat Δ were analyzed relative to each other. The relative luc/Rluc activity was calculated by dividing the luc values by the Rluc values (was done for all following experiments).

In 293T cells for the experiment 6.1.5.2 (Figure 6.1.5.2) the luciferase expressing reporter plucF6lefOtxb was used (includes 6 Otxb Tcf/Lef binding motifs). In the performed experiments the addition of β -cat Δ did not lead to any luciferase induction. This reflects the very weak interaction between the HMG box of Lef1 and the Armadillo repeats of β -catenin. Probably, this interaction on its own is too weak for a clear detection. Therefore, in this experiment an improvement of Lef HMG box-DNA binding by β -cat Δ could not be shown.

Cell line: HELA

Format: 24 well

Reporter: 300ng plucF6Lef

Background (luc): 373 RLU

Background (Rluc): 787 RLU

		pKCRlucLefΔ293m65		
		0	3ng	30ng
100ngpKC	RLU (luc)	4553	621563	1913488
	relative luc activity	1.0	147.0	452.8
	RLU (Rluc)	734	171488	1036066
	relative luc/Rluc activity		0.8	0.4
100ngpKCβcatΔ	RLU (luc)	7953	1497342	3227346
	relative luc activity	1.7	354.3	763.7
	RLU (Rluc)	700	231395	1315355
	relative luc/Rluc activity		1.3	0.5

Figure 6.1.5.3. Wnt pathway upregulation caused by the combined expression of β -cat Δ and Lef Δ 293 in HELA cells. In cotransfections 3ng or 30ng pKCRlucLef Δ 293m65 with or without 100ng pKC β cat Δ were combined with 100ng of the reporter plucF6Lef. The resulting firefly luciferase/Renilla luciferase activity of the RlucLef Δ 293m65 with β -cat Δ was calculated relative to cells which produce only the fusion protein without β -cat Δ .

Using the HELA cell line, slightly higher luc/Rluc values of the reporter activity could be observed (up to 62 percent) upon expression of high amounts of β -cat Δ (Figure 6.1.5.3). Lower concentrations of β -cat Δ did not show any effect (data not shown). However, summarizing the experiments in HELA cells, a weak and therefore non-significant reporter activation through the interaction of the HMG box and β -catenin Δ could be shown. In order to prove that β -catenin can improve the DNA binding efficiency of the Lef HMG domain, further experiments and strategies had to be performed to achieve reliable results.

6.1.6 Activation through the fusion of β -catenin Δ to the HMG boxes of Lef1, Tcf3 and Tcf1

The coexpression of Lef Δ 293 with β -cat Δ resulted in an extremely weak induction of activity in 293T and HELA cells. Furthermore, high concentrations of β -catenin had to be used confirming the weak nature of this interaction. A possible model could therefore be that the high affinity interaction of the Lef N-terminus with β -catenin is essential before a DNA binding effect can be transmitted by the second interaction inter-

phase at the HMG domain. Since in the truncated protein Lef Δ 293 this primary well known interaction motif is missing, a significant reporter activation cannot be observed. For this reason as a next step the construct pKCRLucLef Δ 293m β cat Δ 65, consisting of the cDNAs of Lef Δ 293, β -cat Δ and the transactivation domain p65 with three myc tag copies in between as a linker (linker m), was created. Through the resulting covalent linkage between Lef Δ 293 and β -cat Δ , an imitation of the primary high affinity interaction domain in the N-terminus of Lef1 would be achieved. The myc linker in this fusion protein together with further adapters (between Rluc and Lef Δ 293 and β -cat Δ and p65) would assure high flexibility of the fusion partners, conserving their natural sterical forms and functions (see Figure 6.1.6.1). Besides, the use of this fusion protein would also exclude misleading results, created by strong overexpression of β -cat Δ . In addition for the creation of a reliable negative control the cDNA of Lef Δ 293 was replaced by the cDNA of the zinc finger homeodomain protein ZFHD-1 (ZF) for cloning the construct pMCRLucZFb6m β cat Δ 65. The ZFHD-1 protein represents an artificial combination of the zinc finger structures of Zif268, a short polypeptide linker and the homeodomain (HD) of the octamer-motif-binding protein Oct-1 (Pomerantz et al., 1995). This artificial DNA binding protein recognizes a specific binding motif consisting of a combination of a ZF and a HD motif not present in the human genome. Zif268 represents a typical transcription factor of the early growth response (EGR) family consisting of zinc finger structures for DNA binding, which is required for cell differentiation, mitogenesis and tumor suppression (Krones-Herzig et al., 2003). The ZF DNA interaction motif is shown in Table 5.1.7.4. β -catenin should not be able to interact with the ZF protein which therefore serves as a negative control. For the analysis the reporter plucF4LefOtxab each with four Tcf/Lef binding sites of the variants Otxa and Otxb was used. The resulting luciferase levels of pMCRLucZFb6m65 or pMCRLucZFb6m β cat Δ 65 were detected by the pSGFluc reporter, including twelve ZF binding sites.

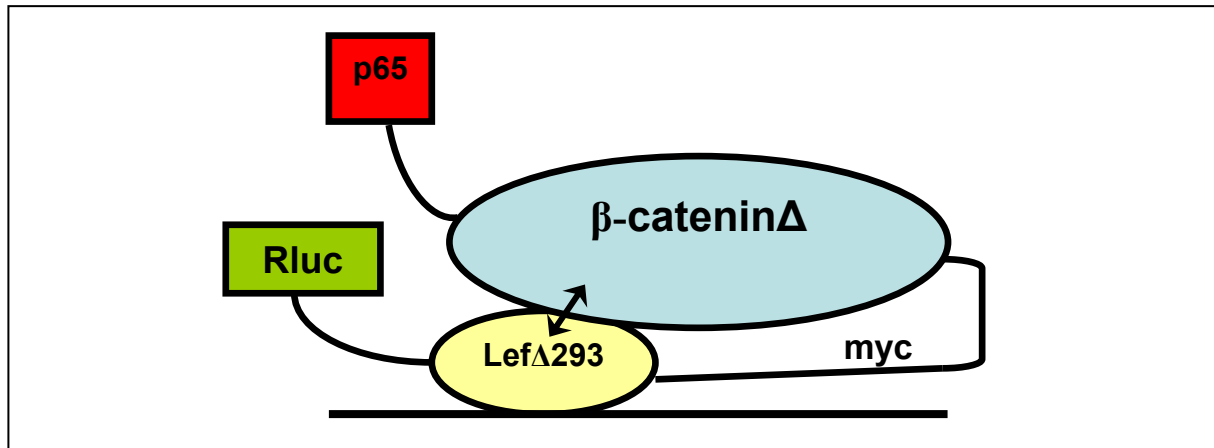


Figure 6.1.6.1. Schematic view of the RlucLef Δ 293m β cat Δ 65 fusion protein. Rluc represents the Renilla luciferase, Lef Δ 293 the HMG box of Lef1, myc a 3x myc tag, β -catenin Δ all Armadillo repeats of β -catenin and p65 the transactivation domain of p65. The lines between the domains indicate linker sequences. The potential interaction of β -catenin and the HMG domain of Lef1 is indicated by an arrow.

Cell line: 293T

Format: 24 well

Reporter: 10ng pSGFluc, 10ng plucF4LefOtxab

Background (luc): 254 RLU

Background (Rluc): 331/256 RLU

	0	0.1ng pMCRlucZFb6m65	3ng pMCRlucZFb6mβcatΔ65	0	0.1ng Rluc- LefΔ293m65	3ng Rluc- LefΔ293mβcat Δ65
RLU (luc)	7350	637794	1102808	10763	20368	36294
relative luc activity	1.0	87.0	150.0	1.0	2.8	4.9
RLU (Rluc)	331	53375	59446	256	24720	12744
relative luc/Rluc activity		1.0	1.5		1.0	3.7

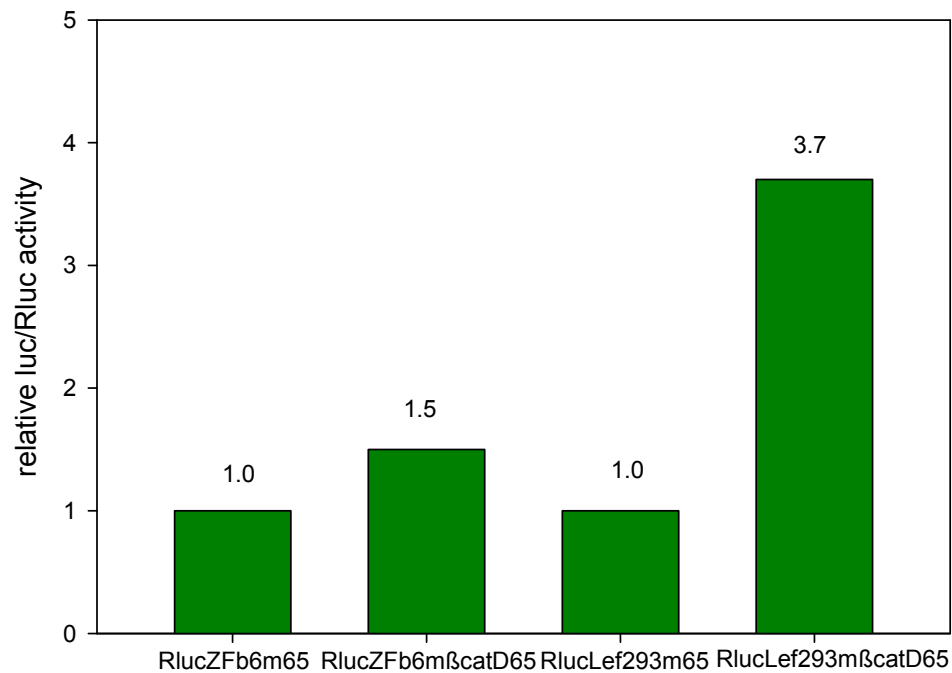


Figure 6.1.6.2. β -catenin-Lef fusion proteins in 293T cells. The truncated protein Lef Δ 293 was N-terminally fused to Rluc, and C-terminally either directly to p65 or first to the Armadillo repeats of β -catenin (β -cat Δ). As a negative control the zinc finger protein ZF was fused to Rluc and to p65 or in addition to β -cat Δ separated by the myc linker. All four fusion protein variants were overexpressed in cells. For luciferase detection the reporters plucF4LefOtxab (four Otxa and Otxb motifs) or pSGFluc (twelve ZF sites) were transfected. The firefly luciferase/Renilla luciferase induction levels of RlucLef Δ 293m β cat Δ 65 or RlucZFb6m β cat Δ 65 were calculated relative to cells which produced RlucLef Δ 293m65 or RlucZFb6m65. All transfected plasmid amounts are shown in the table (see text for more details). The names of the fusion proteins can include a “D” instead of “ Δ ”.

In 293T cells cotransfection of pKCLef Δ 293m β cat Δ 65 with the reporter plucF4LefOtxab resulted in a convincing relative upregulation of the luciferase gene (3.7 fold), compared to pKCLef Δ 293m65, indicating an interaction of Lef Δ 293 (containing the HMG box) with the Armadillo repeats of β -catenin. The elevated luciferase activity indicates that the Armadillo repeats of β -catenin are responsible for a stronger DNA binding of Lef1. In a control experiment RlucZF6m65 and RlucZFb6m β cat Δ 65 under the same conditions showed almost the same activity. In contrast to the results for Lef Δ 293 expressing constructs a binding of the ZF protein on its binding motif at the pSGFluc reporter, was only weakly activated (1.5 fold) by addition of β -cat Δ .

Cell line: HELA

Format: 24 well

Reporter: 300ng plucF4LefOtxb/300ng plucZF24

Background (luc): 303 RLU

Background (Rluc): 424 RLU

	0	0.3ng pMCRLucZF6m65	3ng pMCRLuc- ZF6mβcatΔ65	0	1ng pKCRluc- LefΔ293m65	10ng pKCRluc- LefΔ293mβcatΔ65
RLU (luc)	1566	12197	352409	590	40899	206880
relative luc activity	1.0	9.4	278.7	1.0	141.0	720.0
RLU (Rluc)	521	794	4205	394	14847	13809
relative luc/Rluc activity		1.0	1.5		1.0	5.4

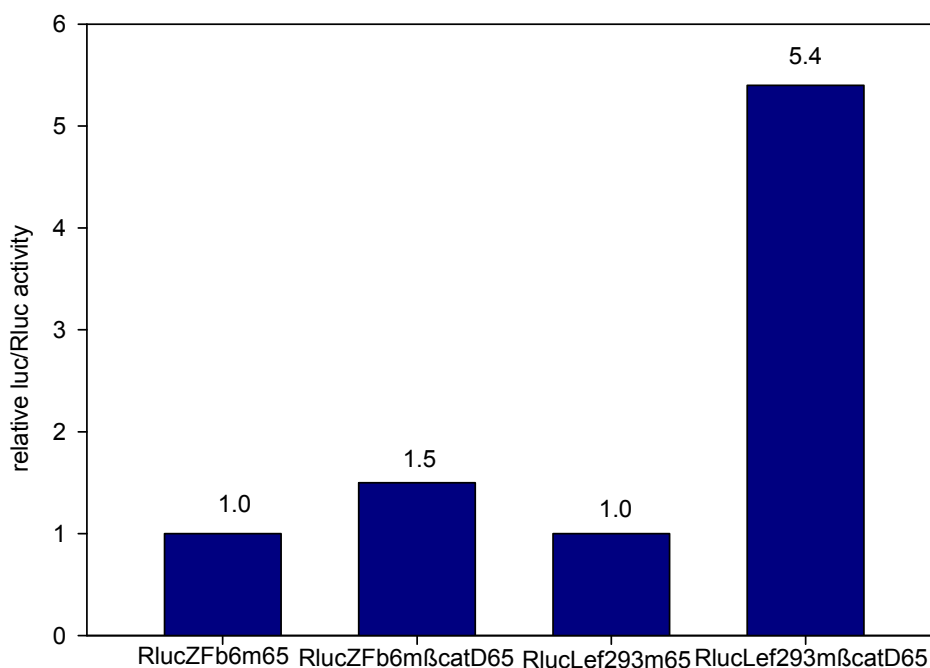


Figure 6.1.6.3. β -catenin-Lef fusion proteins in HELA cells. In cotransfections 1ng pKCRlucLefΔ293m65, 0.3ng pMCRLucZFb6m65, 10ng pKCRlucLefΔ293mβcatΔ65 or 3ng pMCRLucZFb6mβcatΔ65 were combined with 300ng of the reporters plucF4LefOtxb or pSGFluc. The firefly luciferase/Renilla luciferase induction caused by the expression of RlucLefΔ293mβcatΔ65 or RlucZFb6mβcatΔ65 were calculated relative to cells which produced RlucLefΔ293m65 or RlucZFb6m65. The names of the fusion proteins can include a "D" instead of "Δ".

In a repetition of the same experiment in HELA cells, the results were much clearer (see experiment shown in Figure 6.1.6.3). An induction of 5.4 fold could be observed by cotransfection of pKCRlucLefΔ293mβcatΔ65 and plucF4LefOtxb. Again, because

of the lower endogenous basal levels of Wnt signaling in HELA cells, a 141 fold higher luciferase activity could be obtained by the addition of pKCRlucLef Δ 293m65 alone. Like in the first experiment, using 293T cells, a relative luc/Rluc upregulation with the ZF protein, instead of the Lef Δ 293 protein, was not seen. For this analysis the reporter plucZF24 with 24 ZF binding sites was generated. Again this experiment supported the hypothesis that β -catenin can improve the binding of Lef1 to DNA. Since the results were clearer in HELA cells we performed the following experiments in this cell line. In addition, in opposition to 293T cells, in HELA cells an application of higher concentrations of expression vectors was possible, leading to more convincing effects.

Cell line: HELA

Format: 24 well

Reporter: 300ng plucF8LefOtxab

Background (luc): 230 RLU

Background (Rluc): 738 RLU

	0	0.03ng pKCRluc- Tcf3Δ(320-434)m65	3ng pKCRluc- Tcf3Δ(320-434)mβcatΔ65
RLU (luc)	19959	70765	11119172
relative luc activity	1.0	3.2	510.2
RLU (Rluc)	820	9267	143618
relative luc/Rluc activity		1.0	9.7

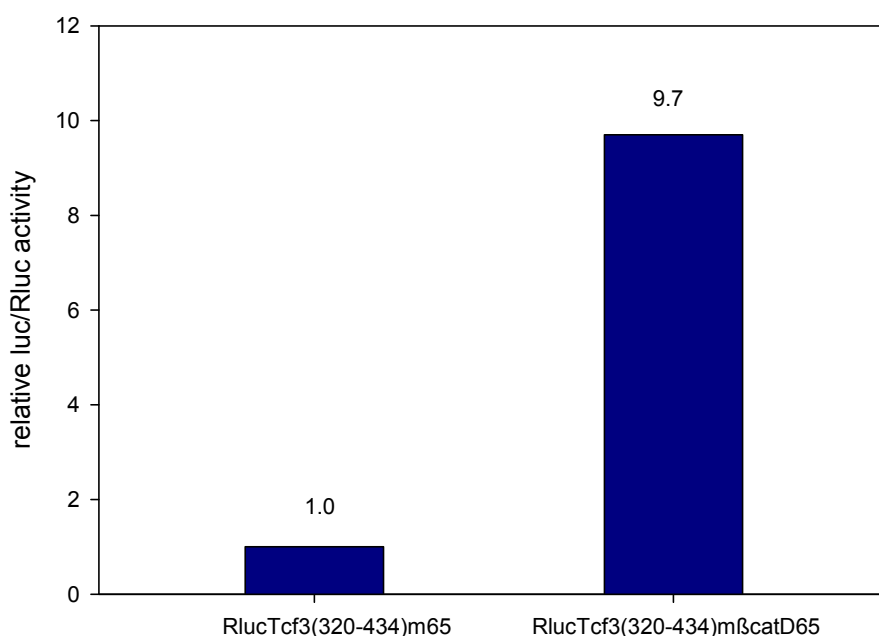


Figure 6.1.6.4. Indication of the interaction between Tcf3Δ(320-434) and β-catΔ by using the fusion protein RlucTcf3Δ(320-434)mβcatΔ65. The fusion proteins RlucTcf3Δ(320-434)m65 or RlucTcf3Δ(320-434)mβcatΔ65, which contain the HMG box of Tcf3, Rluc, p65 and optionally β-catΔ were expressed. For luciferase detection the reporter plucF8LefOtxab was used. For transfection the DNA amounts were used as indicated. The resulting firefly luciferase/Renilla luciferase induction of RlucTcf3Δ(320-434)mβcatΔ65 was compared relative to the levels of RlucTcf3Δ(320-434)m65. The names of the fusion proteins can include a “D” instead of “Δ”.

As a next step I tested the Tcf3 DNA binding domain instead of that of Lef1. In this experiment (Figure 6.1.6.4) a higher relative induction than in the other experiment (part of Figure 6.1.6.3) was achieved by using the truncated form Tcf3Δ(320-434) of the Tcf3 protein, which includes the LefΔ293 analogous amino acids 320-434, in combination with β-catΔ65, expressed as a fusion protein. RlucTcf3Δ(320-

434)m β cat Δ 65 led to a 9.7 fold higher luciferase activity, indicating that the Tcf3 HMG domain also interacts with β -catenin. Interestingly, the observed effect was even stronger compared to Lef1. Again in this experiment for the analysis of the effect of β -cat Δ on the DNA binding of Tcf3, the reporter plucF8LefOtxab was cotransfected with the constructs Rluc Tcf3 Δ (320-434)m65 or Rluc Tcf3 Δ (320-434)m β cat Δ 65.

Cell line: HELA

Format: 24 well

Reporter: 300ng plucF8LefOtxab

Background (luc): 261 RLU

Background (Rluc): 4670 RLU

	0	0.3ng pKCRluc-Tcf1 Δ (269-398)m65	3ng pKCRluc-Tcf1 Δ (269-398)m β cat Δ 65
RLU (luc)	6119	266499	10248181
relative luc activity	0.8	34.3	1320.8
RLU (Rluc)	6907	28563	166107
relative luc/Rluc activity		1.0	5.8

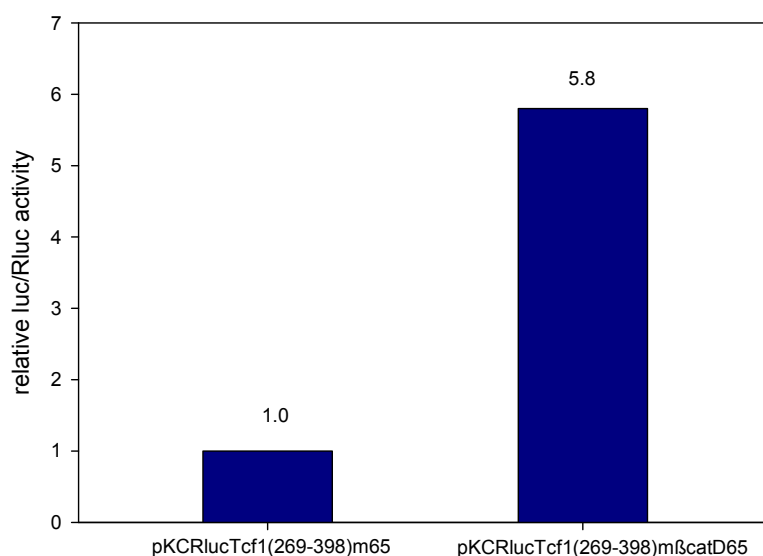


Figure 6.1.6.5. Luciferase induction caused by the fusion protein RlucTcf1 Δ (269-398)m β cat Δ 65. The fusion proteins RlucTcf1 Δ (298-398)m65 or RlucTcf1 Δ (269-398)m β cat Δ 65, which contain the HMG box of Tcf3, Rluc, p65 and optionally β -cat Δ were expressed. For luciferase detection the reporter plucF8LefOtxab was used. The resulting firefly luciferase/Renilla luciferase induction of RlucTcf1 Δ (269-398)m β cat Δ 65 was compared relative to the level of RlucTcf1 Δ (269-398). The

amounts of plasmids for expressing the fusion proteins are shown in the table. The names of the fusion proteins can include a “D” instead of “Δ”.

Furthermore, I tested for an interaction of Tcf1 with β -catenin by using the corresponding region of Tcf1 fused with the Armadillo repeats of β -catenin, representing Tcf1 Δ (269-398)m β cat Δ 65 (see experiment shown in Figure 6.1.6.5). The impact of β -catenin on Tcf1 DNA binding was similar to that of Lef1. Without β -cat Δ , the addition of Tcf1 Δ (269-398)m65 led to a luciferase induction of 34.3 fold, but considering the produced amount of protein (measuring Rluc), Tcf1 Δ (269-398)m β cat Δ 65 increased the luciferase activity 5.8 times higher. Thus, β -cat Δ exhibits a similar effect on Tcf1 compared to Lef1.

6.1.7 The Lef1 HMG domain itself is responsible for the interaction with β -catenin Δ

So far it was only an assumption that β -cat Δ interacts with the HMG domain of Lef1, Tcf3 and Tcf1, influencing their efficiency for DNA binding. Although, it would be very unlikely, β -cat Δ could also interact with sequences outside the HMG box, comprising the amino acids 380 to 397. To find out whether the interaction takes place within the HMG box, constructs were designed, containing exclusively the HMG domain. One is called RlucLef Δ HMGm65 and the other one RlucLef Δ HMGm β cat Δ 65. In the first case, the region of the HMG box of Lef1 (amino acids 293 to 380) and in the other case the HMG box of Lef1 together with β -cat Δ was included (see Chapter 5.1.7 for detailed cloning steps).

Cell line: HELA

Format: 24 well

Reporter: 300ng plucF8LefOtxab

Background (luc): 360 RLU

Background (Rluc): 861 RLU

	0	0.1ng pKCRlucLefΔ293m65	30ng pKCRlucLefΔ293mβcatΔ65
RLU (luc)	2157	30019	2133079
relative luc activity	1.0	13.9	989.0
RLU (Rluc)	861	15016	130228
relative luc/Rluc activity in relation to RlucLefΔ293m65		1.0	4.1

	1ng pKCRlucLefΔHMGm65	1ng pKCRluc- LefΔHMGmβcatΔ65
RLU (luc)	3581	7149
relative luc activity	1.7	3.3
RLU (Rluc)	442683	41970
relative luc/Rluc activity in relation to RlucLefΔ293mβcatΔ65	0.002	0.04
relative luc/Rluc activity in relation to RlucLefΔHMGm65	1.0	20.0

Figure 6.1.7.1. The second interaction domain between Lef1- β -cat Δ is located in the HMG box of Lef1. The fusion proteins RlucLef Δ HMGm65, RlucLef Δ HMGm β cat Δ 65, containing exactly the Lef1 HMG box, RlucLef Δ 293m65 and RlucLef Δ 293m β cat Δ 65 were expressed. The reporter plucF8LefOtxab was used for the detection of luciferase activities. For transfection the DNA amounts were used as indicated. The relative firefly luciferase/Renilla luciferase induction caused by the expression of the β -cat Δ containing fusion proteins were calculated relative to cells which produced proteins without β -cat Δ (see text for more details).

The relative luc/Rluc induction of RlucLef Δ 293m β cat Δ 65 was compared to the relative activations of the proteins RlucLef Δ HMGm65 and RlucLef Δ HMGm β cat Δ 65 (Figure 6.1.7.1). The first protein showed again a clear luciferase upregulation up to 4. In contrary, both HMG proteins showed extremely low activation of the reporter, which is due to the lack of the B- box at the C-terminus of the HMG box. This additional region comprises 9 amino acids, which are important for DNA binding (Carlsson et al., 1993; Read et al., 1994). Nevertheless, the expression of RlucLef Δ HMGm β cat Δ 65 led to a 20 fold higher luciferase induction compared to RlucLef Δ HMGm65, which does not include β -cat Δ . These results indicate that the HMG box itself is able to interact with β -catenin.

6.1.8 Point mutations within the Lef1 HMG domain for the identification of amino acids, involved in the β -catenin interaction

To confirm the potential interaction between β -cat Δ and the HMG domain of Lef1, an additional strategy was performed to identify the amino acids within the HMG box, which mediate the interaction. For this, data from the X ray structure and sequence comparisons were used as a basis for creating point mutations within the HMG domain. Fifteen different mutants (MI to MXV) were generated (see Figure 6.1.8.1). In most cases just one amino acid was replaced by alanine. For the synthesis of the mutants IV, VII, XII or XIII two amino acids and in mutant XV four amino acids were exchanged by alanines. Mutant III is a combination of the mutants IX and X. All these mutants were cloned as fusion proteins (in connection with β -cat Δ , Renilla luciferase and the transactivation domain of p65) to show their effect on DNA and β -catenin binding (the cloning strategies of all constructs are described in Chapter 5.1.7).

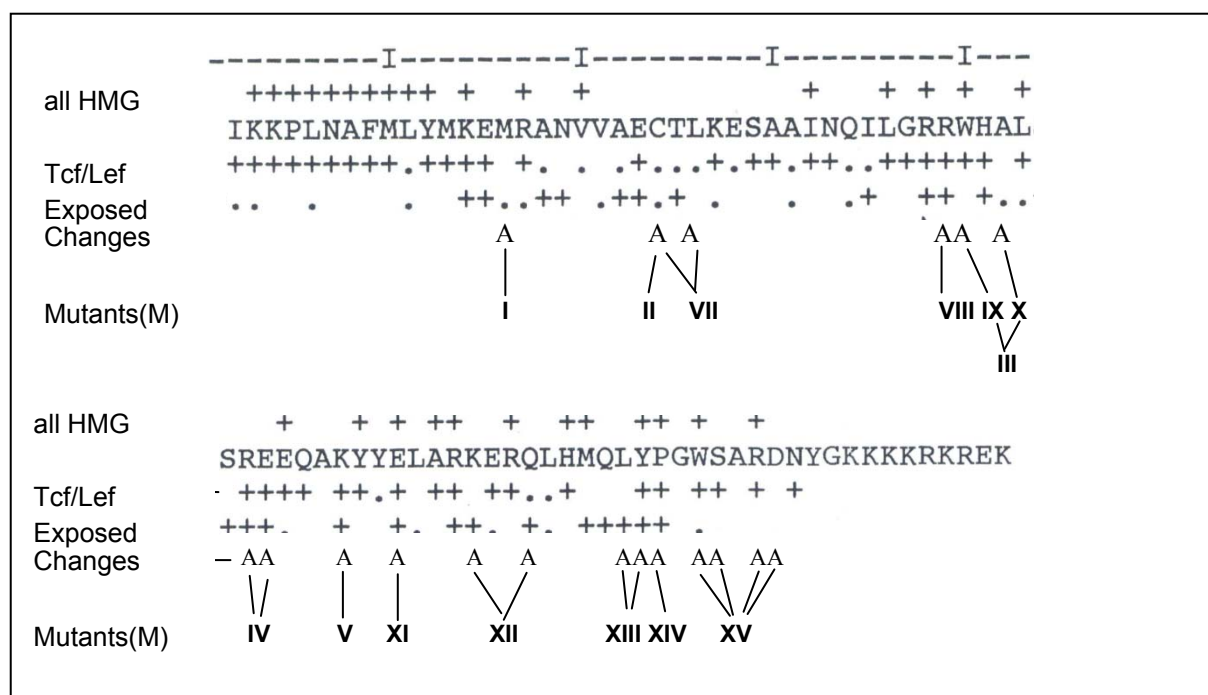


Figure 6.1.8.1. Linear representation of the amino acid sequence of the HMG box of murine Lef1 (amino acids 293 to 380), including the amino acids, which are changed in the point mutations (M). Line "all HMG", "+" represents all highly conserved amino acids of HMG domains. Line "Tcf/Lef", "+" indicates highly conserved and "." weakly conserved amino acids in the HMG domains of Tcf/Lef proteins. Non-conserved amino acids are indicated by empty spaces. The same scheme is used for the category "exposed", which indicates exposed amino acids in the Tcf/Lef HMG boxes. A represents alanine.

The experiments were performed in HELA cells in the 96 well format. For the analysis of the relative luciferase activity, mutated Lef Δ 293 compared to β cat Δ fusion proteins the reporter pMluc24Lefnkd was used. The relative luc/RLuc induction of each of the different fusion proteins was calculated as a mean value. The reporter contains one Tcf/Lef binding site, which was inserted 24 times within the construct (Figure 6.1.11.3.1). This particular binding motif, which possesses a weak binding affinity for Tcf/Lef is derived from the *naked cuticle* (nkd) enhancer of *Drosophila*. The reporter was particularly efficient for these experiments (data not shown).

Cell line: HELA

Format: 96 well

Reporter: 90ng pMluc24Lefnkd

Background (luc): 0.0001 RLU

Background (RLuc): 0.0009 RLU

		pKCRLucLef Δ 293m65			pKCRLucLef Δ 293m β cat Δ 65		
	0	0.6ng	2ng	6ng	0.2ng	0.6ng	2ng
RLU (luc)	0.0025	0.045	0.4179	1.2163	0.0214	0.0694	0.1025
relative luc activity	1.2	18.0	167.16	486.52	8.6	27.76	41.0
RLU (RLuc)	0.0004	0.1659	0.5699	1.3936	0.0088	0.0236	0.0341
relative luc/RLuc activity		0.4	1.2	1.4	3.9	4.6	5.1
mean value		1.0			4.5		

	pKCRLucLef Δ 293Mllm65			pKCRLucLef Δ 293Mllm β cat Δ 65			pKCRLucLef Δ 293Mllm65		
	0.6ng	2ng	6ng	0.2ng	0.6ng	2ng	0.6ng	2ng	6ng
RLU (luc)	0.0384	0.119	0.5418	0.0049	0.006	0.0601	0.0023	0.0066	0.0076
relative luc activity	19.0	58.0	265.0	2.0	3.0	29.0	1.1	3.2	3.7
RLU (RLuc)	0.0250	0.0940	1.8943	0.0109	0.0206	0.0759	0.5678	2.1885	4.8673
relative luc/RLuc activity	1.5	1.3	0.3	1.0	1.9	7.0	1.4	1.0	0.5
mean value	1.0			3.5			1.0		

	pKCRIuc-LefΔ293MIllmβcatΔ65			pKCRIucLefΔ293MIVm65				pKCRIucLefΔ293MI VmβcatΔ65		
	0.2ng	0.6ng	2ng	0.6ng	2ng	6ng	0.2ng	0.6ng	2ng	
RLU (luc)	0.0055	0.0083	0.022	0.0045	0.0776	0.3685	0.0147	0.0348	0.1994	
relative luc activity	2.7	4.0	11.0	2.2	38.0	180.0	7.2	17.0	97.0	
RLU (Rluc)	0.0381	0.0773	0.2219	0.0514	0.1841	0.5571	0.086	0.0142	0.0604	
relative luc/Rluc activity	49.0	36.0	36.0	0.2	0.8	1.2	4.3	8.3	6.3	
mean value	40.0			1.0			5.1			

	pKCRIucLefΔ293MVllm65				pKCRIucLefΔ293MVllmβcatΔ65			pKCRIucLefΔ293MVllm65			
	0.2ng	0.6ng	2ng	6ng	0.2ng	0.6ng	2ng	0.2ng	0.6ng	2ng	6ng
RLU (luc)	0.0101	0.032	0.0856	0.2755	0.0069	0.0512	0.1193	0.0091	0.0169	0.0837	0.1203
relative luc activity	4.9	16.0	42.0	135.0	3.4	25.0	58.0	4.4	8.0	41.0	59.0
RLU (Rluc)	0.0857	0.218	0.6895	1.0286	0.004	0.0246	0.0487	0.048	0.1457	0.4169	0.7113
relative luc/Rluc activity	0.7	0.9	0.7	1.6	10.7	13.0	14.4	1.0	0.7	1.3	1.1
mean value	1.0				12.7			1.0			

	pKCRIuc-LefΔ293MVllmβcatΔ65			pKCRIucLefΔ293MIXm65			pKCRIucLefΔ293MIXmβcatΔ65		
	0.2ng	0.6ng	2ng	0.6ng	2ng	6ng	0.2ng	0.6ng	2ng
RLU (luc)	0.0063	0.0266	0.0523	0.0076	0.0526	0.1442	0.0213	0.0444	0.1039
relative luc activity	3.1	13.0	26.0	4.0	26.0	70.0	10.0	22.0	51.0
RLU (Rluc)	0.0038	0.0151	0.0362	0.3015	0.9558	1.3772	0.0168	0.0572	0.1
relative luc/Rluc activity	14.2	11.0	8.5	0.4	0.9	1.7	18.8	12.2	15.9
mean value	11.3			1.0			15.6		

Figure 6.1.8.1. Point mutations in the HMG domain of Lef1. Mutated versions of pKCRIucLefΔ293(M)m65, pKCRIucLefΔ293(M)mβcatΔ65 (M from MI to MXIV) were cotransfected in combination with the reporter pMlucF24Lefnkd. The values RLU (luc) and RLU (Rluc) represent an average of six firefly or Renilla luciferase measurements subtracted by background (luc) or (Rluc). All transfected amounts of plasmids are indicated in the tables. The relative firefly luciferase/Renilla luciferase induction levels based on the expression of RlucLefΔ293(M)mβcatΔ65 was calculated relative to cells which produced RlucLefΔ293(M)m65.

For analysis the relative luc/Rluc induction of the fusion proteins including β-catΔ in comparison to the fusion proteins without β-catΔ were calculated. The wild type form RlucLefΔ293mβcatΔ65 exhibited the best DNA binding capacity compared to all mutated forms (mutants MII, MIII, MIV, MVII and MVIII with and without β-catΔ; see Figure 6.1.8.1). In all cases the mutated Lef1 proteins showed impaired DNA interaction. The addition of β-catΔ as a fusion protein led to varying relative luciferase/Renilla

luciferase inductions, but no mutant showed a loss of the β -catenin induction. The highest induction was reached with the mutant MIII (40 fold), followed by MIX (16 fold) and MVII (13 fold). This means that β -cat Δ was particularly efficient to improve the weak DNA binding of these HMG box mutants.

Cell line: HELA

Format: 96 well

Reporter: 90ng pMluc24Lefnkd

Background (luc): 0.0001 RLU

Background (Rluc): 0.0074 RLU

		pKCRIucLef Δ 293m65				pKCRIucLef Δ 293m β cat Δ 65		
	0	0.2ng	0.6ng	2ng	6ng	0.2ng	0.6ng	2ng
RLU (luc)	0.0042	0.1708	0.6425	1.6811	5.4367	0.0382	0.386	0.1347
relative luc activity	1.0	41.0	156.0	407.0	1317.0	9.0	9.0	33.0
RLU (Rluc)	0.0074	1.0634	3.2295	6.9687	18.069	0.0302	0.0499	0.1613
relative luc/Rluc activity		0.6	0.8	1.0	1.2	3.5	3.2	3.4
mean value		1.0				3.4		

	pKCRIucLef Δ 293MIm65				pKCRIuc-Lef Δ 293MIm β cat Δ 65			pKCRIucLef Δ 293MVm65			
	0.6ng	2ng	6ng	20ng	0.2ng	0.6ng	2ng	0.6ng	2ng	6ng	20ng
RLU (luc)	0.0423	0.2321	1.05	3.4161	0.0513	0.1122	0.4846	0.0151	0.0279	0.0358	0.0215
relative luc activity	10.0	56.0	254.0	827.0	12.0	27.0	117.0	3.6	6.6	8.5	5.1
RLU (Rluc)	0.2442	1.3705	3.4295	11.839	0.0786	0.0982	0.3511	3.8517	9.5833	20.911	25.811
relative luc/Rluc activity	0.8	0.8	1.4	1.3	3.0	5.1	6.5	1.7	1.3	0.7	0.4
mean value	1.0				4.9			1.0			

	pKCRIucLef Δ 293MVm β cat Δ 65			pKCRIucLef Δ 293MXm65				pKCRIuc-Lef Δ 293MXm β cat Δ 65	
	0.2ng	0.6ng	2ng	0.6ng	2ng	6ng	20ng	0.2ng	0.6ng
RLU (luc)	0.1621	0.1859	0.3302	0.0065	0.0193	0.0581	0.153	0.0211	0.0502
relative luc activity	38.6	44.3	78.6	2.0	5.0	14.0	37.0	5.0	12.0
RLU (Rluc)	0.8852	1.5398	2.5535	0.2199	1.0261	3.266	6.857	0.051	0.151
relative luc/Rluc activity	84.0	53.0	55.0	1.5	0.9	0.9	1.1	19.5	16.2
mean value	64.0			1.0				17.8	

	pKCRlucLefΔ293MXIIIm65				pKCRlucLefΔ293MXIIImβcatΔ65		pKCRlucLefΔ293MXIIIm65			
	0.6ng	2ng	6ng	20ng	0.6ng	2ng	0.6ng	2ng	6ng	20ng
RLU (luc)	0.2378	0.6855	1.8686	4.6916	0.1485	0.3385	0.0289	0.1202	0.2973	0.8245
relative luc activity	58.0	166.0	453.0	1136.0	36.0	82.0	6.9	28.6	70.8	196.3
RLU (Rluc)	0.7689	2.7187	6.5148	15.766	0.1145	0.288	1.65	7.2983	15.374	27.986
relative luc/Rluc activity	1.1	0.9	1.0	1.1	4.6	4.2	0.9	0.8	1.0	1.2
mean value	1.0				4.4		1.0			

	pKCRlucLefΔ293MXIIImβcatΔ65			pKCRlucLefΔ293MXIVm65				pKCRlucLefΔ293MXIVmβcatΔ65		
	0.2ng	0.6ng	2ng	0.6ng	2ng	6ng	20ng	0.2ng	0.6ng	2ng
RLU (luc)	0.1373	0.3591	0.7403	0.0426	0.1677	0.5228	1.8664	0.0209	0.028	0.0797
relative luc activity	32.7	85.5	176.3	10.0	41.0	127.0	452.0	5.0	7.0	19.0
RLU (Rluc)	0.2827	0.6030	1.6297	1.1985	4.415	11.6112	34.4193	0.0641	0.0888	0.1758
relative luc/Rluc activity	23.8	29.5	22.3	0.9	0.9	1.2	1.4	7.7	8.0	10.6
mean value	25.2			1.0				9.3		

Figure 6.1.8.2. β -cat Δ in combination with modified Lef1 HMG boxes induces stronger luciferase activity. Variants of RlucLef Δ 293(M)m65 and RlucLef Δ 293(M)m β cat Δ 65 were expressed in cells. For luciferase detection the reporter pMlucF24Lefnkd was transfected. All transfected amounts of plasmids are indicated in the tables. The relative firefly luciferase/Renilla luciferase induction levels based on the expression of RlucLef Δ 293(M)m β cat Δ 65 was calculated relative to cells which produced RlucLef Δ 293(M)m65.

Subsequently, the impacts of Lef mutants MI, MV, MX, MXII, MXIII and MXIV in combination with β -cat Δ on relative luciferase/Renilla luciferase inductions compared to the Lef mutant versions without β -cat Δ were examined (experiment in Figure 6.1.8.2). The mutation MV resulted in a strong loss of DNA binding affinity in opposition to the mutants I and XII, which obtained similar luciferase inductions like the unmodified protein. Again the weaker the DNA interaction of the mutated HMG box proteins the better β -cat Δ could increase the DNA binding capacities (MV: 64 fold) and vice versa (MXII: 4 fold MI: 5 fold). But in no case the potential interaction between Lef Δ 293 and β -cat Δ could be completely destroyed.

Summarizing all data for the Lef HMG point mutants, all variants led to a decrease in DNA binding affinity. Therefore the wild type HMG box sequence of Lef1 possesses the ideal amino acid sequence for efficient DNA interaction. The increases in activity of the β -cat Δ fusion proteins (Figures 6.1.8.1 and 6.1.8.2) strongly differed and were in several cases higher than that of the wild type HMG domain of Lef1. A tendency was seen that the activation by β -cat Δ was higher for mutants with reduced DNA binding affinity. Consequently the luciferase induction of the HMG variants (representing the relative DNA binding affinity) was correlated to the relative luciferase/Renilla luciferase upregulation of the corresponding HMG mutants with β -cat Δ . This resulted in a highly significant negative relationship ($r_s = -0.723$; $p \leq 0.002$). In a diagram the values were plotted on logarithmic axes to show the smaller values in higher detail (see Figure 6.1.8.3).

Cell line: HELA								
Format: 96 well								
Reporter: 90ng pMluc24Lefnkd								
Background (luc): 0.0001 RLU								
Background (Rluc): 0.0004- 0.0074 RLU								
	experiment 1		experiment 2		experiment 3		experiment 4	
Lef Δ 293 mutants	without β -catenin	with β -catenin	without β -catenin	with β -catenin	without β -catenin	with β -catenin	without β -catenin	with β -catenin
WT	0.39	2.9	0.71	4.35	0.25	3.4	0.41	3.7
MI	0.37	5.5			0.22	4.9		
MII	0.22	3.5	0.42	4.9				
MIII	0.003	40.0	0.004	29.0	0.002	31.9		
MIV	0.55	5.1	0.26	5.0				
MV					0.002	28.4	0.002	64.0
MVII	0.17	12.7	0.19	9.0				
MVIII	0.17	11.3	0.19	7.5			0.204	8.2
MIX	0.065	15.6	0.116	7.1			0.099	6.8
MX	0.052	10.0			0.021	17.8		
MXI					0.158	6.2	0.179	7.1
MXII	0.32	3.8			0.282	4.4		
MXIII	0.028	12.2			0.013	19.9	0.02	25.2
MXIV	0.057	16.6			0.039	9.3	0.053	16.2

Lef Δ 293 mutants	experiment 5		experiment 6				β -catenin induction mean
	without β -catenin	with β -catenin	without β -catenin	with β -catenin	without β -catenin mean	with β -catenin mean	
WT	0.35	4.3	0.23	5.3	0.39	1.56	4.0
MI			0.23	5.4	0.273	1.44	5.3
MII			0.21	3.1	0.283	1.09	3.8
MIII			0.002	35.0	0.003	0.1	34.0
MIV			0.26	3.4	0.357	1.61	4.5
MV	0.001	88.0			0.002	0.1	60.1
MVII			0.15	6.5	0.17	1.6	9.4
MVIII					0.188	1.69	9.0
MIX			0.07	8.5	0.088	0.83	9.5
MX					0.037	0.51	13.9
MXI	0.247	5.2			0.195	1.2	6.2
MXII					0.301	1.23	4.1
MXIII	0.03	20.0			0.023	0.44	19.3
MXIV					0.05	0.7	14.0

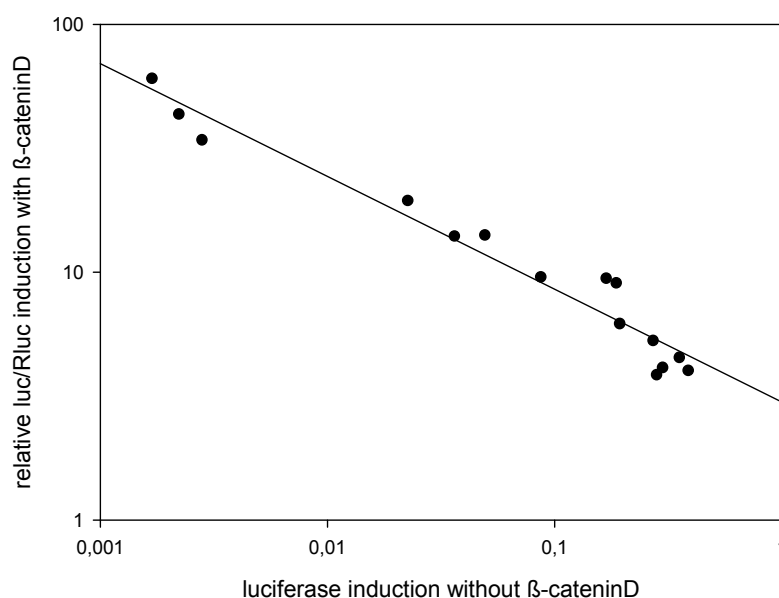


Figure 6.1.8.3. Improvement of Lef1-DNA binding by β -catenin for different point mutations in the HMG box. The luciferase activities of the fusion proteins (contain Lef Δ 293 or Lef Δ 293 mutants) without β -cat Δ were correlated to the relative luciferase/Renilla luciferase inductions of the corresponding fusion proteins with β -cat Δ ($p_s = -0.723$; $p \leq 0.002$). The statistical analysis was done by the Pearson test for parametric deviations. Each point was calculated as a mean value of six (Lef Δ 293), four (MIII, MIX, MXIII) or three (MI, MII, MIV, MV, MVII, MVIII, MX, MXI, MXII, MXIV) experiments. All values were plotted on logarithmic axes.

However, none of the point mutations within the HMG box resulted in a reduced β -catenin induction (compared to the wild type protein). A possible reason for this would be that other amino acids than I have analyzed are involved in β -cat Δ binding

or a combination of several mutations would be necessary to block the interaction significantly. Generally, the effect of β -catenin, which could be explained by a better HMG box-DNA binding, could be further supported by these results. I next concentrated on the interaction interface of β -catenin.

6.1.9 The Armadillo repeats 10 to 12 of β -catenin are most important for an interaction with Lef Δ 293

Hurlstone and Clevers (Hurlstone and Clevers, 2002) and Graham and colleagues (Graham et al., 2000) analyzed the interaction between Tcf/Lef and β -catenin and found that primarily the Armadillo repeats 3 to 10 of β -catenin and the N-terminal part of Tcf/Lef exhibit this process. Most important seem to be the repeats 8 and 9 (von Kries et al., 2000). Furthermore Daniels and Weis (Daniels and Weis, 2005) identified a possible second β -catenin binding domain of Lef1, located close to the HMG domain. Dworak (Dworak, 2006) and Winkler (Winkler, 2008) showed that the region of β -catenin which is responsible for the second interaction with Lef1 seems to be located on C-terminal Armadillo repeats. A further goal was to analyse precisely which Armadillo repeats of β -catenin are involved in an interaction with Lef Δ 293.

Based on experiments of Winkler (Winkler, 2008) several deletion mutants of β -catenin combined with Rluc (N-terminal) and the transactivation domain of p65 (C-terminal) were created (for details see Figure 6.1.9.1).

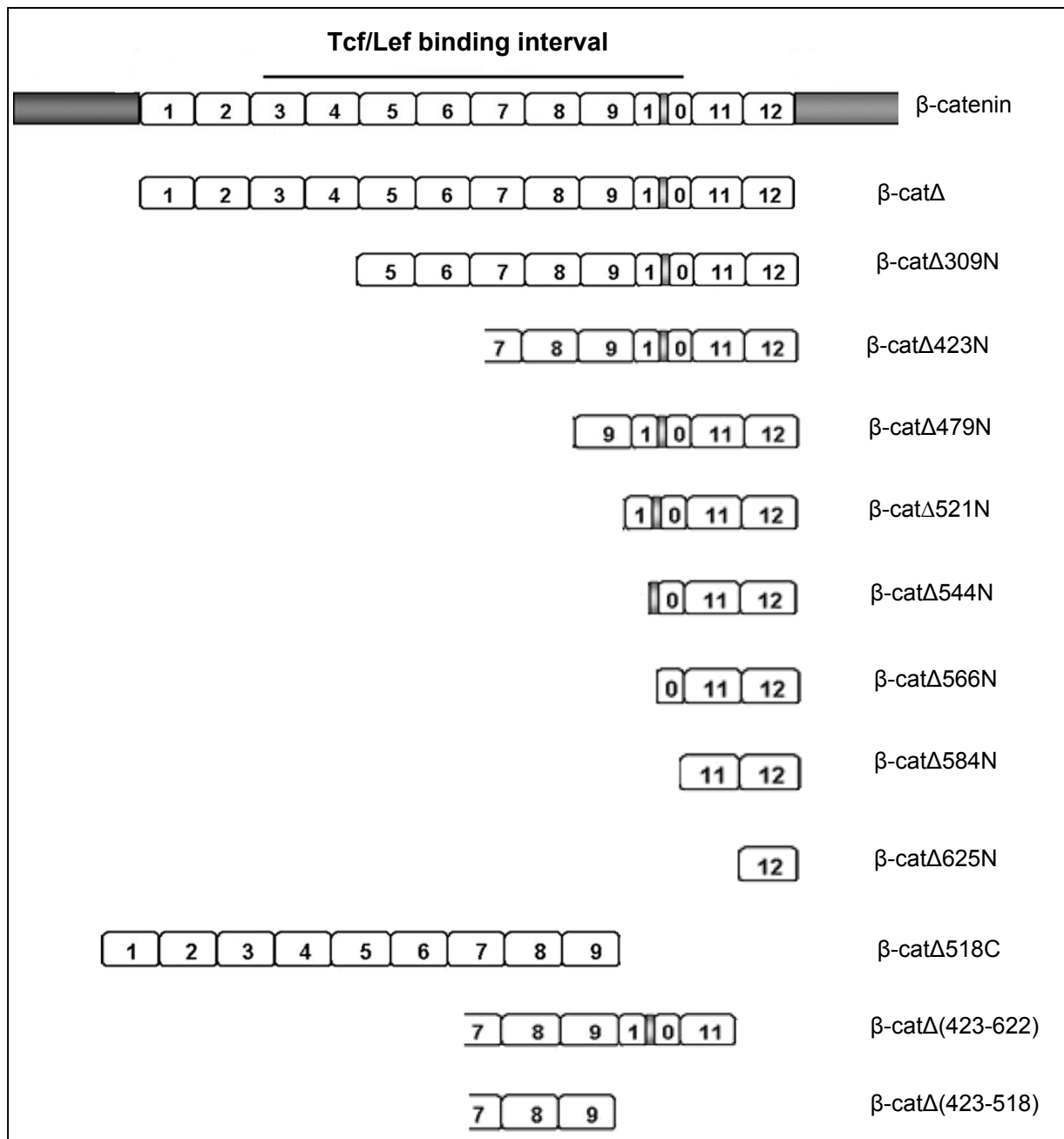


Figure 6.1.9.1: Schematic presentation of all C- and N-terminal β-catenin deletion mutants for the corresponding fusion proteins and the examination of the interaction region between β-catenin and LefΔ293 (adapted from Hurlestone and Clevers, 2002; see text for more details).

6.1.9.1 Neither C- nor N-terminal Armadillo repeats seem to be essential for the Lef HMG box / β-catenin interaction

For examinations in 293T cells the β-catenin deletion mutants β-catΔ, β-catΔ309N and β-catΔ518C were used. For this, β-catΔ was shortened N-terminally to Armadillo repeats 5 to 12 (β-catΔ309N) and C-terminally to 1 to 9 (β-catΔ518C) (see Figure 6.1.9.1). Additionally, these β-catenin deletion mutants were again fused to Renilla luciferase, to LefΔ293, separated by the myc linker and the transactivation domain of

p65. In all experiments firefly luciferase and Renilla luciferase values were measured for normalization. To make all results comparable, similar Rluc values (protein expression levels) were selected for the interpretations.

Cell line: 293T Format: 24 well Reporter: 10ng plucF4LefOtxab Background (luc): 233 RLU Background (Rluc): 352 RLU					
	0	1ng pKCRluc- LefΔ293m65	10ng pKCRluc- LefΔ293mβcatΔ65	10ng pKCRluc- LefΔ293mβcatΔ518C65	10ng pKCRluc- LefΔ293mβcatΔ309N65
RLU (luc)	63328	7646667	19559508	4011982	18038037
relative luc activity	1.0	120.7	308.9	63.4	284.8
RLU (Rluc)	398	938917	356236	172284	570878
relative luc/Rluc activity		1.0	6.5	2.7	7.4

Cell line: 293T Format: 24 well Reporter: 10ng plucF4LefOtxab Background (luc): 237 RLU Background (Rluc): 528 RLU					
	0	1ng pKCRluc- LefΔ293m65	10ng pKCRluc- LefΔ293mβcatΔ65	10ng pKCRluc- LefΔ293mβcatΔ518C65	10ng pKCRluc- LefΔ293mβcatΔ309N65
RLU (luc)	124319	3652958	7212078	2403520	9898938
relative luc activity	1.0	29.4	58.0	38.3	157.6
RLU (Rluc)	528	569897	146026	186120	338160
relative luc/Rluc activity		1.0	7.5	1.9	4.2

Cell line: 293T					
Format: 24 well					
Reporter: 10ng plucF4LefOtxab					
Background (luc): 242 RLU					
Background (Rluc): 343 RLU					
	0	1ng pKCRluc- LefΔ293m65	3ng pKCRluc- LefΔ293mβcatΔ65	3ng pKCRluc- LefΔ293mβcatΔ518C65	3ng pKCRluc- LefΔ293mβcatΔ309N65
RLU (luc)	46614	117268	53428	745757	6377688
relative luc activity	1.0	2.5	1.1	11.9	101.5
RLU (Rluc)	343	105395	2818	150171	428683
relative luc/Rluc activity		1.0	18.5	1.0	2.7

Figure 6.1.9.1.1. Localization of the central β -catenin Armadillo repeats for the potential interaction with LefΔ293. LefΔ293 and LefΔ293 with β -catΔ, β -catΔ518C or β -catΔ309N were overexpressed as fusion proteins. For luciferase detection the reporter plucF4LefOtxab was used. The resulting firefly luciferase/Renilla luciferase induction of the β -catenin truncations were calculated relative to the levels of RlucLefΔ293m65. The transfected amounts of the plasmids, on which the protein expression levels are based, are shown in the table (see text for more details).

Using the reporter 4LefOtxab (four Otxa and Otxb Tcf/Lef binding sites with a weak affinity for Lef1), high luciferase expression levels in combination with β -catΔ could be observed. In addition in the same experiments the expression of the deletion mutant β -catΔ309N led to a slightly weaker luciferase activation compared to β -catΔ. The induction for β -catΔ518C was apparently the weakest one.

Cell line: 293T					
Format: 24 well					
Reporter: 30ng plucF6LefOtxb					
Background (luc): 340 RLU					
Background (Rluc): 547 RLU					
	0	1ng pKCRluc- LefΔ293m65	1ng pKCRluc- LefΔ293mβcatΔ65	1ng pKCRluc- LefΔ293mβcatΔ518C65	1ng pKCRluc- LefΔ293mβcatΔ309 N65
RLU (luc)	32271	27602	130856	96481	54620
relative luc activity	1.0	0.9	4.1	3.0	1.7
RLU (Rluc)	574	54578	5577	5202	1990
relative luc/Rluc activity		1.0	45.6	36.4	65.3

Cell line: 293T					
Format: 24 well					
Reporter: 30ng plucF6LefOtxb					
Background (luc): 272 RLU					
Background (Rluc): 4369 RLU					
	0	1ng pKCRluc- LefΔ293m65	1ng pKCRluc- LefΔ293mβcatΔ65	1ng pKCRluc- LefΔ293mβcatΔ518C65	1ng pKCRluc- LefΔ293mβcatΔ309N 65
RLU (luc)	899	3759	3062	428	1474
relative luc activity	1.0	4.2	3.4	0.5	1.6
RLU (Rluc)	4369	314558	4886	1042	11347
relative luc/Rluc activ- ity		1.0	40.6	8.2	9.3

Figure 6.1.9.1.2. Armadillo repeats 5 to 9 of β -catenin seem to be important for a potential interaction with LefΔ293. In experiments pKCRlucLefΔ293m65 and pKCRlucLefΔ293m65 fused to β -catΔ, β -catΔ518C or β -catΔ309N were cotransfected in combination with the reporter plucF6LefOtxb. The DNA amounts were used as indicated. The relative firefly luciferase/Renilla luciferase induction caused by the expression of the β -catenin fusion protein variants was calculated relative to cells which produced RlucLefΔ293m65.

The experiments with the β -catenin deletion mutants were repeated with the reporter plucF6LefOtxb (based on six Otxb instead of four Otxa and Otxb Tcf/Lef binding sites). In these experiments the effects of β -catenin were stronger and the results more convincing. The average values of the experiments with the expression of β -catΔ and β -catΔ309N showed 48 fold and 44 fold luciferase inductions, respectively. These observations supported my hypothesis that the Armadillo repeats which are close to the N-terminus of β -catenin are not essential for the interaction with LefΔ293. Again, the expression of the deletion mutant β -catΔ518C led to luciferase activations 2 times lower than for the other two proteins.

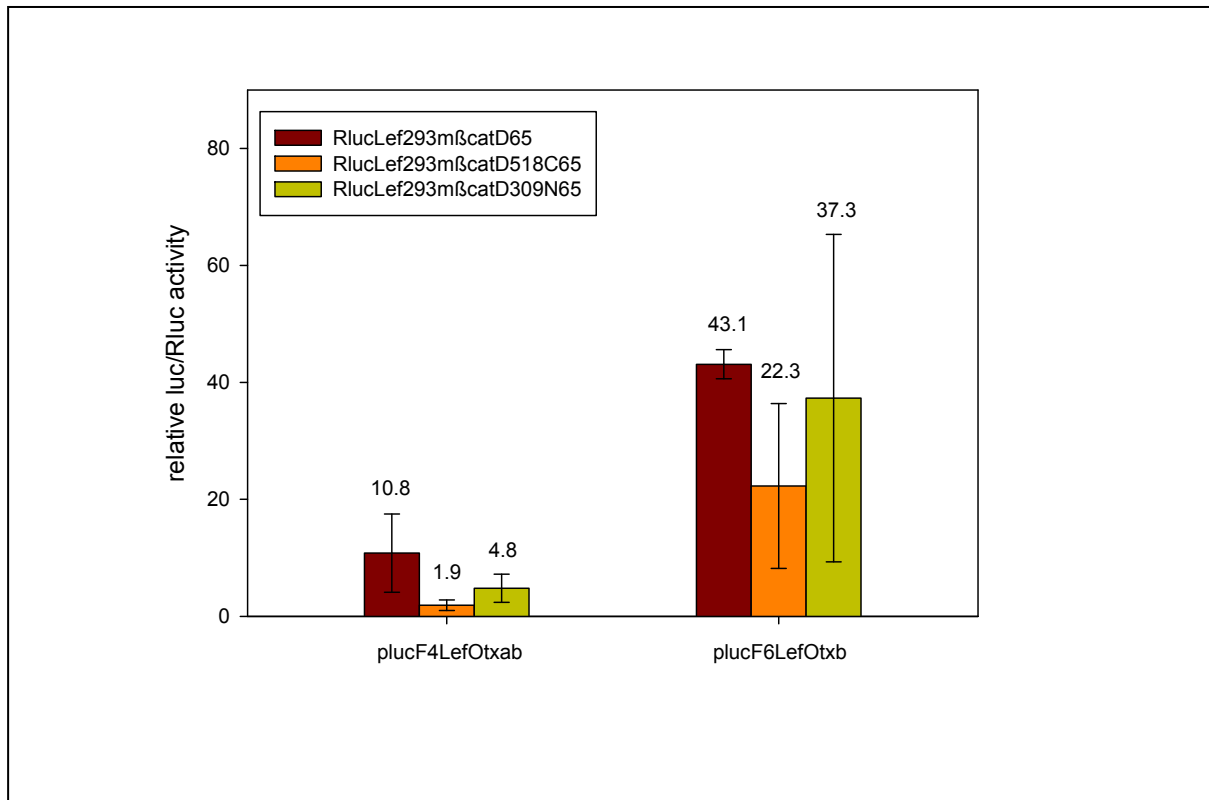


Figure 6.1.9.1.3. Experiments suggesting an interaction between Lef Δ 293 and the Armadillo repeats 5 to 9 of β -catenin in 293T cells. The relative luciferase/Renilla luciferase induction levels arose from the expression of β -cat Δ , β -cat Δ 518C or β -cat Δ 309N as fusion proteins in relation to RlucLef Δ 293m65 (see text for more details). The data represent mean relative luciferase/Renilla luciferase values of two (with reporter plucF6LefOtxb), respectively three experiments (with reporter plucF4LefOtxab). The names of the fusion proteins include a “D” instead of “ Δ ”.

Looking at the experiments with the two different reporters in 293T cells a clear hypothesis arose, namely that the second interaction domain of β -catenin with Lef Δ 293 is located within the Armadillo repeats 5 to 9. This is based on the fact that the expression of β -cat Δ 518C as well as β -cat Δ 309N resulted in a clear luciferase induction (see Figure 6.1.9.1.3). In this context it is interesting that a similar region (Armadillo repeats 3 to 10) is responsible for the strong primary interaction with the N-terminus of Lef1.

6.1.9.2 The potential Lef HMG box- β -catenin interaction region is located at C-terminal Armadillo repeats

The first experiments with deletion mutants indicated a location of the interaction interface on the central Armadillo repeats of β -catenin. Additional experiments with further β -catenin deletion mutants were performed to localize the interaction more precisely. Deletion mutants of β -catenin were created, beginning with N-terminal dele-

tions up to Armadillo repeat 10 (exact cloning descriptions in Chapter 5.1.7). Thus, the following proteins were obtained: β -cat Δ 423N (Armadillo repeats 7 to 12), β -cat Δ 479N (Armadillo repeats 9 to 12) and β -cat Δ 521N (Armadillo repeats 10 to 12). All these β -catenin versions were again expressed as fusion proteins, including Renilla luciferase, Lef Δ 293, the myc linker and the transactivation domain of p65.

In the previous experiments, in 293T cells high levels of Lef1 activation were achieved by β -cat Δ overexpression. (see Figures 6.1.9.1.1. and 6.1.9.1.2.). But for the following experiments HELA cells were utilized, which exhibit a lower basal level of the Wnt pathway than 293T cells. In addition, all transfections were performed in 96 well instead of 24 well format, allowing more meaningful statistical analysis (six instead of four repeats and automatic instead of manual measurement, due to different instruments). In the 24 well format in 293T cells higher relative luciferase/Renilla luciferase levels could be obtained at the expense of high statistical fluctuations (data not shown).

The experiments were carried out using the reporter pLucF8LefOtxab which includes eight Otxa and Otxb Tcf/Lef binding sites. As before the relative luc/RLuc induction of each of the different fusion proteins in combination with the β -catenin deletion mutants were calculated as mean values and compared to that of RlucLef Δ 293m65.

Cell line: HELA

Format: 96 well

Reporter: 90ng plucF 8Lef Otxab

Background (luc): 0.0001 RLU

Background (Rluc): 0.0002 RLU

		pKCRlucLefΔ293m65				pKCRlucLefΔ293mβcatΔ65			
	0	0.02ng	0.06ng	0.2ng	0.06ng	0.2ng	0.6ng	2ng	
RLU (luc)	0.0055	0.0516	0.043	0.2317	0.2697	0.3965	0.2657	1.1678	
relative luc activity	0.8	8.0	7.0	35.0	41.0	60.0	40.0	177.0	
RLU (Rluc)	0.0002	0.005	0.0076	0.0529	0.0095	0.0143	0.0164	0.1294	
relative luc/Rluc activity		1.1	0.6	1.3	4.5	4.0	2.7	1.8	
mean value		1.0			3.3				

	pKCRIucLefΔ293m65			pKCRIucLefΔ293mβcatΔ423N65				pKCRIucLefΔ293mβcatΔ479N65			
	0.02ng	0.06ng	0.2ng	0.06ng	0.2ng	0.6ng	2ng	0.06ng	0.2ng	0.6ng	2ng
RLU (luc)	0.0642	0.1603	0.4737	0.4135	0.5686	0.1387	1.3578	2.0882	1.5729	3.3687	3.8288
relative luc activity	10.0	24.0	72.0	63.0	86.0	21.0	206.0	317.0	239.0	512.0	582.0
RLU (Rluc)	0.0038	0.0082	0.0425	0.0088	0.0135	0.0056	0.0431	0.044	0.0549	0.1348	0.2148
relative luc/Rluc activity	1.5	1.0	0.5	2.2	2.9	1.3	1.6	2.2	1.4	1.2	1.0
mean value	1.0			2.0				1.4			

Figure 6.1.9.2.1. Fine mapping of the Armadillo repeats of β -catenin involved in the interaction with LefΔ293. The reporter plucF8LefOtxab was transfected for luciferase detection. In fusion proteins LefΔ293 and LefΔ293 fused to β -catΔ, β -catΔ423N or β -catΔ479N were expressed. The values RLU (luc) and RLU (Rluc) represent an average of six firefly or Renilla luciferase measurements subtracted by background (luc) or (Rluc). The calculation schemes were applied to all experiments in the 96 well format. The expression of β -catenin variants resulted in relative firefly luciferase/Renilla luciferase induction which was compared to the levels of RlucLefΔ293m65. The amounts of plasmids for protein expression are indicated in the table.

The effect of β -catΔ on LefΔ293 could be shown again by measuring a 3.3 fold up-regulation of luciferase levels (Figure 6.1.9.2.1.). Subsequently, the β -catenin deletion mutant β -catΔ423N was expressed and its effect on Wnt pathway activation was measured. Only a weak luciferase induction (2 fold) could be observed in comparison to β -catΔ, which includes all β -catenin Armadillo repeats. Finally, the evoked luciferase induction levels of β -catΔ479N were related to the luciferase upregulation of β -catΔ and β -catΔ423N. The luciferase induction of the first one accounted for 1.4 fold, which represented a further decrease of activity compared to the luciferase levels of β -catΔ423N. The most likely interpretation of these observations was that the interaction region of β -catenin with LefΔ293 is mainly located on Armadillo repeat 9 (previous experiments indicated that repeats 10-12 are not essential, see Chapter 6.1.9.1).

Cell line: HELA

Format: 96 well

Reporter: 90ng plucF8LefOtxab

Background (luc): 0.0001 RLU

Background (Rluc): 0.0002 RLU

	0	pKCRlucLefΔ293m65				pKCRlucLefΔ293mβcatΔ65		
		0.002ng	0.006ng	0.02ng	0.06ng	0.06ng	0.2ng	0.6ng
RLU (luc)	0.0139	0.0351	0.1212	0.2935	0.5201	0.0652	0.2040	0.4938
relative luc activity	1	3	9	21	38	5	15	36
RLU (Rluc)	0.0001	0.0097	0.0384	0.1170	0.1849	0.0048	0.0143	0.0397
relative luc/Rluc activity		1.2	1.0	0.8	0.9	4.9	4.7	4.1
mean value		1.0				4.6		

	pKCRluc- LefΔ293mβcatΔ423N65		pKCRlucLefΔ293mβcatΔ(423-622)65			
	0.2ng	0.6ng	0.02ng	0.06ng	0.2ng	0.6ng
RLU (luc)	0.0397	0.0920	0.0188	0.0139	0.0561	0.1350
relative luc activity	3	7	1	1	4	10
RLU (Rluc)	0.0024	0.0076	0.0359	0.0492	0.2349	0.6158
relative luc/Rluc activity	6.5	4.4	0.2	0.1	0.1	0.1
mean value	5.4		0.1			

Cell line: HELA

Format: 96 well

Reporter: 90ng plucF8LefOtxab

Background (luc): 0.0001 RLU

Background (Rluc): 0.0003 RLU

	0	pKCRlucLefΔ293m65			pKCRlucLefΔ293mβcatΔ65		
		0.002ng	0.006ng	0.02ng	0.06ng	0.2ng	0.6ng
RLU (luc)	0.0087	0.0485	0.2207	3.1488	0.0396	0.1972	0.4098
relative luc activity	1	3	16	227	3	14	30
RLU (Rluc)	0.0004	0.0029	0.0066	0.1254	0.0009	0.0029	0.0074
relative luc/Rluc activity		0.7	1.3	1.0	2.5	2.9	2.3
mean value		1.0			2.5		

	pKCRlucLefΔ293mβcatΔ479N65				pKCRlucLefΔ293mβcatΔ521N65			
	0.02ng	0.06ng	0.2ng	0.6ng	0.02ng	0.06ng	0.2ng	0.6ng
RLU (luc)	0.0565	0.2874	0.8549	0.3155	0.4047	0.6533	0.5634	0.6621
relative luc activity	4	21	62	23	29	47	41	48
RLU (Rluc)	0.0012	0.0030	0.0086	0.0029	0.0026	0.0066	0.0100	0.0191
relative luc/Rluc activity		4.1	3.9	4.5	6.4	4.0	2.2	1.4
mean value	4.2				3.5			

Figure 6.1.9.2.2 Comparison of the luciferase induction levels of the different fusion proteins with variable lengths of β -cat Δ . Constructs expressing fusion proteins with Lef Δ 293 and β -cat Δ 423N, β -cat Δ 479N, β -cat Δ 521N or β -cat Δ (423-622) were cotransfected in combination with the reporter plucF8LefOtxab (exact amounts indicated in the tables). The firefly luciferase/Renilla luciferase induction caused by the expression of the β -catenin variants were calculated relative to cells which produced RlucLef Δ 293m65.

The results of the previous experiment with β -cat Δ 423N and β -cat Δ 479N were not fully convincing. Therefore, I repeated the experiment using β -cat Δ 423N and β -cat Δ 479N again. In addition, for further analysis the β -catenin deletion mutants β -cat Δ (423-622) and β -cat Δ 521N were expressed. The deletion mutant β -cat Δ (423-622) was shortened from both the C- and the N-terminus, containing the Armadillo repeats 7 to 11 (see Figure 6.1.9.2.1), the expression of β -cat Δ 423N and β -cat Δ 479N caused luciferase inductions, which resemble or even weakly exceed those of β -cat Δ . These results would support the finding that the Armadillo repeat 9 is responsible for the potential Lef Δ 293 interaction. However, the application of β -cat Δ (423-622)N, which contains one Armadillo repeat less than β -cat Δ 479N (7 to 11), led to a complete breakdown of luciferase inductions. This would indicate an essential function of C-terminal repeats, which is contradictory to the results for β -cat Δ 518C (see Figure 6.1.9.1.2). Possibly the interaction interface is divided in two separate parts.

Subsequently the effect of β -cat Δ 521N, encompassing Armadillo repeats 10 to 12 was examined. The expression of this deletion mutant achieved a high luciferase induction (3.4 fold), slightly higher compared to β -cat Δ . These results suggest that primarily the C-terminal Armadillo repeats are responsible for the Lef HMG interaction. Therefore indeed a second weakly supporting region might exist in Armadillo repeats 1-9 (β -cat Δ 518C), whereas the main interaction maps to repeats 10-12 (β -cat Δ 521N). For obtaining clearer results, the experiments with β -cat Δ 518C were re-

peated and in addition deletion mutants including the Armadillo repeats 10, 11 or 12, were used.

Cell line: HELA

Format: 96 well

Reporter: 90ng plucF8LefOtxab

Background (luc): 0.0001 RLU

Background (Rluc): 0.0006 RLU

		pKCRIucLefΔ293m65			pKCRIucLefΔ293mβcatΔ65		
	0	0.006ng	0.02ng	0.06ng	0.06ng	0.2ng	0.6ng
RLU (luc)	0.0130	0.1113	0.3774	0.6918	0.3478	1.5880	0.2787
relative luc activity	1	9	29	54	27	123	22
RLU (Rluc)	0.0003	0.0085	0.0238	0.0447	0.0078	0.0322	0.0120
relative luc/Rluc activity		0.9	1.1	1.0	3.3	3.2	1.5
mean value		1.0			2.7		

	pKCRIucLefΔ293mβcatΔ423N65				pKCRIucLefΔ293mβcatΔ(423-622)65			
	0.02ng	0.06ng	0.2ng	0.6ng	0.02ng	0.06ng	0.2ng	0.6ng
RLU (luc)	0.6974	1.5141	2.4322	7.2990	0.0293	0.0713	0.1215	0.2604
relative luc activity	54	117	187	563	2	5	9	20
RLU (Rluc)	0.0146	0.0377	0.0705	0.2005	0.0118	0.0614	0.1004	0.2340
relative luc/Rluc activity	3.1	2.5	2.1	2.3	0.2	0.1	0.1	0.1
mean value	2.5				0.1			

	pKCRIucLefΔ293mβcatΔ309N65				pKCRIucLefΔ293mβcatΔ518C65			
	0.02ng	0.06ng	0.2ng	0.6ng	0.02ng	0.06ng	0.2ng	0.6ng
RLU (luc)	0.0011	0.2885	0.9305	1.2225	0.2247	0.4916	1.2377	1.8353
relative luc activity	0	22	72	94	17	38	95	141
RLU (Rluc)	0.0008	0.0069	0.0237	0.0301	0.0211	0.0518	0.1236	0.2016
relative luc/Rluc activity		3.0	2.8	2.9	0.7	0.6	0.6	0.6
mean value	2.9				0.6			

	pKCRIucLefΔ293mβcatΔ(423-518)65			
	0.02ng	0.06ng	0.2ng	0.6ng
RLU (luc)	0.0453	0.0707	0.1589	0.4923
relative luc activity	3	5	12	38
RLU (Rluc)	0.0094	0.0224	0.0618	0.1789
relative luc/Rluc activity	0.3	0.2	0.2	0.2
mean value	0.2			

Cell line: HELA

Format: 96 well

Reporter: 90ng plucF8LefOtxab

Background (luc): 0.0001 RLU

Background (Rluc): 0.0008 RLU

		pKCRIucLefΔ293m65			pKCRIucLefΔ293mβcatΔ65			
	0	0.002ng	0.006ng	0.02ng	0.06ng	0.2ng	0.6ng	2ng
RLU (luc)	0.0185	0.0457	0.1172	0.2050	0.5626	1.7452	2.5210	5.9683
relative luc activity	1	4	9	16	43	135	194	460
RLU (Rluc)	0.0004	0.0030	0.0133	0.0334	0.0218	0.0575	0.1116	0.2522
relative luc/Rluc activity		1.8	0.7	0.5	2.0	2.7	1.7	1.8
mean value		1.0			2.0			

	pKCRIucLefΔ293mβcatΔ479N65			pKCRIucLefΔ293mβcatΔ521N65		
	0.02ng	0.06ng	0.2ng	0.02ng	0.06ng	0.2ng
RLU (luc)	0.1175	0.4146	0.9015	0.6758	1.2221	2.7988
relative luc activity	9	32	69	52	94	216
RLU (Rluc)	0.0057	0.0147	0.0388	0.0124	0.0263	0.0606
relative luc/Rluc activity	1.6	2.1	1.8	4.3	3.6	3.5
mean value	1.8			3.8		

Figure 6.1.9.2.3. C-terminal Armadillo repeats of β -catenin are important for the interaction with LefΔ293. LefΔ293, β -catΔ, β -catΔ479N, β -catΔ521N, β -catΔ423N, β -catΔ(423-622), β -catΔ(423-518), β -catΔ309N or β -catΔ518C were expressed as fusion proteins. The reporter plucF8LefOtxab was used for luciferase detection. For transfection the DNA amounts indicated in the table were used. The relative firefly luciferase/Renilla luciferase induction arising from the β -catenin variants was compared to the levels of RlucLefΔ293m65.

In a next series of experiments the luciferase induction levels arising from β -catΔ, β -catΔ423N, β -catΔ479N, β -catΔ521N, β -catΔ(423-622), β -catΔ309N, β -catΔ518C and β -catΔ(423-518) were compared (Figure 6.1.9.2.3). The deletion mutant β -catΔ(423-518) included the Armadillo repeats 7 to 9. The expression of β -catΔ, β -catΔ479N, β -catΔ423N and β -catΔ521N resulted in high luciferase upregulation. The induction of β -catΔ521N even exceeded that of β -catΔ. In addition, the expression of β -catΔ309N led to a similar induction compared to β -catΔ, β -catΔ479N, and β -catΔ423N. For comparison, the addition of the deletion mutants β -catΔ(423-622) and β -catΔ(423-518), which are C-terminally truncated (repeats 12 or 10 to 12 are deleted, respectively) did not show any induction. In almost the same manner no luciferase induction

was measured with β -cat Δ 518C. This strongly indicates that the weak luciferase induction seen before (Figure 6.1.9.1.2) had only artificial character, thus probably did not reflect a possible interaction region. Another explanation would be that this effect is limited to 293T cells. However, again in this experiment all deletion mutants, which include the three C-terminal Armadillo repeats were able to interact with Lef Δ 293.

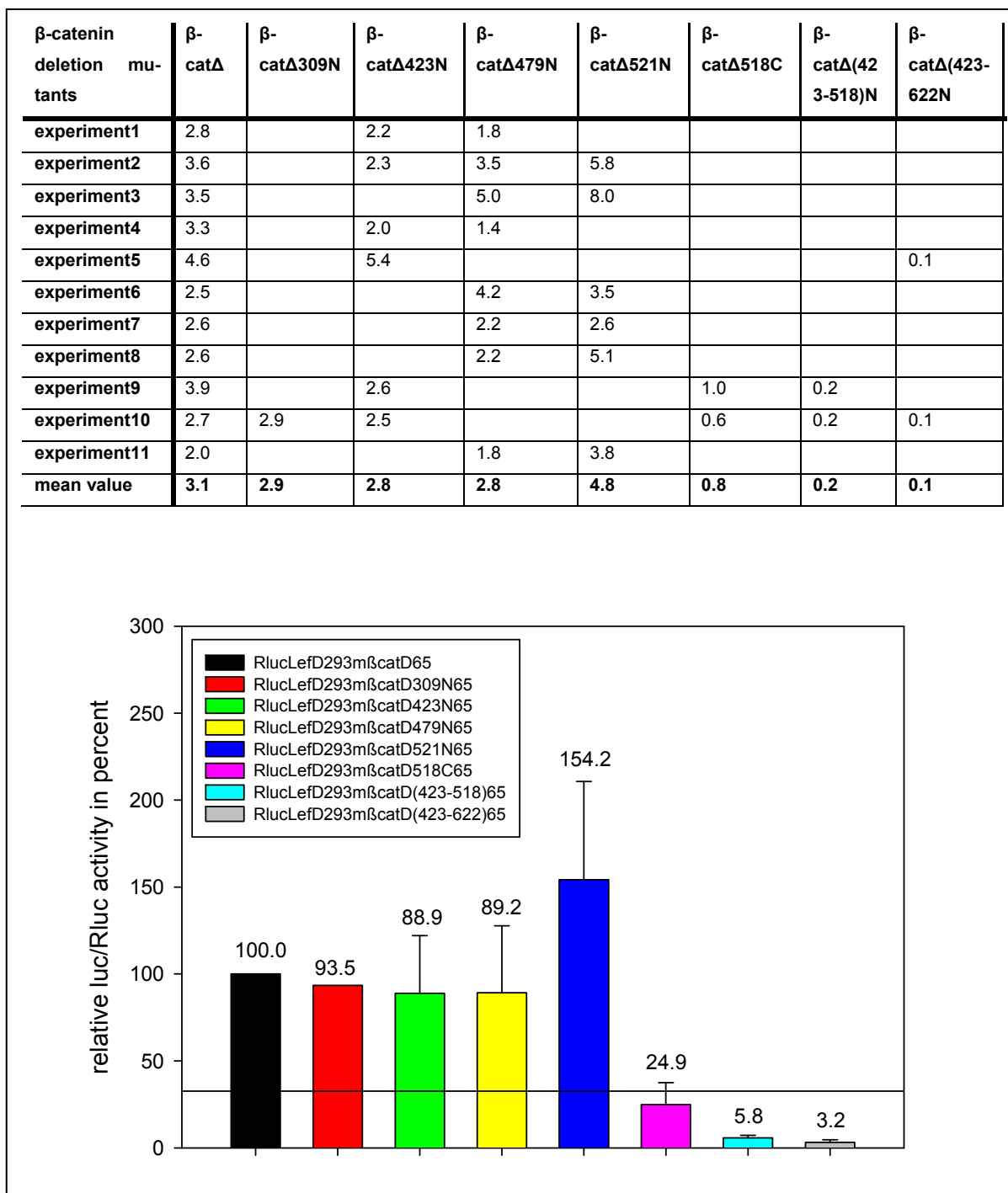


Figure 6.1.9.2.4. The last three C-terminal Armadillo repeats of β -catenin are involved in the interaction with Lef Δ 293. The β -catenin deletion mutants β -cat Δ (n=11), β -cat Δ 518C (n=2), β -cat Δ 309N (n=1), β -cat Δ 423N (n=6), β -cat Δ 479N (n=8), β -cat Δ 521N (n=6), β -cat Δ (423-518) (n=2) and β -cat Δ (423-622) (n=2) were expressed in HELA cells. The firefly luciferase/Renilla luciferase induction

caused by the expression of the β -catenin fragments were compared to the levels of RlucLef Δ 293m β cat Δ 65 (see table). Mean values in percent of n numbers of experiments. To account for the different experimental conditions, the results were calculated in percent of activity of the complete Armadillo repeats (β -cat Δ). The reference line (100/3.1=32.6) determines the level of Lef293 without β -catenin. The names of the fusion proteins include a "D" instead of " Δ ".

Summarizing all experiments, the deletion mutant β -cat Δ 521N, which expresses the Armadillo repeats 10 to 12, seems to contain an interaction domain for the HMG box of Lef1. In comparison to β -cat Δ , β -cat Δ 423N and β cat Δ 479N, which generate in combination with Lef Δ 293 similar luciferase induction levels, it accounted for a 50% stronger effect. It is not clear why the isolated Arm repeats 10-12 showed higher activity compared to all repeats. The deletion mutant β -cat Δ 518C (Arm repeats 1 to 9) did not result in any Wnt pathway upregulation. Therefore the first experiments with β -cat Δ 518C were misleading (Chapter 6.1.9.1) and could not be reproduced in the following experiments. The other deletion mutants, which lack one or all three C-terminal Armadillo repeats (β -cat Δ (423-622) and β -cat Δ (423-518)) showed no luciferase induction levels, indicating that all three C-terminal Armadillo repeats are responsible for the interaction with Lef Δ 293.

6.1.9.3 Fine mapping of the C-terminal Armadillo repeats of β -catenin interacting with the Lef1 HMG box

After locating the potential interaction with C-terminal β -catenin Armadillo repeats, a fine mapping of the Armadillo repeats 10 to 12 was performed. Therefore the C-terminus was divided into regions including the Armadillo repeats 11 to 12 (β -cat Δ 584N), only 12 (β -cat Δ 625N) and 11 to 12 including a part of the repeat 10, starting directly before (β -cat Δ (544N) or after the sequence insertion in this repeat (β -cat Δ (566N)).

Cell line: HELA

Format: 96 well

Reporter: 90ng plucF 8Lef Otxab

Background (luc): 0.0001 RLU

Background (Rluc): 0.0004 RLU

		pKCRIucLefΔ293m65				pKCRIucLefΔ293mβcatΔ65		
	0	0.006ng	0.02ng	0.06ng	0.2ng	0.06ng	0.2ng	0.6ng
RLU (luc)	0.0057	0.0137	0.0302	0.1701	0.6991	0.1838	0.7859	1.2929
relative luc activity	1	3	7	41	169	44	190	312
RLU (Rluc)	0.0005	0.0065	0.0205	0.0840	0.2567	0.0229	0.0960	0.1476
relative luc/Rluc activity		0.6	0.5	0.6	0.8	2.5	2.6	2.7
mean value		0.6				2.6		

	pKCRIucLefΔ293mβcatΔ544N65				pKCRIucLefΔ293mβcatΔ566N65			
	0.006ng	0.02ng	0.06ng	0.2ng	0.006ng	0.02ng	0.06ng	0.2ng
RLU (luc)	0.043	0.124	0.3619	0.8821	0.0248	0.0697	0.1591	0.4755
relative luc activity	8	22	64	155	4	12	28	83
RLU (Rluc)	0.0025	0.0069	0.0234	0.0682	0.0026	0.007	0.0150	0.042
relative luc/Rluc activity	5.9	5.5	4.7	4.2	3.2	3.2	3.4	3.8
mean value	5.1				3.4			

	pKCRIucLefΔ293mβcatΔ584N65			pKCRIucLefΔ293mβcatΔ625N65			
	0.02ng	0.06ng	0.2ng	0.006ng	0.02ng	0.06ng	0.2ng
RLU (luc)	0.0179	0.0250	0.1272	0.0109	0.0195	0.0444	0.2615
relative luc activity	3	4	22	2	3	8	46
RLU (Rluc)	0.0064	0.0186	0.0737	0.0037	0.0101	0.0248	0.0926
relative luc/Rluc activity	0.9	0.4	0.5	1.1	0.7	0.6	0.9
mean value	0.6			0.8			

	pKCRIucLefΔ293mβcatΔ479N65				pKCRIucLefΔ293mβcatΔ521N65			
	0.006ng	0.02ng	0.06ng	0.2ng	0.006ng	0.02ng	0.06ng	0.2ng
RLU (luc)	0.0107	0.0461	0.0884	0.4248	0.0936	0.4155	1.0896	2.2419
relative. luc activity	23	3	11	21	102	100	263	541
RLU (Rluc)	0.0017	0.0068	0.0171	0.0672	0.0077	0.0296	0.0587	0.1351
relative luc/Rluc activity	2.5	2.6	1.7	1.9	4.7	4.5	5.7	5.4
mean value	2.2				5.1			

Figure 6.1.9.3.1. Highest luciferase induction levels by all the C-terminal Armadillo repeats. LefΔ293 and LefΔ293 in combination β-catΔ, β-catΔ479N, β-catΔ521N, β-catΔ544N, β-catΔ566N, β-catΔ584N or β-catΔ625N were overexpressed. For luciferase detection the reporter plucF8LefOtxab was transfected. The transfected DNA amounts are indicated in the table. The resulting firefly luciferase/Renilla

luciferase induction of the β -catenin variants as a part of fusion proteins was calculated relative to the levels of RLucLef Δ 293m65 (see text for more details).

A first analysis showed that β -cat Δ 521N as well as β -cat Δ 544N are responsible for the highest luciferase induction levels, exceeding those of β -cat Δ and β -cat Δ 479N (Figure 6.1.9.3.1.). In comparison, the application of β -cat Δ 566N reduced these high levels of upregulation to the normal levels seen for β -cat Δ and β -cat Δ 479N. However, the expression of the other two deletion mutants led to a complete drop of the β -catenin effect on Lef1.

Cell line: HELA

Format: 96 well

Reporter: 90ng plucF 8Lef Otxab

Background (luc): 0.0001 RLU

Background (RLuc): 0.0002 RLU

	pKCRlucLef Δ 293m65				pKCRlucLef Δ 293m β cat Δ 65		
	0	0.006ng	0.02ng	0.06ng	0.02ng	0.06ng	0.2ng
RLU (luc)	0.0022	0.0010	0.0206	0.0455	0.0128	0.0588	0.1486
relative luc activity	1	0	5	12	3	16	39
RLU (RLuc)	0.0003	0.0004	0.0043	0.0106	0.0014	0.0036	0.0159
relative luc/RLuc activity		1.0	0.8	0.5	3.0	3.2	1.7
mean value		0.8			2.6		

	pKCRlucLef Δ 293m β cat Δ 544N65		pKCRlucLef Δ 293m β cat Δ 566N65	
	0.02ng	0.06ng	0.02ng	0.06ng
RLU (luc)	0.0813	0.1134	0.0297	0.0552
relative luc activity	37	52	14	25
RLU (RLuc)	0.0030	0.0081	0.0027	0.0046
relative luc/RLuc activity	6.2	2.6	1.8	2.1
mean value	4.4		2.0	

	pKCRlucLef Δ 293m β cat Δ 584N65			pKCRlucLef Δ 293m β cat Δ 625N65		
	0.02ng	0.06ng	0.2ng	0.02ng	0.06ng	0.2ng
RLU (luc)	0.0078	0.0236	0.1078	0.0212	0.0234	0.0148
relative luc activity	4	11	49	10	11	7
RLU (RLuc)	0.0039	0.0118	0.0342	0.0081	0.0097	0.0050
relative luc/RLuc activity	0.4	0.4	0.6	0.4	0.4	0.6
mean value	0.5			0.5		

	pKCRIucLefΔ293mβcatΔ479N65				pKCRIucLefΔ293mβcatΔ521N65			
	0.006ng	0.02ng	0.06ng	0.2ng	0.002ng	0.006ng	0.02ng	0.06ng
RLU (luc)	0.0221	0.0981	0.1602	0.4082	0.0207	0.0232	0.1402	0.3092
relative luc activity	6	26	42	108	5	6	37	82
RLU (Rluc)	0.0015	0.0067	0.0203	0.0511	0.0021	0.0024	0.0093	0.0168
relative luc/Rluc activity	2.6	3.1	1.6	1.6	1.9	1.7	3.2	3.6
mean value	2.2				2.6			

Figure 6.1.9.3.2. A limited region of Armadillo repeat 10 is important for a LefΔ293 interaction. The constructs including LefΔ293 or LefΔ293 with β-catΔ, β-catΔ479N, β-catΔ584N, β-catΔ625N, β-catΔ521N, β-catΔ544N or β-catΔ566N were cotransfected in combination with the reporter plucF8LefOtxab. The firefly luciferase/Renilla luciferase induction caused by the expression of the β-catenin fragments was calculated relative to cells which produced RlucLefΔ293m65. The amounts of plasmids in the transfection are shown in the tables.

A further confirmation of an involvement of the three C-terminal Armadillo repeats in an interaction with LefΔ93 showed the repetition of the experiment 6.1.9.3.1, using the same constructs like before (see Figure 6.1.9.3.2). Here, the expression of β-catΔ544N resulted in the strongest luciferase upregulation.

β-catenin deletion mutants	β-cat Δ	β-catΔ309 N	β-catΔ423 N	β-catΔ479 N	β-catΔ521 N	β-catΔ544 N	β-catΔ566 N	β-catΔ584 N	β-catΔ625 N
experiment1	2.8		2.2	1.8					
experiment2	3.6		2.3	3.5	5.8				
experiment3	3.5			5.0	8.0				
experiment4	3.9		2.6						
experiment5	3.3		2.0	1.4					
experiment6	4.6		5.4						
experiment7	2.5			4.2	3.5				
experiment9	2.7	2.9	2.5						
experiment9	2.0			1.8	3.8				
experiment10	2.6			2.2	5.1	5.1	3.4	0.6	0.8
experiment11	2.6			2.2	2.6	4.4	2.0	0.5	0.5
mean value	3.1	2.9	2.8	2.8	4.8	4.8	2.7	0.6	0.7

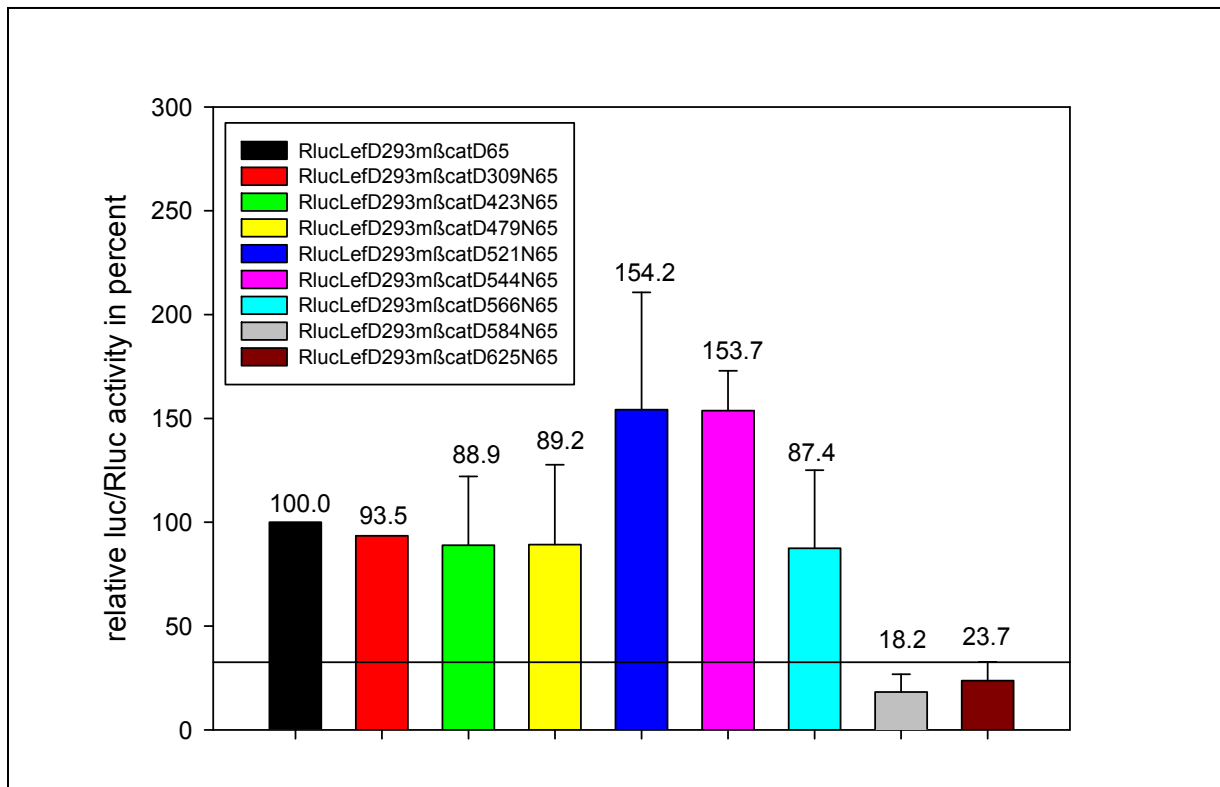


Figure 6.1.9.3.3. A part of the Armadillo repeat 10 in combination with the Armadillo repeats 11 and 12 of β -catenin are essential for the interaction with Lef Δ 293. In fusion proteins the β -catenin variants β -cat Δ (n=11), β -cat Δ 309N (n=1), β -cat Δ 423N (n=4), β -cat Δ 479N (n=8), β -cat Δ 521N (n=6), β -cat Δ 544N (n=2), β -cat Δ 566N (n=2), β -cat Δ 584N (n=2) and β -cat Δ 625N (n=2) were expressed in HELA cells. The expression of the different β -catenin fragments resulted in relative firefly luciferase/Renilla luciferase induction levels which were compared to the levels of RlucLef Δ 293m β cat Δ 65 (see table). Mean values in percent of n numbers of experiments. The reference line (100/3.1=32.6) determines the level of Lef293 without β -catenin. The names of the fusion proteins include a "D" instead of " Δ ".

A direct comparison of the induced luciferase levels of all N-terminally truncated deletion mutants of β -catenin, show highest activity for the Armadillo repeats 10 to 12, for the interaction with Lef Δ 293 (see Figure 6.1.9.3.3). Therefore the short N-terminal region of Armadillo repeat 10 does not participate in the interaction. This is underlined by the highest luciferase induction levels, caused by β -cat Δ 521N and β -cat Δ 544N.

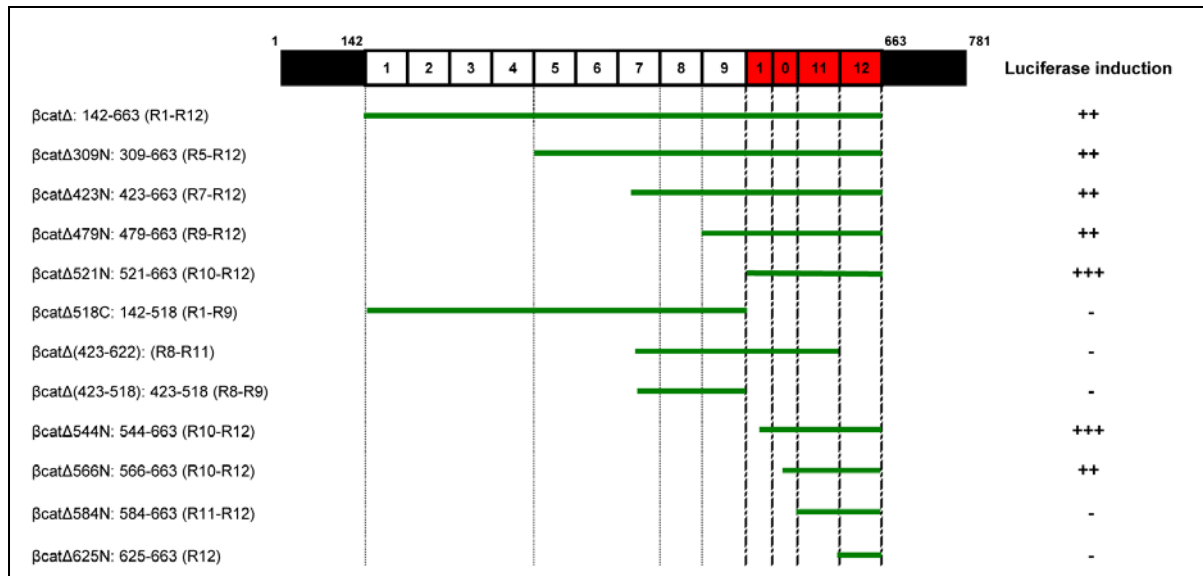


Figure 6.1.9.3.4. Relative luciferase/Renilla luciferase induction of all β -catenin deletion mutants (expressed as fusion proteins together with Rluc, Lef Δ 293 and p65) in relation to RlucLef Δ 293m65, summarizing experiments in 293T and HELA cells. The “+” indicates measured luciferase induction. For β -cat Δ 518C a weak induction in 293T, but not in HELA cells was observed, indicating an artificial character or an effect limited to 293T cells (-/+?).

All experiments performed in 293T cells as well in HELA cells, using the different β -catenin deletion mutants result in a clear picture of the β -catenin regions important for the interaction with Lef Δ 293. All N-terminally truncated β -catenin deletion mutants (having a complete C-terminus) up to amino acid 566 led to convincing luciferase upregulation. The C-terminal deletion β -cat Δ (423-622), which contains the Armadillo repeats 10 and 11, but not 12 resulted in a complete break down of luciferase induction. Therefore the Armadillo repeats 11 and 12 together with part of 10 are involved in an interaction with Lef Δ 293. The existence of a second interaction domain, located on the Armadillo repeats 1 to 9 (based on weak luciferase levels of β -cat Δ 518C, see Chapter 6.1.9.1) could not be verified in the experiments again.

6.1.10 Plakoglobin is able to interact with the HMG domain of Lef1

In vertebrates Plakoglobin or γ -catenin, a second member of the catenin family, can fulfill similar functions like β -catenin in Wnt signaling, concerning upregulation of special target genes (Kolligs et al., 2002; Williams et al., 2006). But, in Plakoglobin only interactions with Lef1 (Behrens et al., 1996; Huber et al., 1996) or Tcf4 (Simcha et al., 1998; Zhurinsky et al., 2000a; Miravet et al., 2002) were examined until now. In combination with Lef1 inhibition or upregulation and in combination with Tcf4 primari-

ly activating processes were described (Kolligs et al, 2002; Williamson et al, 2006). The interactions are achieved by the Plakoglobin Armadillo repeats, which are in structure and sequence highly homologous to that of β -catenin (83% sequence similarity; Zhurinsky et al., 2000a, Huber et al., 1997; see Figure 4.2.2.). These facts are the reason why we additionally tried to analyze whether Plakoglobin is able to interact with Lef Δ 293. For this, two deletion mutants were developed, including all 12 Armadillo repeats of Plakoglobin, Pla Δ , and a N-terminally further truncated version Pla Δ 473N, containing the Armadillo repeats 7 to 12 (for more details see Chapter 6.1.7). In the same manner as before done for β -catenin, the two Plakoglobin variants were expressed as fusion proteins in combination with Renilla luciferase, Lef Δ 293, the myc linker and the transactivation domain of p65. The two representative experiments 6.1.10.1 and 6.1.10.2 are shown.

Cell line: HELA

Format: 96 well

Reporter: 90ng plucF 8Lef Otxab

Background (luc): 0.0001 RLU

Background (Rluc): 0.0002 RLU

		pKCRlucLefΔ293m65			pKCRlucLefΔ293mβcatΔ65			
		0	0.002ng	0.006ng	0.02ng	0.06ng	0.2ng	0.6ng
RLU (luc)	0.0177	0.0582	0.1885	3.5019	0.1011	0.2892	0.6798	1.3435
relative luc activity	1.0	4.0	14.0	253.0	7.0	21.0	49.0	97.0
RLU (Rluc)	0.0004	0.0052	0.0288	0.5167	0.0031	0.0076	0.0214	0.0410
relative luc/Rluc activity		1.4	0.8	0.8	3.8	4.5	3.8	3.9
mean value		1.0			4.1			

	pKCRlucLef Δ 293mPla Δ 65				pKCRlucLef Δ 293mPla Δ 473N65			
	0.006ng	0.02ng	0.06ng	0.2ng	0.02ng	0.06ng	0.2ng	0.6ng
RLU (luc)	0.0467	0.0660	0.2146	0.4857	0.0200	0.0469	0.0602	0.8142
relative luc activity	3.0	5.0	15.0	35.0	1.0	3.0	4.0	59.0
RLU (Rluc)	0.0017	0.0043	0.0113	0.0242	0.0011	0.0015	0.0028	0.0304
relative luc/Rluc activity	4.0	1.7	2.4	2.4	2.9	4.4	2.7	3.1
mean value	2.6				3.3			

	0	pKCRIucLefΔ293m65				pKCRIucLefΔ293mβcatΔ65		
		0.002ng	0.006ng	0.02ng	0.06ng	0.06ng	0.2ng	0.6ng
RLU (luc)	0.0139	0.0351	0.1212	0.2935	0.5201	0.0652	0.2040	0.4938
relative luc activity	1.0	3.0	9.0	2.01	38.0	5.0	15.0	36.0
RLU (Rluc)	0.0001	0.0097	0.0384	0.1170	0.1849	0.0048	0.0143	0.0397
relative luc/Rluc activity		1.2	1.0	0.8	0.9	4.9	4.7	4.1
mean value		1.0				4.6		

	pKCRIucLefΔ293mβcatΔ423N65	
	0.2ng	0.6ng
RLU (luc)	0.0397	0.0920
relative luc activity	3.0	7.0
RLU (Rluc)	0.0024	0.0076
relative luc/Rluc activity	6.5	4.4
mean value	5.4	

Figure 6.1.10.1. Effects of Plakoglobin on the DNA binding of the Lef HMG domain. Fusion proteins LefΔ293 between Rluc, p65, the myc linker and β-catΔ, β-catΔ423N, PlaΔ or PlaΔ473N were expressed together with the reporter plucF8LefOtxab (see tables for the transfected DNA amounts). The firefly luciferase/Renilla luciferase induction caused by the expression of the β-catenin or Plakoglobin variants was calculated relative to cells which produced RlucLefΔ293m65 (see text for more details).

The luciferase induction levels for β-catΔ, β-catΔ423N, PlaΔ and Pla473ΔN were analyzed (Figure 6.1.10.1). Both Plakoglobin constructs showed a clear upregulation of LefΔ293 DNA binding. The β-catenin constructs (β-catΔ and β-catΔ423N) used as a reference behaved as before (Figure 5.1.9.2.2). The inductions were slightly higher as seen for Plakoglobin. But clearly C-terminal Armadillo repeats of Plakoglobin showed the same effect on the HMG domain as that of β-catenin.

Cell line: HELA

Format: 96 well

Reporter: 90ng plucF 8Lef Otxab

Background (luc): 0,0001 RLU

Background (Rluc): 0,0006 RLU

	0	pKCRIucLefΔ293m65			pKCRIucLefΔ293mβcatΔ65			
		0.002ng	0.006ng	0.02ng	0.06ng	0.2ng	0.6ng	2ng
RLU (luc)	0.0192	0.0471	0.0806	0.2982	0.5505	2.0699	0.7505	2.0342
relative luc activity	1	4	6	23	42	160	58	157
RLU (Rluc)	0.0006	0.0048	0.0154	0.0513	0.0238	0.0838	0.0568	0.1508
relative luc/Rluc activity		1.5	0.7	0.8	3.0	3.2	1.8	1.8
mean value		1.0			2.4			

	pKCRIucLefΔ293mPlaΔ65				pKCRIucLefΔ293mPlaΔ473N65			
	0.006ng	0.02ng	0.06ng	0.2ng	0.02ng	0.06ng	0.2 ng	0.6ng
RLU (luc)	0.0206	0.3056	0.4902	2.9442	0.0668	0.3398	1.5117	2.0836
relative luc activity	2	24	38	227	5	26	117	161
RLU (Rluc)	0.0017	0.0131	0.0394	0.1761	0.0063	0.0205	0.0781	0.1228
relative luc/Rluc activity	2.7	2.4	1.7	2.4	1.5	2.3	2.6	2.2
mean value	2.3				2.2			

	0	pKCRIucLefΔ293m65			pKCRIucLefΔ293mβcatΔ65		
		0.006ng	0.02ng	0.06ng	0.06ng	0.2ng	0.6ng
RLU (luc)	0.0130	0.1113	0.3774	0.6918	0.3478	1.5880	0.2787
relative luc activity	1	9	29	54	27	123	22
RLU (Rluc)	0.0003	0.0085	0.0238	0.0447	0.0078	0.0322	0.0120
relative luc/Rluc activity		0.9	1.1	1.0	3.3	3.2	1.5
mean value		1.0			2.7		

	pKCRIucLefΔ293mβcatΔ423N65			
	0.02ng	0.06ng	0.2ng	0.6ng
RLU (luc)	0.6974	1.5141	2.4322	7.2990
relative luc activity	54	117	187	563
RLU (Rluc)	0.0146	0.0377	0.0705	0.2005
relative luc/Rluc activity	3.1	2.5	2.1	2.3
mean value	2.5			

Figure 6.1.10.2. Clear but weak potential of Plakoglobin for a LefΔ293 interaction. LefΔ293 and LefΔ293 with the β-catenin variants β-catΔ, β-catΔ423N or the Plakoglobin fragments PlaΔ and PlaΔ473N were overexpressed. The reporter plucF8LefOtxab was used for luciferase detection. The expression of the β-catenin and Plakoglobin variants in fusion proteins led to relative firefly lucife-

rase/Renilla luciferase induction which was calculated relative to the levels of RlucLef Δ 293m65. The transfected amounts of plasmids are listed in the tables.

A repetition of this experiment (Figure 6.1.10.2) gave the same result for Pla Δ and Pla Δ 473N (2.3 fold, respectively 2.2 fold). Again the induction levels with the reporter plucF8LefOtxab were lower than those for β -cat Δ or β -cat423N. Therefore, the potential of Plakoglobin in controlling the DNA binding of Lef1 is weaker compared to β -catenin.

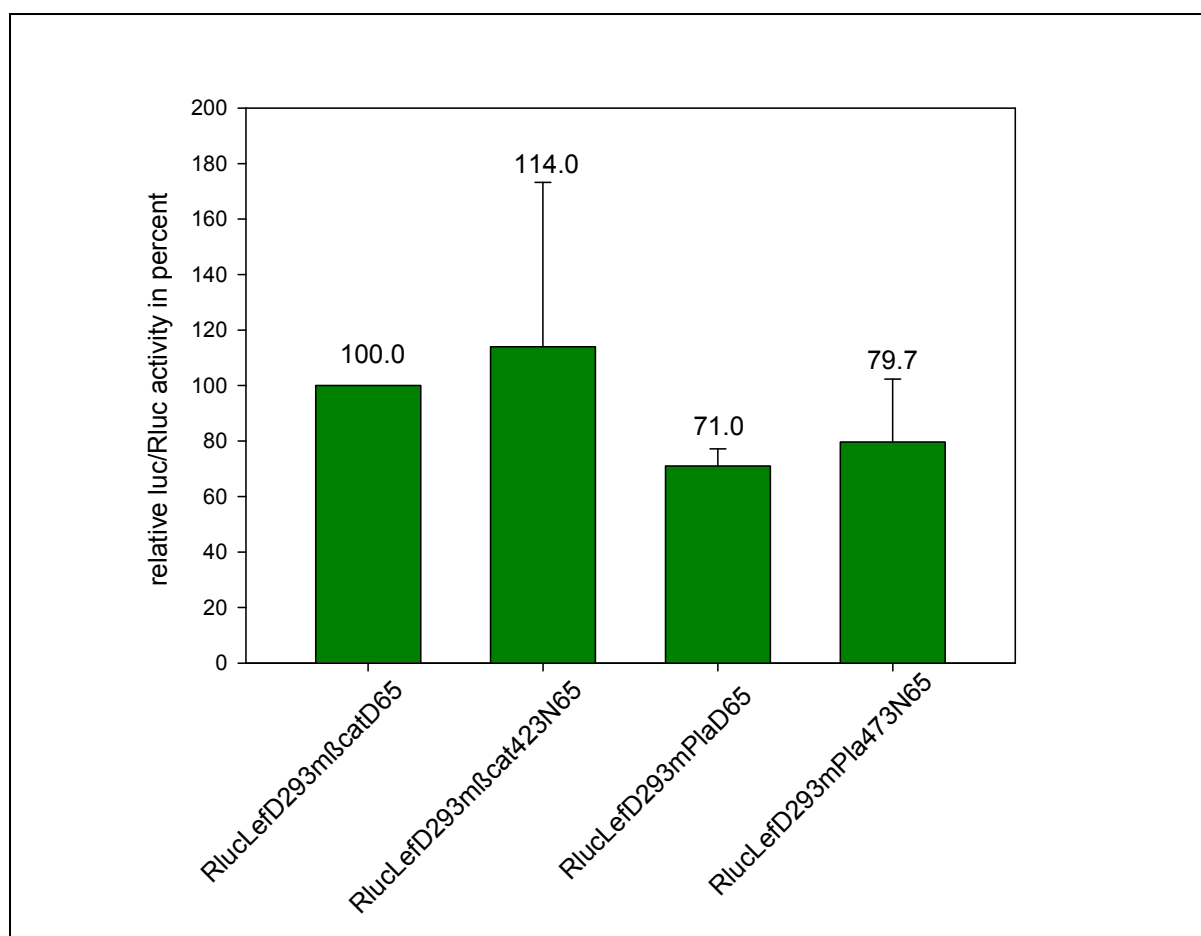


Figure 6.1.10.3. The Armadillo repeats of Plakoglobin and β -catenin seem to have similar potential for the interaction with Lef Δ 293. The fusion proteins including β -cat Δ 423N or Pla Δ 473N, β -cat Δ and Pla Δ were expressed in HELA cells (see text for more details). The firefly luciferase/Renilla luciferase inductions caused by the expression of the β -catenin or Plakoglobin variants were compared relative to firefly luciferase/Renilla luciferase inductions of RlucLef Δ 293m β cat Δ 65, respectively RlucLef Δ 293mPla Δ 65. The points represent mean values of four experiments in percent. The names of the fusion proteins include a “D” instead of “ Δ ”.

Summarizing the data, Plakoglobin performs a similar function like β -catenin reinforcing the DNA binding of Lef1 (see Figure 6.1.10.3). Whether this process results in activation or repression, depends on the target genes and the interaction partners Lef1 or Tcf4.

6.1.11 The effect of β -catenin Δ on Lef1-DNA binding is specific for defined Tcf/Lef binding motifs

6.1.11.1 β -catenin Δ -Lef Δ 293 interaction in combination with *Otx2* enhancer binding motifs

For the analysis of the Wnt pathway, several different reporters were designed, containing multimerized Tcf/Lef binding motifs which differ in their sequences. The sequence composition of the Tcf/Lef binding sites had both pure artificial or natural character. Highest attention was turned to artificial reporters with high affinity binding sites, which were designed for strong activation. At the beginning of this thesis, a widely used luciferase reporter, named TopFlash (Korinek et al., 1997) was used for our experiments, containing consensus binding sites for Tcf/Lef. In addition, another reporter was also used (pLefluc), possessing also artificial Tcf/Lef binding sites, but with a slightly reduced affinity compared to TopFlash. This reporter was designed by Grosschedl and colleagues (Hsu et al., 1998) (for further details see Chapter 5.1.1). In first experiments, a comparison of these two reporters showed higher luciferase upregulation with the Lefluc reporter than with the TopFlash reporter when β -cat Δ was expressed (data not shown). This fact, together with the idea that reporters with natural Tcf/Lef binding motifs, might result in a stronger effect of β -catenin on Lef HMG box DNA binding, led to the design of reporters with modified Tcf/Lef consensus sequences. As a first step I used the core region of the mid-hindbrain specific enhancer of the Wnt target gene *Otx2*, which is here referred as EOtxFMcore. This region includes two Tcf/Lef binding sites (Otxa and Otxb), one Pax and one Otx binding motif. The *Otx2* enhancer fulfills its function during early brain development in vertebrates by regulating the expression of *Otx2* (Kurokawa et al., 2004). For my experiments I cloned the core enhancer region upstream of a Fos minimal promoter for firefly luciferase expression.

Based on the results of the previous experiments (see Chapter 6.1.9), the constructs RlucLef Δ 293m65 and RlucLef Δ 293m β cat Δ 65 were transfected into HELA cells, together with different reporters. Again the Rluc values were measured and used as an internal reference for the expression level. The firefly luciferase/Renilla luciferase inductions caused by the expression of RlucLef Δ 293m β cat Δ 65 were calculated relative

to cells which produce RlucLef Δ 293m65. For a correct interpretation the corresponding relative luciferase/Renilla luciferase inductions arising from the expression of RlucLef Δ 293m65 and RlucLef Δ 293m β cat Δ 65 were evaluated and compared among each other within one experiment.

Cell line: HELA

Format: 96 well

Reporter: 90ng pMlucF 16EotxFMcore

Background (luc): 0.0001 RLU

Background (Rluc): 0.0003 RLU

	0	pKCRlucLef Δ 293m65			pKCRlucLef Δ 293m β cat Δ 65			
		0.02ng	0.06ng	0.2ng	0.06ng	0.2ng	0.6ng	2ng
RLU (luc)	0.0255	0.0401	0.0528	0.1864	0.0483	0.0585	0.0971	0.2353
relative luc activity	1.0	1.6	2.1	7.3	1.9	2.3	3.8	9.3
RLU (Rluc)	0.000	0.0077	0.0288	0.1754	0.0030	0.0066	0.0251	0.0751
relative luc/Rluc activity		2.0	0.7	0.4	6.4	3.1	1.4	1.0
mean value		1.0			3.0			

Cell line: HELA

Format: 96 well

Reporter: 90ng pMlucF 16EotxFMcore

Background (luc): 0.0001 RLU

Background (Rluc): 0.0004 RLU

	0	pKCRlucLef Δ 293m65			pKCRlucLef Δ 293m β cat Δ 65			
		0.02ng	0.06ng	0.2ng	0.06ng	0.2ng	0.6ng	2ng
RLU (luc)	0.0094	0.0359	0.0509	0.2082	0.0484	0.0769	0.1307	0.1256
relative luc activity	1.0	3.9	5.5	22.5	5.2	8.3	14.1	13.6
RLU (Rluc)	0.000	0.0297	0.0877	0.4044	0.0076	0.0251	0.0507	0.1362
relative luc/Rluc activity		1.6	0.7	0.7	8.1	4.0	3.2	1.1
mean value		1.0			4.1			

Figure 6.1.11.1.1. Improved Lef Δ 293-DNA binding caused by β -cat Δ within the Otx2 enhancer. The reporter pMlucF16EotxFMcore reflects the natural situation of the full Otx2 enhancer. The constructs pKCRlucLef Δ 293m65 or pKCRlucLef Δ 293m β cat Δ 65 were cotransfected with the reporter pMlucF16EotxFMcore (used DNA amounts as indicated).

The reporter pMlucF16EOtxFMcore, which was used for the first analysis, reflects the natural situation of the human *Otx2* enhancer with two antiparallel Tcf/Lef binding sites, Otxa and Otxb, respectively. Thus, each of the different *Otx* Tcf/Lef binding sites was present 16 times as part of the reporter (see Chapter 5.1.7 for design). In the course of the experiment (Figure 6.1.11.1.1) an increase of luciferase expression could be observed, when RlucLef Δ 293m β cat Δ 65 was expressed. Compared to RlucLef Δ 293m65 the luciferase expression was elevated 3 to 4 fold. This means that under transient conditions β -cat Δ contributes to a more efficient Lef1-DNA binding, suggesting a similar situation for the *Otx2* enhancer.

Cell line: HELA

Format: 96 well

Reporter: 90ng plucF 8Lef Otxab

Background (luc): 0.0001 RLU

Background (Rluc): 0.0003 RLU

	0	pKCRIucLef Δ 293m65		pKCRIucLef Δ 293m β cat Δ 65			
		0.06ng	0.2ng	0.06ng	0.2ng	0.6ng	2ng
RLU (luc)	0.0176	0.0208	0.6966	0.1086	0.3358	0.7920	1.7268
relative luc activity	1.0	1.2	40	6.2	19	45	99
RLU (Rluc)	0.0000	0.0005	0.0818	0.0038	0.0150	0.0383	0.0910
relative luc/Rluc activity			1.0	4.2	3.6	3.1	2.9
mean value		1.0		3.4			

Cell line: HELA

Format: 96 well

Reporter: 90ng plucF 8Lef Otxab

Background (luc): 0.0001 RLU

Background (Rluc): 0.0004 RLU

	0	pKCRIucLef Δ 293m65			pKCRIucLef Δ 293m β cat Δ 65			
		0.02ng	0.06ng	0.2ng	0.06ng	0.2ng	0.6ng	2ng
RLU (luc)	0.0055	0.0516	0.0430	0.2317	0.2697	0.3965	0.2657	1.1678
relative luc activity	0.8	8	7	35	41	60	40	177
RLU (Rluc)	0.0002	0.0050	0.0076	0.0529	0.0095	0.0143	0.0164	0.1294
relative luc/Rluc activity		1.1	0.6	1.3	4.5	4.0	2.7	1.8
mean value		1.0			3.3			

Figure 6.1.11.1.2. Improved Lef Δ 293-DNA binding caused by β -cat Δ on Otxa and Otxb Tcf/Lef motifs. The reporter plucF8LefOtxab consists of 8 Otxa and 8 Otxb Tcf/Lef binding sites, which are alternatively arranged. The fusion proteins which include Lef Δ 293 or Lef Δ 293 in combination with β -cat Δ

were overexpressed. The reporter plucF8LefOtxab was transfected for luciferase detection. The transfected amounts of plasmids are shown in the table.

Subsequently, the two different Tcf/Lef binding sites Otxa and Otxb of the *Otx2* enhancer were separated from the remaining part of the enhancer and were repeated 8 times, resulting in the luciferase reporter plucF8LefOtxab with 16 binding motifs. In comparison to the reporter pMlucF16EOtxFMcore the enhancer sequence was thus reduced to the Tcf/Lef binding sites. The natural state of the *Otx2* enhancer should be kept by this but with more binding sites and different flanking sites. The application of the construct pKCRlucLef Δ 293m β cat Δ 65 led to a clear luciferase induction of around 3.3 fold in comparison to Lef Δ 293 without β -catenin, which is in the same range as the luciferase upregulation with the pMlucF16EOtxFMcore reporter (Figure 6.1.11.1.2). Therefore the Tcf/Lef sites in the context of the *Otx* enhancer behave in the same way as the isolated artificial binding sites Otxa and Otxb.

6.1.11.2 A potential β -catenin effect on Lef-DNA binding in the natural promoters of *Axin2* and *c-myc*

To test my findings on the β -cat Δ -Lef Δ 293-DNA interaction in a natural promoter context, I selected the Wnt target gene *c-myc*. The reason for this was the result of ChIP experiments, in which it could be shown that in the case of Wnt upregulation, special parts of the human *c-myc* enhancers (consisting of five different Tcf/Lef binding sites) were occupied by Tcf4, but are devoid of it during Wnt downregulation (Yochum et al., 2008). This fact provides a basis for a possible β -catenin effect on Lef1-DNA binding and for the analysis of the situation in the *c-myc* regulatory regions. For this approach (Figure 6.1.11.2.1) I used the reporter pMyc5'3'luc with the four most prominent *c-myc* Tcf/Lef binding sites, generated by Yochum et al. (2008), stimulating the natural event.

Cell line: HELA

Format: 96 well

Reporter: 90ng pMyc5'3'luc

Background (luc): 0.0001 RLU

Background (Rluc): 0.0005 RLU

	0	pKCRlucLefΔ293m65			pKCRlucLefΔ293mβcatΔ65		
		0.01ng	0.02ng	0.06ng	0.06ng	0.2ng	0.6ng
RLU (luc)	0.0653	0.0748	0.0922	0.1096	0.0493	0.1121	0.2363
relative luc activity	1.0	1.1	1.4	1.7	0.8	1.7	3.6
RLU (Rluc)	0.0005	0.0807	0.2929	0.6239	0.0293	0.1832	0.5822
relative luc/Rluc activity		2.0	0.7	0.4	3.4	1.2	0.8
mean value		1.0			1.8		

Figure 6.1.11.2.1. Improved LefΔ293-DNA binding caused by β-catΔ within the *c-myc* regulatory regions. The relative luciferase/Renilla luciferase induction levels arising from the expression of β-catΔ were compared to those of RlucLefΔ293m65. For luciferase detection the reporter pMyc5'3'luc was used. All amounts of the transfected constructs are listed in the table.

In this experiment, again a luciferase upregulation could be observed (1.8 fold) by expressing RlucLefΔ293mβcatΔ65. This effect is weaker as seen for the artificial reporters used before. However the effect is strong compared to the low induction levels of the *c-myc* gene when activated by Wnt signaling (Yochum et al., 2008).

Cell line: HELA

Format: 96 well

Reporter: 90ng plucAxin2

Background (luc): 0.0001 RLU

Background (Rluc): 0.0002 RLU

	0	pKCRlucLefΔ293m65			pKCRlucLefΔ293mβcatΔ65	
		0.01ng	0.02ng	0.06ng	0.2ng	0.6ng
RLU (luc)	0.0760	0.0612	0.1725	0.2069	0.2269	0.2282
relative luc activity	1.0	0.8	2.3	2.7	3.0	3.0
RLU (Rluc)	0.0002	0.0048	0.0130	0.0357	0.0067	0.0080
relative luc/Rluc activity		1.3	1.2	0.5	2.4	2.3
mean value		1.0			2.3	

Cell line: HELA

Format: 96 well

Reporter: 90ng plucAxin2

Background (luc): 0.0001 RLU

Background (Rluc): 0.0011 RLU

	0	pKCRIucLefΔ293m65			pKCRIucLefΔ293mβcatΔ65		
		0.01ng	0.02ng	0.06ng	0.06ng	0.2ng	0.6ng
RLU (luc)	0.0388	0.3278	0.4090	1.0726	0.3362	0.5809	1.3672
relative luc activity	1.0	8.5	10.6	27.7	8.7	15.0	35.4
RLU (Rluc)	0.0017	0.1299	0.4378	1.3228	0.0903	0.2197	0.6336
relative luc/Rluc activity		1.8	0.7	0.6	2.6	1.9	1.5
mean value		1.0			2.0		

Figure 6.1.11.2.2. An improved LefΔ293-DNA binding caused by β-catΔ within the *Axin2* promoter. The constructs pKCRIucLefΔ293m65 and pKCRIucLefΔ293mβcatΔ65 were cotransfected with the reporter plucAxin2. In the tables the DNA amounts of the constructs for expressing the fusion proteins are shown.

Based on ChIP experiments of Wöhrle and colleagues (Wöhrle et al., 2007), in which a variable occupation of Tcf/Lef binding sites in the *Axin2* enhancer could be observed, I tried to find a second example for testing the β-catenin effect in a natural regulatory region (in addition to the *c-myc* promoter). Therefore a luciferase expressing reporter, under control of the *Axin2* promoter was used (Wöhrle et al., 2007). The luciferase expression was again driven by the natural promoter. By using this reporter, named plucAxin2, the same β-catenin effect on Lef-DNA binding could be demonstrated in combination with RlucLefΔ293mβcatΔ65, supporting my hypothesis again (see Figure 6.1.11.2.2). The induction (around two fold) was not as high as with the *Otx2* enhancer, but stronger than with the *c-myc* enhancer.

6.1.11.3 Artificial luciferase reporters identify specific Tcf/Lef binding motifs which preferentially confer the β-catenin effect

After demonstrating the β-catenin effect in the regulatory regions of *Otx2*, *c-myc* and *Axin2*, I separately analyzed individual Tcf/Lef binding motifs. Firstly, the two different Tcf/Lef binding motifs (*Otxa* and *Otxb*) of the *Otx2* enhancer were examined separately within different reporters. Subsequently, one Tcf/Lef binding motif of the *c-myc* enhancer was chosen, which has similar sequence modifications as the two *Otx* sites in comparison to the ideal binding sequence of the TopFlash reporter. For creation of

an additional reporter, a Tcf/Lef binding site of the enhancer of the Wingless (Wg) target gene naked cuticle (nkd) in *Drosophila* was used. The basis for the choice of this Tcf/Lef binding motif were ChIP experiments (Parker et al., 2007). Finally, as a “negative control” a reporter, including the ideal high affinity Tcf/Lef binding motif of the TopFlash reporter was cloned (Korinek et al., 1997). All reporters constructs were designed in a way that no differences in size, flanking sequences and distance between separate binding sites were existent (see Figure 6.1.11.3.1). Furthermore, in all cases the luciferase expression was driven by a Fos minimal promoter. The exact design of all used reporters is shown in Chapter 5.1.7.

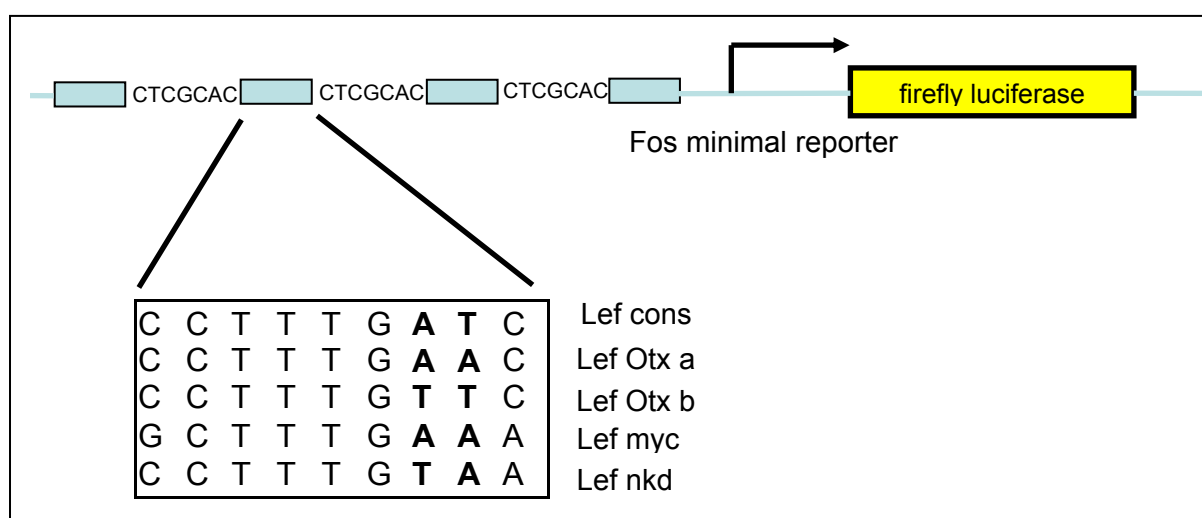


Figure 6.1.11.3.1. Used firefly luciferase expressing reporters with the different Tcf/Lef binding sites. Lef cons represents the ideal Tcf/Lef binding motif. Lef Otxa and Otxb describe the two different Tcf/Lef consensus sequences, derived from the *Otx2* enhancer, Lef myc represents one Tcf/Lef binding site originating from the *c-myc* promoter and Lef nkd is derived from the *naked cuticle* enhancer. The sequence CTCGCAC was used as an intermediate linker. The expression of the firefly luciferase was driven by a Fos minimal promoter.

Cell line: HELA

Format: 96 well

Reporter: 90ng pMlucF 24LefOtxa

Background (luc): 0.0001 RLU

Background (Rluc): 0.0003 RLU

	0	pKCRIlucLefΔ293m65		pKCRIlucLefΔ293mβcatΔ65		
		0.01ng	0.02ng	0.06ng	0.2ng	0.6ng
RLU (luc)	0.0261	0.0175	0.0465	0.0391	0.1418	0.3649
relative luc activity	4	3	7	6	22	58
RLU (Rluc)	0.0009	0.0018	0.0092	0.0057	0.0213	0.0618
relative luc/Rluc activity		1.2	0.9	1.5	1.3	1.1
mean value		1.1		1.3		

Cell line: HELA

Format: 96 well

Reporter: 90ng pMlucF 24LefOtxa

Background (luc): 0.0001 RLU

Background (Rluc): 0.0003 RLU

	0	pKCRIlucLefΔ293m65		pKCRIlucLefΔ293mβcatΔ65		
		0.01ng	0.02ng	0.06ng	0.2ng	0.6ng
RLU (luc)	0.0223	0.0247	0.0454	0.0436	0.2135	0.4186
relative luc activity	4	4	7	7	34	66
RLU (Rluc)	0.0008	0.0028	0.0079	0.0055	0.0312	0.0615
relative luc/Rluc activity		1.3	0.7	1.8	1.5	1.4
mean value		1.0		1.5		

Figure 6.1.11.3.2. The LefΔ293-β-cateninΔ interaction on Otxa binding sites. The reporter pMlucF24LefOtxa contains 24 Otxa Tcf/Lef binding sites. This reporter was used for luciferase detection. The fusion proteins LefΔ293 or LefΔ293mβcatΔ were cotransfected with the indicated amounts.

Otxa was inserted 24 times in a luciferase expressing reporter, having the same distances between all binding sites. The high number of Tcf/Lef binding sites should allow clearer results. But this approach failed (no higher relative luciferase/Renilla luciferase levels could be achieved by using reporters with more binding motifs, data not shown). However, in the experiment (see Figure 6.1.11.4) the luciferase upregulation with RlucLefΔ293mβcatΔ65 was weak, in comparison to the reporters combining Otxa/Otxb sites (like plucF8LefOtxab or pMlucF16EOtxFMcore). This result indicated that possibly the strong influence of β-catenin on Lef-DNA binding on the Otx2 enhancer primarily depends on the Otxb site. Furthermore, Otxb represents a Tcf/Lef

binding site with more deviations from the consensus sequence compared to Otxa (see comparison of the Tcf/Lef binding sites in Figure 6.1.11.3.1).

Cell line: HELA							
Format: 96 well							
Reporter: 90ng pMlucF 24LefOtxb							
Background (luc): 0.0001 RLU							
Background (Rluc): 0.0005 RLU							
		pKCRIlucLefΔ293m65			pKCRIlucLeΔf293mβcatΔ65		
	0	0.02ng	0.06ng	0.06ng	0.2ng	0.6ng	
RLU (luc)	0.0860	0.0171	0.2092	0.3258	0.1132	1.8527	
relative luc activity	3	1	8	12	4	67	
RLU (Rluc)	0.0005	0.0151	0.1751	0.0493	0.0293	0.2685	
relative luc/Rluc activity		1.1	1.0	5.6	3.3	5.6	
mean value		1.0			4.9		

Cell line: HELA							
Format: 96 well							
Reporter: 90ng pMlucF 24LefOtxb							
Background (luc): 0.0001 RLU							
Background (Rluc): 0.0003 RLU							
		pKCRIlucLefΔ293m65			pKCRIlucLefΔ293mβcatΔ65		
	0	0.01ng	0.02ng	0.06ng	0.06ng	0.2ng	0.6ng
RLU (luc)	0.0066	0.0107	0.0179	0.0861	0.0434	0.0444	0.2025
relative luc activity	0	1	1	6	3	3	13
RLU (Rluc)	0.0004	0.0079	0.0211	0.1200	0.0122	0.0208	0.0936
relative luc/Rluc activity		1.5	0.8	0.7	3.1	2.1	2.1
mean value		1.0			2.4		

Figure 6.1.11.3.3. The LefΔ293-β-cateninΔ interaction on Otxb binding sites. The reporter pMlucF24LefOtxb contains 24 Otxb Tcf/Lef binding sites. The constructs which include LefΔ293 or LefΔ293 with β-catΔ were cotransfected in combination with the reporter pMlucF24LefOtxb. For transfection the DNA amounts were used as indicated.

Therefore, the reporter pMlucF24LefOtxb with 24 Otxb Tcf/Lef binding sites was generated and analyzed by expressing RlucLefΔ293mβcatΔ65. Indeed (Figure 6.1.11.3.3) the luciferase induction levels resulted in 2.5 to 5 fold activation similar to the pMlucF16EOtxFMcore and plucF8LefOtxab reporters. This observation indicates that the influence of β-catΔ on Lef1-DNA binding relies on its interaction with specific binding motifs, like Otxb in this case.

Cell line: HELA

Format: 96 well

Reporter: 90ng plucF 24ZFLeFotxb

Background (luc): 0.0001 RLU

Background (Rluc): 0.0005 RLU

	0	pKCRlucLefΔ293m65			pKCRlucLefΔ293mβcatΔ65		
		0.01ng	0.02ng	0.06ng	0.06ng	0.2ng	0.6ng
RLU (luc)	0.0143	0.0177	0.0301	0.0799	0.0303	0.0379	0.0714
relative luc activity	1	1	2	5	2	3	5
RLU (Rluc)	0.0005	0.0178	0.0169	0.1799	0.0095	0.0391	0.0724
relative luc/Rluc activity		0.9	1.7	0.4	2.9	0.8	0.9
mean value		1.0			1.6		

Cell line: HELA

Format: 96 well

Reporter: 90ng plucF 24ZFLeFotxb

Background (luc): 0.0001 RLU

Background (Rluc): 0.0004 RLU

	0	pKCRlucLefΔ293m65			pKCRlucLefΔ293mβcatΔ65		
		0.006ng	0.02ng	0.06ng	0.06ng	0.2ng	0.6ng
RLU (luc)	0.0083	0.0131	0.0329	0.0599	0.0597	0.0834	0.3375
relative luc activity	2	3	8	14	14	20	81
RLU (Rluc)	0.0004	0.0132	0.0554	0.1516	0.0476	0.1123	0.3212
relative luc/Rluc activity		1.5	1.0	1.0	3.0	1.9	2.6
mean value		1.2			2.5		

Figure 6.1.11.3.4. Variations of the linker size in Lef reporter construct. The reporter plucF24ZFLeFotxb is composed of 24 Otxb Tcf/Lef binding sites with a big linker in between. The fusion proteins RlucLefΔ293m65 RlucLefΔ293mβcatΔ65 were overexpressed. For luciferase detection the reporter plucF24ZFLeFotxb was transfected. In the tables the amounts of transfected DNA, which led to the protein expression are listed.

In order to compare different linker lengths between the Tcf/Lef binding sites, a further reporter was created. This reporter plucF24ZFLeFotxb consists of 24 Otxb Tcf/Lef and in between a linker with 18 nucleic acids, compared to the reporter pMlucF24LefOtxb, which contains a linker of 7 nucleotides. In the experiment (Figure 6.1.11.3.4) the expression of RlucLefΔ293mβcatΔ65 led to a luciferase upregulation of around two fold (in comparison to RlucLefΔ293m65). This result showed that the distance between the Tcf/Lef binding sites plays a role, but the effect of β-catenin on

Lef1-DNA binding stronger depends on the Tcf/Lef binding sites, whereas the linker plays a minor role.

Cell line: HELA							
Format: 96 well							
Reporter: 90ng pMlucF 24Lef myc							
Background (luc): 0.0001 RLU							
Background (Rluc): 0.0005 RLU							
		pKCRIucLefΔ293m65			pKCRIucLefΔ293mβcatΔ65		
	0	0.01ng	0.02ng	0.06ng	0.06ng	0.2ng	0.6ng
RLU (luc)	0.0033	0.0451	0.0327	0.2783	0.0294	0.1735	0.2087
relative luc activity	0	3	2	19	2	12	14
RLU (Rluc)	0.0005	0.0108	0.0077	0.0768	0.0033	0.0179	0.0241
relative luc/Rluc activity		1.2	1.1	1.0	2.9	2.7	2.4
mean value		1.0			2.7		

Cell line: HELA							
Format: 96 well							
Reporter: 90ng pMlucF 24Lef myc							
Background (luc): 0.0001 RLU							
Background (Rluc): 0.0005 RLU							
		pKCRIucLefΔ293m65			pKCRIucLefΔ293mβcatΔ65		
	0	0.01ng	0.02ng	0.06ng	0.06ng	0.2ng	0.6ng
RLU (luc)	0.0030	0.0159	0.0080	0.1248	0.0241	0.2058	0.5166
relative luc activity	0	1	1	8	2	14	34
RLU (Rluc)	0.0006	0.0042	0.0038	0.0329	0.0030	0.0189	0.0514
relative luc/Rluc activity		1.0	0.7	1.1	2.4	3.1	2.6
mean value		0.9			2.7		

Figure 6.1.11.3.5. Analysis of one isolated Tcf/Lef binding site of the *c-myc* enhancer. The pMlucF24Lefmyc reporter was constructed with one specific Tcf/Lef binding site from the *c-myc* enhancer, multimerized 24 times. The fusion protein expressing constructs pKCRIucLefΔ293m65 or pKCRIucLefΔ293mβcatΔ65 were cotransfected with the reporter pMlucF24Lefmyc. For transfection the DNA amounts were used as indicated.

To analyze one further Tcf/Lef binding sequence for the β-catenin effect, a Tcf/Lef binding motif of the *c-myc* 3' enhancer, which shows similar sequence modifications like the Otxb site (in comparison to the ideal high affinity binding site of the TopFlash reporter; see Figure 6.1.11.3.1) was multimerized. The resulting reporter pMlucF24Lefmyc consists of 24 repeats of this particular low affinity Tcf/Lef binding site. In the experiment (Figure 6.1.11.3.5) an increase in luciferase expression could

be seen (around 2.7 fold), evoked by RlucLef Δ 293m β cat Δ 65. This induction is similar to that seen for the pMyc5'3'luc construct.

Cell line: HELA								
Format: 96 well								
Reporter: 90ng pMlucF 24Lefnkd								
Background (luc): 0.0001 RLU								
Background (Rluc): 0.0007 RLU								
	0	pKCRIlucLef Δ 293m65				pKCRIlucLef Δ 293m β cat Δ 65		
		0.2ng	0.6ng	2ng	6ng	0.2ng	0.6ng	2ng
RLU (luc)	0.0089	0.0022	0.045	0.4179	1.2163	0.0214	0.0694	0.1025
relative luc activity	2.1	0.5	10	97	283	5	16	24
RLU (Rluc)	0.0007	0.0216	0.1659	0.5699	1.3936	0.0088	0.0236	0.0341
relative luc/Rluc activity		0.4	1.2	1.4	0.8	3.9	4.6	5.1
mean value		1.0				4.5		

Cell line: HELA						
Format: 96 well						
Reporter: 90ng pMlucF 24Lefnkd						
Background (luc): 0.0001 RLU						
Background (Rluc): 0.0012 RLU						
	0	pKCRIlucLef Δ 293m65		pKCRIlucLef Δ 293m β cat Δ 65		
		2ng	6ng	0.2ng	0.6ng	2ng
RLU (luc)	0.0017	0.1416	0.3566	0.0103	0.0071	0.02
relative luc activity	0.8	69	173	5	3	10
RLU (Rluc)	0.0012	0.3472	0.2666	0.0020	0.0031	0.006
relative luc/Rluc activity		0.4	1.3	5.2	2.3	3.3
mean value		0.9		3.6		

Figure 6.1.11.3.6. The β -cat Δ effect for an improved Lef Δ 293-DNA binding for a specific Tcf/Lef motif within the *nkd* enhancer. The pMlucF24Lefnkd reporter contains one specific Tcf/Lef binding site from the naked cuticle enhancer, multimerized 24 times. Lef Δ 293m65 and Lef Δ 293 in combination with β -cat Δ were overexpressed as fusion proteins. The reporter pMlucF24Lefnkd was used for luciferase detection. The transfections were done by using the indicated DNA amounts.

Searching for a further example in *Drosophila*, the sequence of the enhancer of the Wingless target gene *naked cuticle* (*nkd*) was scanned in the region where a Wnt activity dependent Tcf/Lef binding could be detected (Parker et al., 2007). Again one specific binding motif was chosen, which shows sequence modifications resembling those of Otxb (see Figure 6.1.11.3.1). In the experiment (Figure 6.1.11.3.6) by adding RlucLef Δ 293m β cat Δ 65 a clear luciferase upregulation in comparison to

Lef Δ 293m65 could be measured. This observed luciferase induction for Lefnkd was even higher compared to those of LefOtxb.

Cell line: HELA								
Format: 96 well								
Reporter: 90ng pMlucF 6Lefcons								
Background (luc): 0.0001 RLU								
Background (Rluc): 0.0004 RLU								
		pKCRIucLef Δ 293m65				pKCRIucLef Δ 293m β cat Δ 65		
	0	0.006ng	0.02ng	0.06ng		0.06ng	0.2ng	0.6ng
RLU (luc)	0.0031	0.0074	0.0480	0.1855		0.0105	0.2123	0.2572
relative luc activity	0	1	7	28		2	33	39
RLU (Rluc)	0.0001	0.0001	0.0041	0.0127		0.0022	0.0588	0.0992
relative luc/Rluc activity		1.1	1.5	1.1		0.4	0.4	0.3
mean value		1.2				0.3		

Cell line: HELA								
Format: 96 well								
Reporter: 90ng pMlucF 6Lefcons								
Background (luc): 0.0001 RLU								
Background (Rluc): 0.0004 RLU								
		pKCRIucLef Δ 293m65				pKCRIucLef Δ 293m β cat Δ 65		
	0	0.002ng	0.01ng	0.02ng	0.06ng	0.06ng	0.2ng	0.6ng
RLU (luc)	0.0060	0.0571	0.0509	0.4543	1.1237	0.0960	0.2937	0.4721
relative luc activity	1	9	8	70	172	15	45	72
RLU (Rluc)	0.0004	0.0044	0.0080	0.0518	0.1655	0.0181	0.0533	0.1615
relative luc/Rluc activity		1.2	0.6	0.9	0.8	0.6	0.6	0.4
mean value		0.9				0.5		

Figure 6.1.11.3.7. No β -cat Δ effect for the high affinity Tcf/Lef binding sites of the TopFlash reporter. The pMlucF6Lefcons reporter contains six high affinity Tcf/Lef binding sites similar to the TopFlash reporter. In cotransfections pKCRIucLef Δ 293m65 or pKCRIucLef Δ 293m β cat Δ 65 were combined with the reporter pMlucF6Lefcons (the amounts were used as indicated).

Finally, the effect of β -catenin on Lef-DNA binding was examined with the reporter pMlucF6Lefcons, consisting of six Tcf/Lef binding sites, reflecting the ideal consensus sequence (as in the TopFlash reporter; Korinek et al., 1997). This reporter contains binding motifs with a high Tcf/Lef binding affinity compared to the reporters, which were used in the experiments before. The expression of RlucLef Δ 293m β cat Δ 65 did not lead to a detectable luciferase induction compared to RlucLef Δ 293 (see Figure 6.1.11.3.7). Since naturally occurring Tcf/Lef sites rarely are

consensus binding sequences, these results support the idea that in natural promoters, β -catenin helps Lef1 and other Tcfs to obtain a more efficient DNA binding.

reporter	pMlucF16EOtxFMcore	plucF8LefOtxab	pMlucF24LefOtxa	pMlucF24LefOtxb	pMlucF24Lefnkd
relative luc/Rluc activity (β -cat Δ expression)	3.0	3.4	1.3	4.9	8.8
	4.1	3.3	1.5	2.4	2.9
	2.8	4.0		3.6	5.1
		3.6		2.4	3.6
		2.0		2.3	2.3
		2.6		1.3	3.4
		2.0		3.6	3.7
		3.4		4.0	4.3
		2.4		3.4	4.7
		3.3			4.0
		3.2			5.3
		3.8			3.3
		3.7			5.8
		4.2			
		3.2			
		4.3			
		1.9			
		3.4			
		3.6			
		3.2			
		1.6			
		2.0			
		3.0			
		3.5			
		2.4			
		5.7			
		1.1			
		2.5			
		3.6			
		3.1			
mean value	3.3	3.1	1.4	3.1	4.4

reporter	pMyc5'3'luc	pMlucF24Lefmyc	plucAxin2	plucF24ZFLeOtxb	pMlucF6Lefcons
relative luc/Rluc activity (β - cat Δ ex- pression)	1.8	2.7	2.0	1.0	0.4
		2.7	1.9	1.7	0.3
		1.6		1.6	0.5
				2.5	
mean value	1.8	2.3	2.0	1.9	0.4

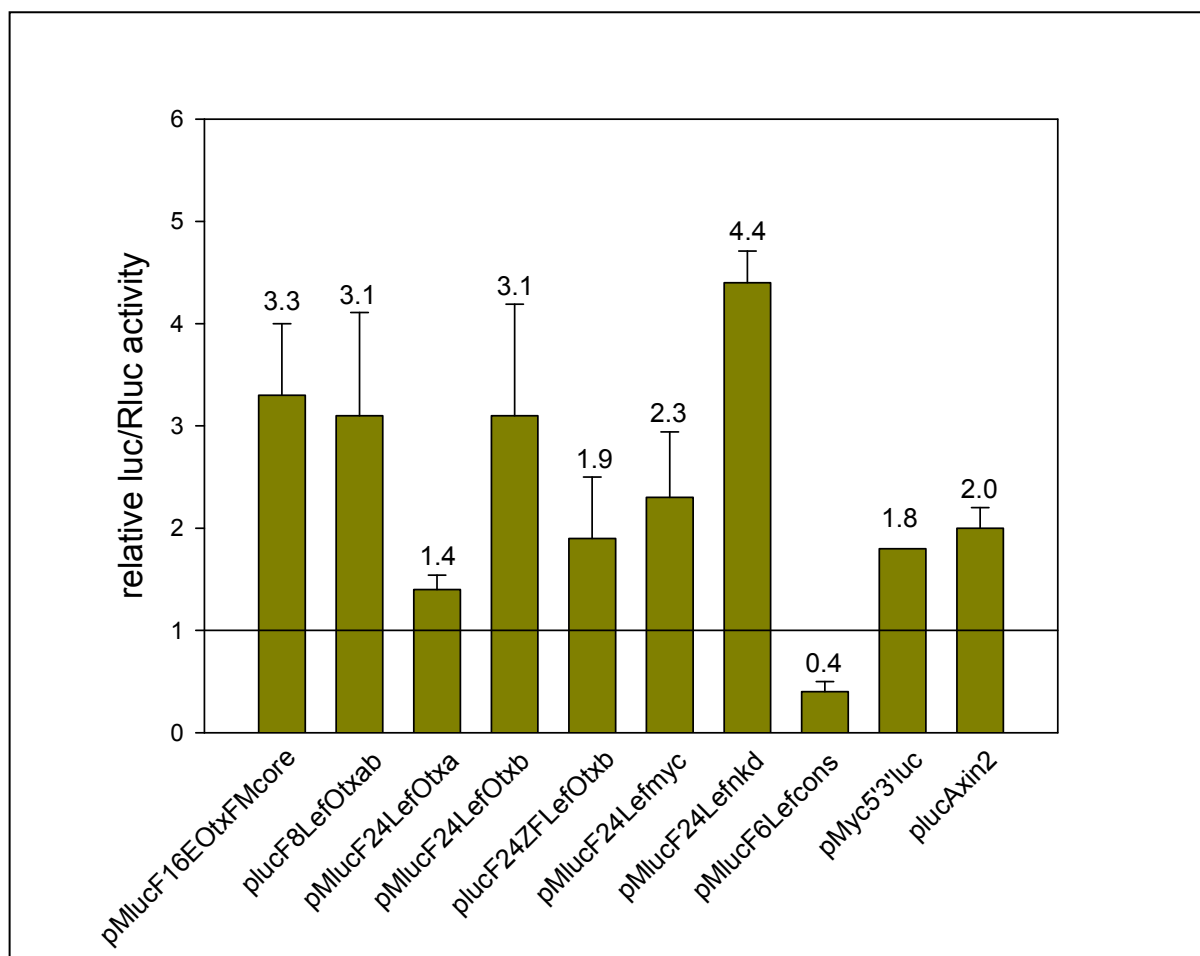


Figure 6.1.11.3.8. Summary of results for different Tcf/Lef binding sites resulting from the expression of β -cat Δ . Mean values were calculated from three experiments with pMlucF16OtxFMcore, 30 with plucF8LefOtxab, two with pMlucF24LefOtxa, nine with pMlucF24LefOtxb, four with plucF24ZFLeOtxb, three with pMlucF24Lefmyc, 13 with pMlucF24Lefnkd, three with pMlucF6Lefcons, one with pMyc5'3'luc and two with plucAxin2 (see the table). The reference line (=1) determines a limit for a detectable induction.

Comparing all results for the different reporters in HELA cells, a summary, how β -catenin works on Lef(Δ 293) could be made. The Armadillo repeats of β -catenin seem to interact with Lef1 within its HMG domain to achieve a stronger Lef-DNA binding. This mechanism can serve as a preparation for the transactivation of some specific Wnt target genes. In this context, the lower the binding affinity for Tcf/Lef is the more eminent this mechanism seems to be. This approach could explain the differences between the various Wnt target genes (*Otx2*, *Axin2*, *c-myc*, *nkd*) with their variable Tcf/Lef binding sequences, resulting in a different influence of β -catenin on Lef-DNA binding (see Figure 6.1.11.3.8). The strongest effect was detected with a *nkd* binding site followed by *Otxb*, *Axin2*, *c-myc* and *Otxa*.

6.2. Antisense function of modified Peptide nucleic acids (PNAs)

Antisense molecules are important tools to selectively block single gene functions. In order to develop an efficient antisense method for Medaka fish we analyzed modified peptide nucleic acids (PNAs) for this purpose. The group of PNAs presents artificially synthesized, organic polymers with similar structures compared to DNA or RNA. However the backbone of these chemical compounds differs fundamentally, because they consist of N-(2-aminoethyl)-glycine or alanyl units, which are linked by uncharged amide bonds. Over methylene carbonyl linkages, every monomer can be linked to the different nucleic bases (Nielsen-PNAs; Wittung et al., 1994; Nielsen and Egholm, 1999; see Figure 6.2.1). This structure leads to high stability against proteases and nucleases, temperature and pH fluctuations (Buchardt et al., 1993). A very important fact displays their ability to bind strongly and efficiently to complementary DNA or RNA sequences. In addition, this entirely neutral molecule demonstrates an advantage in case of hybridization with DNA or RNA, due to its lower probability for forming secondary structures (Egholm et al., 1993; Buchardt et al., 1993). But, this fact means a big problem for water solubility and membrane permeability. Due to their high affinity for DNA and RNA, in general the PNAs are used in shorter lengths (13-18 bps) in comparison to other oligonucleotides (e.g. morpholinos normally are used with 22 to 25 monomeres). In cell culture experiments the gene silencing potential of the PNAs could be demonstrated, leading for example to a reduced proliferation of cancer cells (Folini et al., 2003; Shiraishi and Nielsen, 2004; Efimov and Chakhmakhcheva, 2005). But for *in vivo* applications the PNAs did not show any knockdown phenotypes in Zebrafish embryos (Urtishak et al., 2003; Wickstrom et al., 2004). Using modified PNAs (HypNa-PNAs) an antisense activity could be shown in *in vitro* experiments and a high knockdown efficiency for targeting certain genes in Zebrafish (Urtishak et al., 2003; Wickstrom et al., 2004; Efimov and Chakhmakhcheva, 2005) and *Xenopus* (Efimov et al., 2006). These molecules are composed of alternating phosphonate and trans-4-hydroxy-L-proline PNA-like monomers (see Figure 6.2.1), leading to an overall negative charge and therefore high water solubility.

In cooperation with a company (Ugichem GmbH), which synthesized the PNAs and Narges Aghaallaei, I tested new modifications of PNAs to reach high water solubility and specificity for effective gene targeting and silencing (the chemical modifications

and synthesis steps using different building blocks are described in the manuscript, Chapter 8). As a first step the Nielsen-PNA backbone was modified using two different side chains R (C2) and R (C3) (see Figure 6.2.1), forming the UgiPNAs. This modification resulted in high water solubility (a stock concentration of 10mM was possible). In a second step the Ugi-PNAs were capped at the 5' end with a trimethyl-(L)-lysine dioxalate group. The trimethyl-(L)-lysine dioxalate group (TML or L) replaced the phosphate group, which includes two ethyl groups (see Figure 6.2.1). This modification led to an overall positive charge and an even better water solubility (data not shown). For the analysis of changes in solubility and knockdown efficiency based on chemical modifications a TML group or a dinitrophenyl acetic acid group (N), being attached to the 5' end were used. Subsequently, a steric hindrance between the phosphonic esters and the oligomer backbone was avoided by the introduction of a second TML group and the reduction of side chains, resulting in mixed Nielsen-PNA-Ugi-PNAs (mixed PNA; see Figure 6.2.1). This composition combined high water solubility and strong DNA/RNA binding affinity (see results). The Ugi-PNAs with one TML group were used to investigate the optimal length and concentration for gene targeting. Mixed PNAs were used *in vivo* (*Six3* knockdown). Finally, another set of oligomeres were synthesized, which include phenyl acetic acid (Pac) as part of every monomere and an additional lysine group (K). This group works as a hydrophobic spacer for Pac to allow a suitable distance to nucleic bases for a stronger Watson Crick base pairing. This chemical modification was used for *Tcf3* knockdown experiments in Medaka embryos.

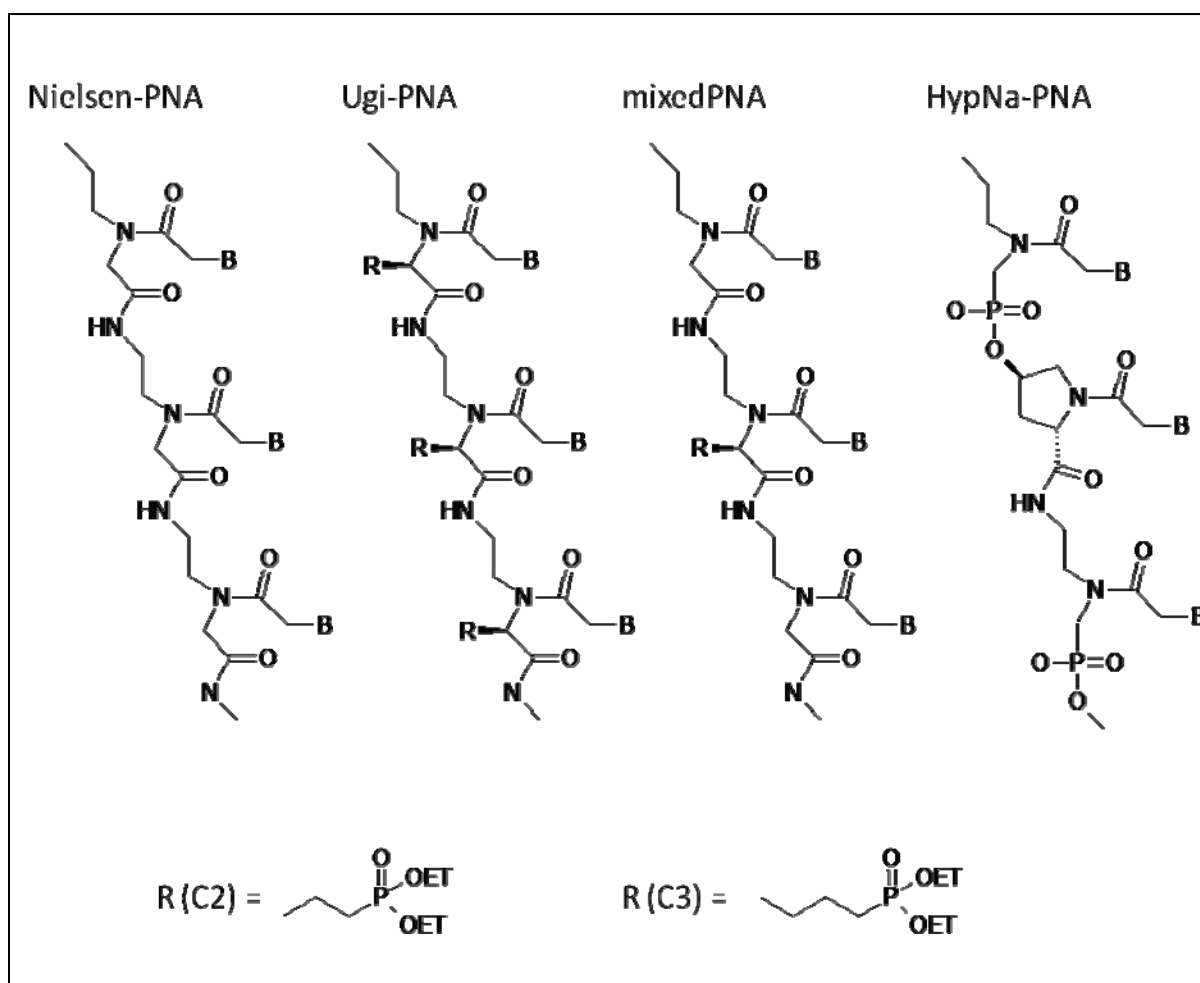


Figure 6.2.1. Schematic presentation of the chemical composition of Nielsen Peptide nucleic acids (PNAs), Ugi-PNAs, mixture of both (mixed PNAs) and HypNa-PNAs. The Nielsen PNAs consist of N-(2-aminoethyl)-glycine units connected by amid bonds and nucleic bases. The Ugi-PNAs or mixed PNAs have additional side chains (R) which include two or three carbon atoms and two ET groups linked by a phosphodiester bond. The HypNa-PNAs are formed by alternated phosphodiester and amid bonds. "B" represents the four possible nucleic bases Adenine, Cytosine, Thymine or Guanine.

6.2.1 Best GFP signal reduction through PNA-16mers

For first experiments on the effectiveness of Ugi-PNA oligomeres on mRNA knock-down, *gfp* mRNA was coinjected in combination with RNase inhibitor and the antisense molecules into Medaka embryos at the one-cell stage. The used PNAs targeted the translational starting point for GFP expression. 24 hours after injection the GFP signal was determined under the fluorescence microscope. These experiments were done in a colloboration with Narges Aghaalaei and are presented in an accompanying manuscript (Chapter 8). In these experiments a 16mer Ugi-PNA was determined as an optimal size for *gfp* knockdown. The sequence of all PNAs, the morpholino oligomer and the *gfp* target mRNA are shown in Figure 6.2.1.1.

<i>gfp</i> mRNA	5' - ccggucgccacc AUG GUGAGCAAGGGCGAGGAGCUGUUCAC - 3'
Morpholino	3' - GTACCACTCGTTCCCGCACTCCTCG - 5'
Gfp12	3' - CGTTCCCGCTCC - L - 5'
Gfp14	3' - CGTTCCCGCTCCTC - L - 5'
Gfp16	3' - CGTTCCCGCTCCTCGA - L - 5'
Gfp17	3' - CGTTCCCGCTCCTCGAC - L - 5'
Gfp15mixL1	3' - CGTTCCCGCTCCTCG - L - 5'
Gfp16mixL1	3' - CGTTCCCGCTCCTCGA - L - 5'
Gfp16mixL4	3' - CGTTCCCGCTCCTCGA - LLLL - 5'
Gfp16mixRho	3' - CGTTCCCGCTCCTCGA - L - SuRho - 5'
Gfp15Flu	3' - CAGCGGTGGTACCAC - Flu - 5'
Ref15mix	3' - TGAGAAACCGTCGT - L - 5'

Figure 6.2.1.1. Sequences of morpholino and PNA A2L4, targeting the translation start site of GFP. The mRNA target is shown in 5'-3'-orientation and the morpholino and the Gfp PNAs in 3'-5' direction. The red overlay represent the start codon for translation. Capital letters in the mRNA indicate the coding region. For all PNAs the black letters represent Nielsen PNAs and the red letters the Ugi-PNA components.

In addition to the experiments presented in the manuscript I performed further experiments comparing different lengths of PNAs (in comparison to the PNA Ref15mix, which contains an unrelated sequence). The knockdown efficiency for GFP expression was compared to that of a morpholino oligo (25mer), which also targets the translational start on the *gfp* mRNA. Morpholino antisense molecules, which include morpholino rings instead of ribose and non-ionic phosphorodiamidate instead of phosphodiester linkages (Summerton, 1999), can efficiently block the translation machinery at the 5' untranslated region through RNA hybridization. The hallmark of morpholino knockdown is a high knockdown efficiency at low concentrations of this antisense molecule. This feature makes morpholino oligos to a tool for analyzing gene functions in Zebrafish, *Xenopus* and Medaka. Thus, a comparison of the knockdown efficiency of Ugi-PNAs to morpholino oligos could evaluate their importance as an alternative for future applications. The experiments were performed by coinjecting the different PNAs and the morpholino oligos with RNase inhibitor and *gfp* mRNA into the Medaka embryos.

	100μM	200μM	400μM	600μM
morpholino 25mer	88	94		
PNA-Gfp12		4		
PNA-Gfp14		45		
PNA-Gfp-16	32	72		
PNA-Gfp17	5	25		
PNA-Ref15mix		0	16	38

Figure 6.2.1.2. 12mers to 17mers of PNAs resulted in different GFP signal reductions in Medaka embryos. The control Ref15mix PNA contains an unrelated sequence. The numbers represent the average percentage of GFP reduction. The injection of PNAs was performed at concentrations of 100μM to 600μM (see text for more details). The highest numbers of GFP signal reduction are shown in bold. As a positive control group an injection with a morpholino 25mer was done. The GFP expression was analyzed after 24 hours.

In addition to the experiments described in the manuscript, experiments were performed with concentrations of 100μM to 200μM of the *gfp* targeting PNAs and 200μM to 600μM of the control PNA (Ref15mix). Experimental details and calculations are described in the manuscript. The analysis of the *gfp* knockdown efficiency depending on their length gave a clear result again (see Figure 6.2.1.2). The injection of a 16mer PNA at 200μM led to the highest activity in impairing the GFP expression. With increasing amounts of the PNA, the efficiency of knockdown improved (32% reduction for 100μM, compared to 72% with 200μM). But at higher concentrations (400 and 600μM) a relevant number of embryos showed reduced GFP expression also for the reference oligo Ref15mix. Overall, Ugi-PNAs clearly work in the knockdown of *gfp* but at lower efficiency compared to morpholinos.

6.2.2 Best GFP blocking through mixed backbone PNAs in Medaka

The attachment of different endgroups to the PNAs could largely eliminate the problem of foam formation in water. In additional experiments mixed backbones were used in which Nielsen PNA components were combined with Ugi-PNA components (for details see manuscript, Chapter). These experiments were performed by Narges Aghaallaei and me in cooperation. The molecules with the mixed backbones (Gfp1mix) and four tri-methyllysine groups were most efficient in *gfp* knockdown

(Gfp16mixL4). A clear reduction of the GFP signal was the result (see manuscript for details).

In addition to the experiments described in the manuscript the knockdown potential of mixed PNAs was compared to that of morpholino oligos and their specificity was evaluated using a 15mer PNA with an unrelated sequence. Moreover, one *gfp* targeting mixed PNA (16Gfp1mixN) with a dinitrophenyl acetic acid endgroup (N) and one without any endgroup (16Gfp1mix) was used in comparison to that with the trimethyllysine group(s). For analyzing the optimal *gfp* targeting sequence (Gfp1) for *gfp* knockdown another mixed PNA oligo, which contains an alternative *gfp* targeting sequence (Gfp2, sequence not shown), was used in the experiments.

	100μM	200μM	400μM	600μM
morpholino 25mer	88	94		
PNA-15Gfp1mixL1	42	41	75	
PNA-16Gfp1mixL1		59	80	
PNA-16Gfp1mixL4		48	86	72
PNA-16Gfp2mixL4		39	85	
PNA-16Gfp1mix		60	85	
PNA-16Gfp1mixN		52	79	
PNA-15mix		0	16	38

Figure 6.2.2.1. GFP signal reduction by PNAs with variable lengths and end groups. The control PNA Ref15mix contains an unrelated sequence. As positive control injections with a morpholino 25mer were done. The numbers represent the percentage of GFP signal reduction. The injection of PNAs was carried out with the concentrations of 100μM to 600μM. The most efficient GFP reductions are shown in bold (see text for more details). The GFP expression was analyzed after 24 hours.

The application of the mixed PNAs with a concentration of 400μM led to the best results (see Figure 6.2.2.1). The mixed PNA without any specific endgroup gave the best results for *gfp* knockdown, but showed the problem of foam formation (data not shown). Four trimethyllysine groups again increased the *gfp* knockdown efficiency, but also resulted in problems in water solubility (data not shown). A direct comparison of the PNAs with different *gfp* targeting sequences (16Gfp1mixL4 and Gfp2mixL4) demonstrated similar results for both PNAs. The control injections of Ref15mix, containing an unrelated sequence, did not cause any reduction of the GFP expression (only in small numbers at high concentrations). Summarizing the results of all ex-

periments (see manuscript and Chapters 6.2.1 and 6.2.2), PNAs achieved similar results as morpholinos, when they consisted of 16mers, a mixed backbone and a trimethyl-lysine group.

6.2.3 Distribution of Ugi-PNAs in HEK293 cells

In the previous experiments the high knockdown potential of the 16Gfp2L4 mixed molecule was shown. In order to examine its permeability through cell membranes a cell culture experiment was performed and their intracellular distribution was tested by using the 16Gfp2L4 PNAs labelled with the dyes sulforhodamine (Rho-PNA) or fluorescein isothiocyanate (FITC-PNA). These experiments were performed in collaboration with Narges Aghaallaei. These dyes can be detected by fluorescence microscopes. In the experiments HEK293 cells were incubated with 16Gfp2L4 PNAs at concentrations of 3 to 300 μ M.

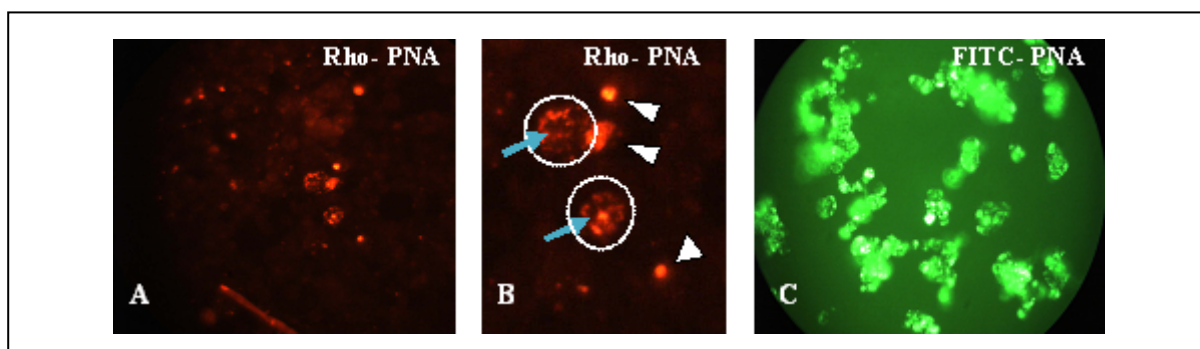


Figure 6.2.3.2. Distribution of 16Gfp2L4 PNAs in 293 cells. The cells are incubated with sulforhodamine (Rho) (pictures A and B) or FITC (C) labelled PNAs. In the magnified picture B the cycles mark two intact, but detached cells with accumulated Rho-PNAs in the cytoplasm (blue arrows) The white arrow heads represent dead cells (B). In C the whole cells are filled with FITC labelled PNAs. For the experiment the cells were incubated for 24 hours with 100 μ M of the PNAs.

The results show an uptake of the labelled PNAs into the cells. It is however not clear whether the uptake was through passive diffusion or through active endocytosis. The PNAs, labelled with rhodamine could be found in the cytoplasm, it remains to be shown whether they localize to certain organelles. In Figure 6.2.3.2B, individual cells are shown with a higher magnification, which allows to differentiate between intact and dead cells. A similar distribution of FITC labelled PNAs can be seen in the cells in Figure 6.2.3.2C. In difference to the rhodamine PNAs, they were better distributed within the cell, which might indicate a better solubility.

stable cell line	A1 (GFP ++)	A1 (GFP +)
	34861488	17462858
	31260998	15867668
	32405816	15288238
MW	32842767	16206255

+200 μ M 16Gfp2L4		
	32967992	21720964
	32752924	19239144
	32803502	21141670
MW	32841473	20700593
% reduction	0	-28

Figure 6.2.3.3. Activity of 16Gfp2L4 PNAs on luciferase expression of HEK 293 cells. The cells had stably integrated the pS(GUAS)CgfpLucdNpuro construct for constitutive luciferase and GFP expression. The activity of PNAs was analyzed after 24 hours incubation time. All values are measured in RLU (luc). The luciferase signal reduction was calculated in percent, subtracting the luciferase expression levels of the cells with PNA incubation from the luciferase expression levels of the cells without PNAs. Thus, the resulting negative value in cell line A1 (GFP+) represents an increase of GFP expression (negative GFP signal decrease). All values represent mean values from four measurements. A1 represent two different GFP and luc expressing cells lines with GFP (+) or strong GFP expression (++)).

After analyzing the intracellular distribution, two stable HEK 293 cell lines, including the construct pS(GUAS)CgfpLucdNpuro were created for examination of the function of *gfp* targeting PNAs (the stable cell line was generated by me and the PNA incubation experiments were done by Narges Aghaallaei). The construct consists of a CMV promoter, which drives the production of a downstream located GFP-luciferase fusion protein. C-terminally a degradation signal decN was inserted, which results in a fast degradation rate of the protein (half life 3-4 hours instead of 6; data not shown). This degradation is achieved by a functional fragment (amino acids 422-461) of the protein ornithine decarboxylase (ODC), which encodes for an enzyme, splitting off CO₂ from ornithine in the urea cycle. The fragment contains recognition sites for fast degradation in an ubiquitin-independent pathway (Zhang et al., 2003). The reason for using this degradation signal was that GFP and luciferase are proteins, which after their production are accumulated in the cell in large amounts. Therefore a reliable measurement of GFP or luciferase degradation rate by an antisense molecule would not have been possible. The 16Gfp2L4 PNAs target the *gfp* sequence and therefore should be able to also impair the expression of the GFP-luciferase fusion protein. But, as shown in Figure 6.2.3.3, the application of the PNAs did not result in any decrease of the luciferase activity. Since the 16Gfp2L4 PNAs worked efficiently in *gfp*

downregulation in injection experiments (see Chapters 6.2.1 and 6.2.2) an insufficient uptake of the PNAs into the cells is the most possible reason for the failure of the experiment. Overall, a sufficient membrane uptake of the mixed PNAs into the cells could not be demonstrated.

6.2.4 PNA distribution in intestine-surrounding cells in Medaka

Subsequently, similar diffusion experiments were performed in Medaka fish. For this purpose, dechorionized embryos were bathed over night in solutions, containing the FITC or sulforhodamine labelled 16Gfp2L4 PNAs (experiments with FITC were done by Narges Aghaallei and those with Rodamine by me). On the next day after washing, the embryos were transferred onto glass slides for analysis in a confocal microscope. During over night incubation the embryos had swallowed the PNA solution, which therefore could be detected in the whole digestive tract, including the swim bladder, which is connected to the digestive tract over a conserved canal in Cypriniformes and Siluriformes (Medaka belongs to the first group).

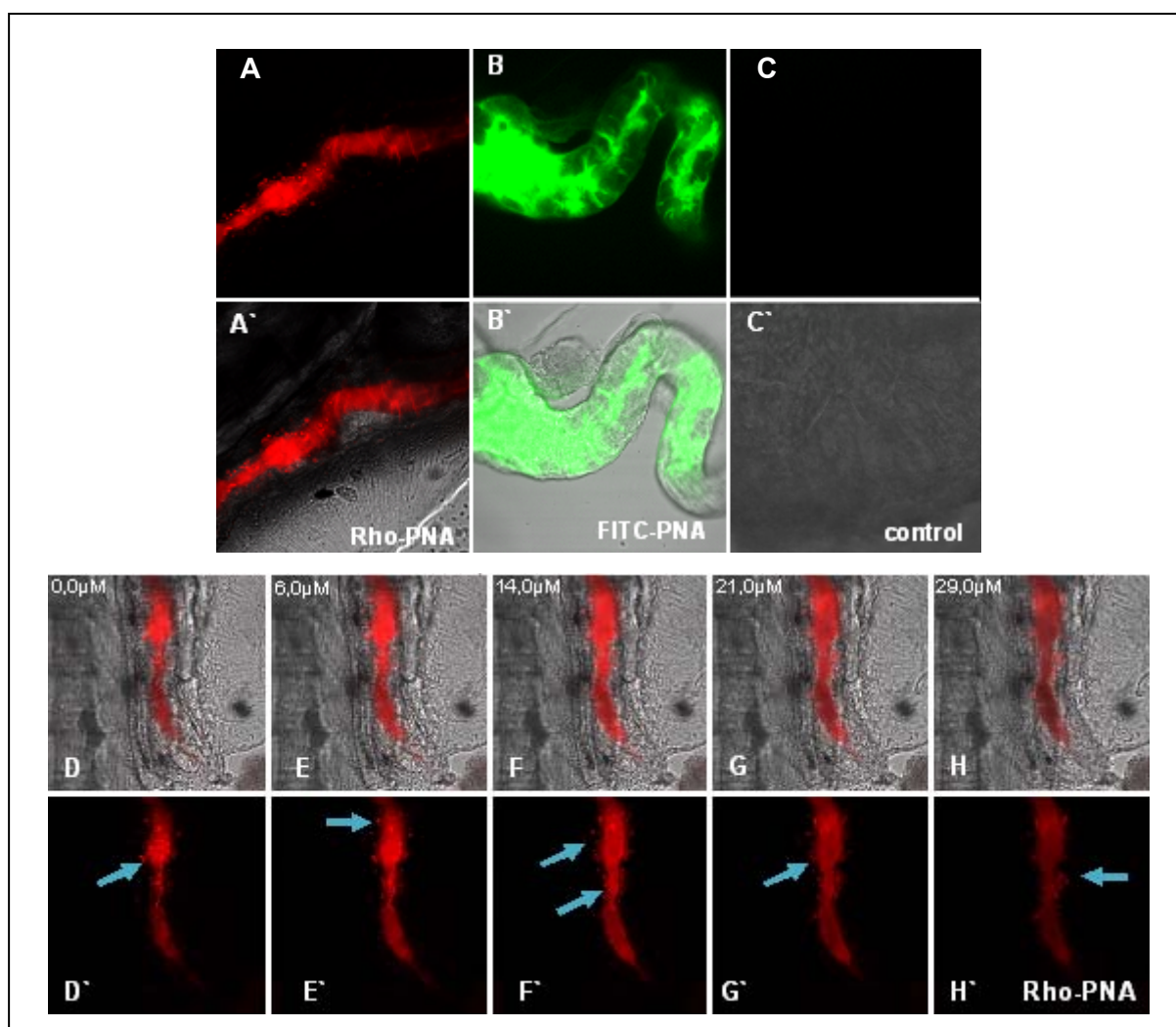


Figure 6.2.4.1. Distribution of FITC and sulforhodamine (Rho) labelled 16Gfp2L4 PNAs in intestine surrounding cells of the mucosa in Medaka. Representative distributions of Rho-PNAs (A, A') and FITC-PNAs (B, B') in the intestine (A and B show fluorescence and A' and B' an overlap of fluorescence and bright field pictures). The control group displays fish areas outside of the intestine (C and C'). The pictures D to H' represent optical sections (from 0.0 μM to 29.0 μM), of the rectum and the distributions of Rho-PNAs. The intestine surrounding cells, containing PNAs are marked with blue arrows. The pictures D to H represent bright field and D' to H' fluorescence pictures. The PNA concentration during incubation was 100 μM and the incubation time 24 hours.

Based on the optical sections, the small intestine, the hindgut and finally the rectum could be very well differentiated. The PNAs were detected in all intestine areas. In the small intestine, which is characterized by its vili, the rhodamine labelled PNAs could be detected directly in the lumen, but also in surrounding cells (shown by the different optical sections in Figure 6.2.4.1). During the process of digestion, carbohydrates, proteins, fatty acids and nucleotides are fragmented into small units to be taken up by the blood or the lymph system. These processes can happen by active transport mechanisms (symport procedures or pumps) or by passive diffusion (fatty acids and nucleotides). The most likely variant for the PNA uptake would be passive

transport processes or diffusion, due to their structure, being similar to nucleotides. In addition, the PNAs are neutral in charge, which is not consistent with most active transport mechanisms.

6.2.5 PNAs efficiently block translation of *Six3*

Targeting endogenous genes for a knockdown during embryonic development in Medaka, PNAs against 5' regions of *Six3* mRNA were designed and injected at the one blastomere stage. Subsequently, in embryonic stage 29 (34 somites stage) the resulting phenotypes were analyzed and compared to uninjected (WT) and morpholino injected embryos. The results of these injection experiments are presented in the accompanying manuscript (all *Six3* injection experiments were done by me).

In addition I tested PNAs with specific end groups: 16mer PNAs Six3L (a trimethyllysine end group), Six3 (no specific end group) and Six3N (dinitrophenyl acetic acid end group) (data not shown). All three PNAs contain the same sequence and only differ in the endgroup. The injection of Six3L resulted in the highest number of embryos with a loss of forebrain structures, a fusion of distal retina structures and strongly reduced eyes (strong defects) (see manuscript for details). For comparison the number of embryos with a cyclopic eye (moderate phenotype) or reduced eyes (weak phenotype) was clearly lower. The application of Six3N or Six3 PNAs resulted in lower numbers of phenotypes (data not shown).

In a next step for the analysis of specificity and efficiency of the *Six3* knockdown, the effects of Six3L and Six3Lmut-T (one base pair mismatch) injections were compared to a 16mer PNA with an unrelated sequence (Ref16mix) and a *Six3* morpholino oligo. The results of these experiments are shown in the manuscript.

In additional experiments another PNA variant, Six3/2L1 which binds to an internal *Six3* sequence was tested. Generally, injections with morpholino oligos which target an internal mRNA sequence are not successful. However, PNAs bind with higher affinity to mRNA and might therefore block the translation machinery also at an internal position. Injections of 400 μ M Six3/2 led in 92 percent and with 900 μ M in 47 percent of the surviving embryos to *Six3* phenotypes (see Figure 6.2.5.1). But, in both cases

the number of dying embryos strongly exceeded that of surviving ones. Thus, the observed effects of Six3/2 were partly unspecific.

	200µMSix3/2L1		400µMSix3/2L1		900µMSix3/2L1	
	number	%	number	%	number	%
dead	6		18		35	
strong defects	0	0	5	38	4	27
moderate defects	0	0	4	31	3	20
weak defects	0	0	3	23	0	0
normal	15	100	1	8	8	53

Figure 6.2.5.1. Internal block of Six3 translation through the Six3/2L1 PNA variant. The phenotypes weak, intermediate and strong represent different levels of eye and brain defects. The surviving embryos are listed in numbers and percent, depending on their phenotypes. The dead embryos are shown in numbers. All described phenotypes were examined at stage 29 (somites 34). The injected concentrations are indicated (see text for more details).

6.2.6 Efficient block of *Tcf3* by PNAs leads to a specific anterior phenotype

For further demonstration of the knockdown activity of the PNAs in Medaka, the gene *Tcf3* was chosen. In Zebrafish two forms exist, *Tcf3a* and *Tcf3b*, which are complementary to each other in function. The overexpression of *Tcf3* promotes head formation and its impairment a loss of brain structures. Thus, the Zebrafish *headless* mutant (point mutation in *Tcf3a*) shows a strong reduction of eyes, forebrain and parts of the midbrain in Zebrafish (Kim et al., 2000). The reason for these defects is that during normal development a gradient of Wnt signaling activity is formed, being responsible for proper axis formation (Kiecker and Niehrs, 2001). Therefore, in case of *Tcf3* downregulation, which acts as a strong anterior repressor for Wnt signaling, anterior structures get lost. An *in silico* search of the Medaka genome using the murine *Tcf3* amino acid sequence resulted in just one *Tcf3* gene. In experiments the mixed PNA Tcf3LL (two trimethyl-lysine end groups, see Figure 6.2.6.1 for sequence details) was injected, which possessed a complementary sequence to the translation start site of *Tcf3* mRNA. The resulting *Tcf3* phenotypes affected the eyes, which were reduced or resulted in a complete loss of one or both eyes. In addition, the development of some parts of the fore-, but not of the midbrain was impaired. Depending on the severity of brain and eye defects the phenotypes were classified as “strong”, “moderate” and “weak”. A strong phenotype is characterized by complete loss of eyes and im-

paired regions of the forebrain. The intermediate phenotype represents embryos with just one eye and weak phenotypes had one reduced eye. All phenotypes were identical to those of *Tcf3*-morpholino injections (see Figure 6.2.6.2). As before with the Six3-PNAs, no unspecific phenotypes could be identified at the concentrations used for the experiments. All phenotype determinations were done at stage 29 (34 somates, according to Iwamatsu, 2004), in which the eyes and the retina were clearly visible due to pigmentation.

<i>Tcf3</i> mRNA	5' -uuugagcugaucgacuggugugcaaac	AUG	CCUCAACUGAA-3'
Morpholino	3' -GACTAGCTGACCACACGTTTGTACG-5'		
<i>Tcf3</i> LL	3' -G A CTAGCTGACCACAC-LL-5'		
<i>Tcf3</i> K	3' -G A CTAGCTGACCACAC-K-5'		
<i>Tcf3</i> Kmut-TG	3' -G A CTAG C GTACCACAC-K-5'		
<i>Tcf3</i> -MO	3' -GACTAGCTGACCACACGTTTGTACG-5'		
<i>Tcf3</i> pre-mRNA	exon 5' -UGACUCCGAGguaaaauuguuauua-3'	intron	
<i>Tcf3</i> SPLL	3' -TGAGG CTCCATTTAAC -LL-5'		

Figure 6.2.6.1. Nucleic acid sequences of the *Tcf3* mRNA including the 5' end of translation start or the boundary of exon1/intron1 (pre-mRNA). The sequences of the used *Tcf3*-PNAs, together with the splicing *Tcf3*-PNA and morpholino oligo. The translation start point of *Tcf3* is presented in red overlay, Exon sequences are shown in capital and intron sequences in small letters. The sequences of all *Tcf3* mRNAs are shown in 5'-3' directions and the antisense molecules in 3'-5' orientations. In PNAs the red letters show Ugi-PNA components, the black letters indicate the Nielsen PNA components and the blue letters Ugi-PNA monomers, containing the lysine spacer K. The underlined letters represent the introduced point mutations in comparison to the original complementary *Tcf3* mRNA sequence.

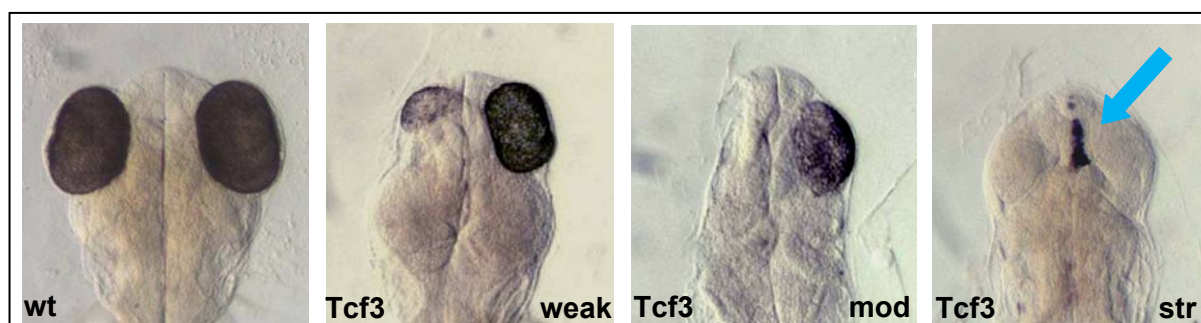


Figure 6.2.6.2. Phenotypes of Medaka embryos injected by *Tcf3* antisense molecules. wt represents a wild type reference embryo and pictures marked by “*Tcf3*” show embryos injected with *Tcf3* PNAs or morpholinos. “weak”, “mod” and “str” mark injected embryos with a weak, moderate or strong phenotype (see text for details). All embryos are at stage 29 (34 somites) and are shown in dorsal views. The arrow indicate remnants of the eyes observed in “strong” embryos.

The application of *Tcf3*LL led to a convincing amount of *Tcf3* phenotypes, but the block was not as efficient as for morpholino oligo injections (phenotypes for *Tcf3*LL

PNA injection at 600 μ M compared to 92% phenotypes for 100 μ M morpholino oligos; see Figure 6.2.6.3). In further experiments Tcf3K (lysine introduced as spacer for better Watson Crick pairing of nucleic bases, see introduction of this Chapter) and Tcf3Kmut-TG (two point mutations) were used, analyzing their knockdown efficiency. Injections of Tcf3K PNAs and *Tcf3* morpholino oligos caused the same levels of *Tcf3* phenotypes (92% at 400 μ M for the PNAs versus 100 μ M for the morpholinos) in combination with a similar death rate (20% versus 13%). In addition, a strong occurrence of *Tcf3* phenotypes (63%) could be already observed with an injected concentration of 50 μ M (see results in Figure 6.2.6.3). The reason for these improvements of knockdown efficiency was probably a stronger PNA-mRNA binding due to the chemical modification. The specificity of the *Tcf3* knockdown was shown by applying a mutated version of Tcf3K, which led to a strong drop of *Tcf3* phenotypes in comparison to Tcf3K (11% versus 93%) with the same amounts injected (see Figure 6.2.6.3).

concentration	0	50μM	100μM	200μM	400μM	600μM	900μM	1200μM
Tcf3LL-PNA								
number of embryos	17		72	72	110	123	52	90
dead	1		5	2	10	8	3	34
death rate	6%		7%	3%	9%	7%	6%	38%
strong defects	0		0	2	10	15	3	24
moderate defects	0		3	7	12	17	10	12
weak defects	0		12	18	29	33	15	12
normal	16		52	43	49	50	21	8
phenotypes in surviv.	0%		22%	39%	51%	57%	57%	86%
Tcf3K-PNA								
number of embryos	19	47	48	45	92	78	42	
dead	3	7	7	8	18	33	30	
death rate	16%	15%	15%	18%	20%	42%	71%	
strong defects	0	15	6	6	26	21	7	
moderate defects	0	6	6	11	26	10	0	
weak defects	0	4	19	12	16	11	0	
normal	16	15	10	8	6	3	5	
phenotypes in surviv.	0%	63%	76%	78%	92%	93%	58%	
Tcf3Kmut-TG-PNA								
number of embryos	25		24	46	49	53	40	
dead	3		2	7	8	17	32	
death rate	12%		8%	15%	16%	32%	80%	
strong defects	0		0	0	1	0	0	
moderate defects	0		0	0	1	1	0	
weak defects	0		0	2	3	3	1	
normal	22		22	37	36	32	7	
phenotypes in surviv.	0%		0%	5%	12%	11%	13%	
Tcf3-MO								
number of embryos	9	171	112	34				
dead	1	17	14	15				
death rate	11%	10%	13%	44%				
strong defects	0	65	66	16				
moderate defects	0	19	19	2				
weak defects	0	25	5	1				
normal	8	45	8	0				
phenotypes in surviv.	0%	71%	92%	100%				

Figure 6.2.6.3. Effects of *Tcf3* antisense molecule injections. The sequences of the PNAs are shown in Figure 6.2.5.2. The Tcf3Kmut PNA includes mutations of two nucleic acids, compared to the complementary mRNA sequence. “MO” indicates the used *Tcf3* morpholino oligo. Untreated embryos are represented in the first column. The total numbers of uninjected/injected embryos are indicated by “number of embryos” and the “death rate” represents all dead embryos in percent. “Normal” stands for embryos without any phenotype and “phenotypes in surviv.” for the total number of *Tcf3* phenotypes

(weak, moderate, strong) of the surviving embryos in percent. All phenotypes were detected at stage 29 (34 somites).

In a next step the PNA Tcf3SPLL with a complementary sequence to a splice donor was generated for blocking splicing. The application of this PNA also resulted in specific *Tcf3* phenotypes, indicating a successful splice blocking of the *Tcf3* pre-mRNA. The numbers of phenotypes was however lower compared to the translation blocking Tcf3LL PNA. However, a combined injection of Tcf3LL and TcfSPLL (concentrations of 600 and 900µM) led to higher levels of overall (67% and 74%) and strong (20% and 24%) *Tcf3* phenotypes, implying a synergistic effect of translational and splice blocking of *Tcf3* expression (see Figure 6.2.6.4).

concentration	0	100µM	200µM	400µM	600µM	900µM	1200µM
Tcf3SPLL-PNA							
number of embryos	45	32	34	59	101	83	55
dead	2	2	2	3	21	7	49
death rate	4%	6%	6%	5%	21%	8%	89%
strong phenotype	0	0	0	2	11	6	1
moderate phenotype	0	0	1	5	8	11	1
weak phenotype	0	2	2	10	20	18	3
normal	43	28	29	39	41	41	1
phenotypes in surviving	0%	7%	9%	30%	49%	46%	83%
concentration				200+ 200µM	300+ 300µM	400+ 400µM	600+ 600µM
Tcf3SPLL+Tcf3LL							
number of embryos	37			45	93	121	42
dead	1			1	15	17	30
death rate	3%			2%	16%	14%	71%
strong phenotype	0			2	20	24	2
moderate phenotype	0			7	18	19	0
weak phenotype	0			13	14	34	9
normal	36			22	26	27	1
phenotypes in surviving	0%			50%	67%	74%	92%

Figure 6.2.6.4. Impact of *Tcf3* splicing PNAs. All injections were done at concentrations of 0, 100, 200, 600, 900 or 1200 µM either with *Tcf3* splicing (Tcf3SPLL) or translation targeting (Tcf3LL) or both (Tcf3SPLL+Tcf3LL) PNAs. The absolute numbers of uninjected/injected embryos are shown in row “number of embryos” and the numbers of phenotypes are indicated in percent (“phenotypes of surviving”). All dead embryos are represented in the row “dead” as total number and in the row “death rate” in percent. Embryos without any phenotype are indicated as “normal” and embryos with any *Tcf3* phenotype are counted as “phenotypes in surviving” in percent. All described phenotypes were detected at stage 29 (somites 34).

During late gastrulation a Wnt gradient, ranging from forebrain to tailbud is a hallmark of vertebrate development (Kiecker and Niehrs, 2001). At this time the Wnt8 proteins are posteriorly expressed in Zebrafish (Lekven et al., 2001) and Medaka embryos (Yokoi et al., 2003). But in anterior direction its expression is blocked by the antagonist Tcf3, necessary for the formation of tel-, diencephalon and eyes. This could be demonstrated by the loss of these brain structures in *Tcf3a headless* mutants in Zebrafish (Kim et al., 2000). Overactivity of *Wnt1*, elicited by a point mutation in Axin, results in an anterior shift of brain regions at expense of the forebrain and an overall caudalized embryo (van de Water et al., 2001; Dorsky et al., 1998). This shift could be demonstrated by midbrain marker genes, whose expression profiles were shifted in anterior direction (e.g. van de Water et al., 2001). Thus, I used the characteristic expression profile of *Wnt1* as a mid-hindbrain specific marker (in Zebrafish at the tailbud stage; Kim et al., 2000) for analyzing the *Tcf3* knockdown in Medaka embryos, which should result in an anteriorly shifted expression. Actually, this picture was observed in the embryos, which have been injected with Tcf3K or Tcf3LL PNAs (the expressions patterns of *Wnt1* in wild type and PNA injected embryos are shown in Figure 6.2.6.5). Thus, this result represents a further indication for the specificity of *Tcf3* knockdown by the modified Ugi-PNAs, targeting the sequence of the translational start point of *Tcf3* in Medaka.

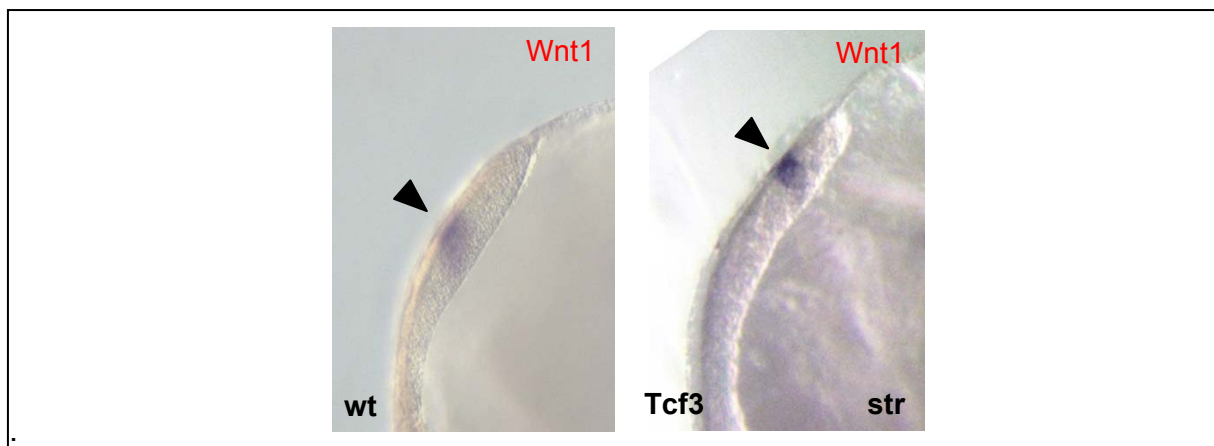


Figure 6.2.6.5. Expression of *Wnt1* in uninjected and *Tcf3* antisense molecule injected embryos. “Wt” stands for a wild-type embryo and “Tcf3 str” for a *Tcf3* antisense injected embryo with a strong phenotype. The arrow heads represent the *Wnt1* expression in the mid-hindbrain boundary. All embryos are fixed and stained at stage 16 and shown in lateral view.

Taken together in an *in vivo* environment a highly specific antisense function of the modified PNAs, targeting mRNAs of *gfp*, *Six3* and *Tcf3* could be demonstrated. Using K-modified mixed Ugi-PNAs almost the same efficiency in gene knockdown experi-

ments can be reached in comparing to morpholino antisense molecules. Therefore, Ugi-PNAs represent an important tool for gene specific downregulation in fish embryos. In addition a splice blocking function could be demonstrated which can be combined synergistically with PNAs blocking translation of the mRNAs.

7. Discussion

The main part of my experiments was focused on the examination of the interaction between the HMG box of Lef1, Tcf3 or Tcf1 and β -catenin. The main interaction between these proteins is mediated by the N-terminal part of Tcf/Lef and the Armadillo repeats 3 to 10 of β -catenin. It represents a strong interaction and plays the most important role for Wnt target gene upregulation. For the detection and analysis of the second weak interaction between the Tcf/Lef HMG domain and β -catenin, the basal transcription levels had to be increased. This was achieved by the transactivation domains of VP16 or p65.

7.1 The transactivation domains of VP16 and p65 turn Tcf/Lef proteins into strong activators

The viral protein VP16 is a strong activator (Cress and Triezenberg, 1991). This feature of VP16 has often been utilized by fusing it to transcription factors (e.g. to Gal4; Hagmann et al., 1997). I used this strategy also for my experiments. VP16 was C-terminally fused to Lef Δ 293, which led to a strong increase of the reporter activity up to 30 fold. This luciferase induction is directly dependent on the interaction of the Lef HMG domain with the Tcf/Lef binding sites. Lef-VP16 fusion proteins can directly activate Wnt target genes independent of the transactivation domain normally provided by β -catenin (Aoki et al., 1999).

Another transactivation domain derived from p65, a subunit of the transcription factor NF- κ B, works even better than VP16 (Ballard et al., 1992). Indeed, the C-terminal addition of p65 to Lef Δ 293 led to 12 times higher luciferase levels compared to VP16 fusion proteins. A high transactivation was important for sensitive detection of DNA binding differences. When fused to β -catenin Δ p65 resulted in 120 times higher luciferase induction levels than β -catenin Δ alone. For further experiments a fusion protein was constructed, which includes Renilla luciferase, Lef Δ 293 and p65 combined with deletion mutants of β -catenin. Thus, by using the transactivation function of p65, an efficient analysis of the DNA binding effects of the weak interaction between Lef Δ 293 and the Armadillo repeats of β -catenin lacking its natural C-terminal transactivation domain of β -catenin was possible.

7.2 Renilla luciferase fusion constructs allow a sensitive quantification of protein expression levels

The firefly luciferase is used for a well established biochemical reaction for the detection of transcription levels (Recchia et al. 2008). In my experiments increasing luciferase activities could be detected by adding increasing amounts of Lef1, Tcf3 or Tcf1 or β -catenin. The luciferase activity represents the transcriptional activity of the reporter constructs in the cell. But, for evaluating the correct influence of all components the cDNA of Renilla luciferase (Rluc) was N-terminally fused to the proteins. Indeed in my experiments the Rluc activity which represents the actual amount of fusion proteins, indicated that large proteins like e.g. RlucLef Δ 293m β cat Δ 65 were generally expressed at lower levels than small proteins like RlucLef Δ 293m65. Furthermore, the internal reference of Rluc strongly reduced the variations between the experiments and therefore improved the experimental set up by correcting for cell viability, cell number, transfection efficiency and protein synthesis.

7.3 The HMG box of Lef1, Tcf3 and Tcf1 weakly interacts with β -catenin

In 2005, a study by Daniels and Weis with purified proteins in pull down and competition assays demonstrated that β -catenin and Groucho directly compete for binding to the Lef1 C-terminal region (Daniels and Weis, 2005). It could be shown that Groucho as well as β -catenin are able to interact with Lef1. The Groucho/ β -catenin interaction site is located in a region, comprising the amino acids 252-397 of Lef1 (HMG domain plus few amino acids in C- and N-terminal directions). Therefore β -catenin has the ability to interact with Lef1 via this C-terminal region, implying the existence of a second low affinity interaction site nearby the primary high affinity N-terminally located binding domain. Indeed this interaction could not be detected by Daniels and Weis in a pull down assay, because they were not able to gain purified Lef Δ (252-397), due to the strong DNA binding of the Lef1 HMG box. Interestingly my results indicate that this interaction maps to the HMG domain of Tcf/Lef proteins. This hypothesis is supported by experiments in which β -catenin Δ (all Armadillo repeats) and Lef Δ 293 (HMG box plus C-terminus), were expressed as a fusion protein, leading to

a 3 fold luciferase upregulation (in comparison to Lef Δ 293 alone). For this experiment, a luciferase reporter with low affinity Tcf/Lef binding sites derived from the *Otx2* enhancer was used. The HMG domain is unstructured in solution and acquires its final structure only when bound to DNA. A protein-protein interaction with the HMG domain would therefore shift the equilibrium between the unfolded and the folded state to the latter. Since only a folded HMG domain can bind DNA efficiently, this would be equivalent to an increase in DNA affinity. An interaction between β -catenin and the HMG domain of Tcf/Lef proteins would therefore suggest a direct effect on DNA binding affinity. However this stabilization of the folded structure needs support by the first interaction at the N-terminal high affinity β -catenin binding domain of Tcf/Lef.

The hypothesis that in case of Wnt activation β -catenin is shuttled in high amounts into the nucleus and Tcf/Lef can be bound to DNA on low affinity Tcf/Lef binding sites with the help of β -catenin, is supported by several studies demonstrating an increasing Tcf/Lef occupancy of their binding sites upon Wnt pathway activation (Wöhrle et al., 2007; Yochum et al., 2008). But, in most cases these observations are explained by chromatin associated effects, which are primarily connected to the last two Armadillo repeats and the C-terminus of β -catenin. Tutter and colleagues (Tutter et al., 2001) could show by using Tcf/Lef consensus binding sites, that β -catenin can indeed support DNA binding of Lef1 but only in connection with chromatin assembly and not on naked DNA. Furthermore, it could be demonstrated that cooperative DNA binding by Lef1 and β -catenin is not dependent on the C-terminal transactivation domain of β -catenin. Several target genes contain high affinity Tcf/Lef binding sequences. However the majority of Tcf/Lef target sequences in the genome show considerable deviations from the consensus sequence. Here I studied mainly low affinity Tcf/Lef binding sites and found a supporting effect of β -catenin. The improved DNA binding observed in the experiments was restricted to low affinity binding sites and might therefore be independent on chromatin effects. The experiments have been performed in cell culture experiments. In these transient transfection experiments naked DNA was transfected into cells and the activation of the reporters was measured 24h after transfection. Under these conditions a prominent role of chromatin is unlikely. In addition the effects were not observed with consensus binding sites.

However, a chromatin independent effect has to be confirmed by further methods (footprint analysis, EMSA).

An interaction between the HMG domain and β -catenin Δ could not only be observed for Lef1 but in addition also for Tcf3 and Tcf1. In combination with Tcf3 the β -catenin effect was even stronger compared to Lef1 or Tcf1. The amino acid sequence of the HMG box in Tcf/Lef proteins is highly conserved (97% identity between the paralogous genes in humans; van de Wetering et al., 1991). However several amino acid changes are typical for the individual paralogs and therefore might directly affect the β -catenin interaction. Interestingly, Tcf3 shows a slightly reduced DNA binding affinity compared to Lef1 and Tcf1 (Figures 6.1.6.3, 6.1.6.4 and 6.1.6.5; Pukrop et al., 2001). An inverse correlation is seen for the β -catenin effect. Tcf3 is better supported in DNA binding by β -catenin than Tcf1 and Lef1, which showed almost identical results (Figures 6.1.6.3 and 6.1.6.5). It therefore remains to be shown whether Tcf3 exhibits a better interaction with β -catenin or simply a reduced affinity for its DNA target sites, where β -catenin exhibits stronger support (see Figure 6.1.6.4).

7.4. Point mutations in the Lef1 HMG box result in a stronger β -catenin effect

To examine the interaction between the Lef1 HMG domain and β -catenin in more detail, several point mutations (MI-MX) were introduced into the coding sequence of the HMG box, so that alanine was expressed instead of the wild type amino acids. The amino acids which were changed are exposed and are not critical for DNA interaction. These informations were derived from the crystal structure of a Lef1 DNA complex (Love et al., 1995). Alanine is an unpolar amino acid and is often used for neutral point mutations. However, in no experiments a loss of β -catenin caused luciferase induction could be obtained for the mutants. It is known from the literature that several negatively charged amino acids are responsible for an interaction with the positively charged groove of β -catenin (Poy et al., 2001). But, the addition of the mutants MI, MII, MIV and MXI which compensated the negatively charged amino acids glutamic acid and aspartic acid, had also no impact. These results might be explained by a wrong choice of amino acids for the mutations. It could also be that only a combination of point mutations would impair the interaction with β -catenin. There-

fore more experiments will be necessary to characterize the interaction interface of the Lef1 HMG domain with β -catenin.

In the course of the experiments a highly significant negative correlation between the binding affinity and the supportive effect of β -catenin could be observed (see Figure 6.1.8.3). The highest luciferase upregulation was observed for the Lef Δ 293 mutants MV and MIII (exchange of the amino acids lysine, arginine or histidine to alanine) and MVIII and MIX (arginine replaced by alanine). On the contrary, point mutations of the amino acids glutamic acid and aspartic acid led to the lowest luciferase induction levels (mutants MII, MI, MXI, MIV). But none of the mutations showed an induction below that of the original β -catenin Δ . These results can be interpreted in a way that the mutations led to a reduced DNA binding function, by impairing the stability of the HMG box. The mutants MIII, MV, MVIII and MIX showed a stronger influence than the mutants MI, MII, MIV and MXI. It is unclear why β -catenin supports weak DNA binding mutants to a higher degree than wild type Lef1. The inverse correlation seen in the experiments however indicates a systematic reason for this observation.

7.5 The C-terminus of β -catenin Δ interacts with the HMG domain of Lef1

The transient cell culture experiments indicate an interaction between the HMG box of Lef1, Tcf3 and Tcf1 and the β -catenin Armadillo repeats 10 (part of it) to 12. This was further supported by the highest induction levels seen for a construct, which includes the β -catenin Armadillo repeats 10 to 12, fused to Lef Δ 293. Initially, weak luciferase inductions were observed for β -cat Δ 518C (Armadillo repeats 1 to 9), suggesting that the Tcf/Lef HMG domain interaction does not depend on the C-terminal Armadillo repeats. However these results could not be confirmed in subsequent experiments. Further experiments will be necessary (in combination with point mutations), to see if an additional interaction is located in the N-terminal Armadillo repeats outside the critical repeats 10-12.

The primary interaction with the N-terminus of Tcf/Lef is mediated by two regions, a more N-terminal part, interacting with the Armadillo repeats 4 to 9 and a more C-terminal region, which binds to the Armadillo repeats 3 to 5. Both sections are found

in the first 65 to 69 amino acids of Tcf/Lef (Poy et al., 2001). The interaction is based on charges, being located on the helices of β -catenin and the Tcf/Lef interaction points (Graham et al., 2000; Hurlstone and Clevers, 2002). Tcf/Lef binds in antiparallel direction to β -catenin. The weak interaction domain of Tcf/Lef maps to the HMG box, close to the C-terminus of the transcription factors. Therefore in a Lef- β -catenin complex the HMG box must be located next to the N-terminus of Lef, because it interacts with Armadillo repeats 10 to 12. This structure could be connected to the strong DNA bending induced by Tcf/Lef (Giese et al., 1992) or the wrapping of Lef around the DNA (Love et al., 1995).

7.6 Plakoglobin is able to interact with the Lef1 HMG domain

Plakoglobin is closely related to β -catenin which can be seen from high sequence homology, especially in the middle part, representing the 12 Armadillo repeats (83% identity). This would imply that Plakoglobin could also interact with the Lef1 HMG domain at a second interaction site. In my experiments the Armadillo repeats of Plakoglobin showed an induction of luciferase activity indicating such an interaction. The absolute numbers of induction were slightly lower compared to β -catenin. Further evidence comes from a deletion variant of Plakoglobin Pla Δ 473N, including the Armadillo repeats 7 to 12 which also shows induction of the Lef HMG domain, similar to β -cat Δ 423N which contains the corresponding C-terminal repeats. Hence these results lead to the conclusion that probably the interaction is mediated by the same regions. This assumption is supported by the fact that the primary interaction domain between Plakoglobin and Lef1 is mediated by the N-terminus of Lef1 and the Armadillo repeats 3 to 10 of Plakoglobin (Behrens et al., 1996; Huber et al., 1996). However other regions seem to be involved in the interaction with Tcf4, because it could be shown that the primary interaction with Tcf4 is mediated by residues 51 to 80 of Tcf4 and the Armadillo repeats 1 to 6 of Plakoglobin (Miravet et al., 2002). These interaction regions differ from those of β -catenin-Tcf4. Therefore an exact mapping must be carried out for the interaction. Nevertheless, the existence of a second weak interaction between Plakoglobin and Lef1 indicates an evolutionary conservation at least back to a time before β -catenin and Plakoglobin were not yet splitted into separate genes.

In this context, another essential question is raised, namely the general function of Plakoglobin in Wnt signaling together with the relevance of the interaction with the HMG domain. The experiments with β -catenin indicate that this interaction can further support the upregulation of the Wnt target genes by improving the DNA binding of Lef1, Tcf3 and Tcf1. In most cases a transcriptional activation takes place, mediated by the C-terminal transactivation domain of β -catenin. But the function of Plakoglobin is different from β -catenin. The function of Plakoglobin depends on the cell type and the interaction partner and is limited to specific target genes. In many cases the formation of the Plakoglobin-Lef1 complex leads to repression (Williamson et al., 2006; Charpentier et al., 2000), whereas an interaction with Tcf4 often results in transcriptional upregulation (Williamson et al., 2006; Kolligs et al., 2000). The difference depends on the variable interaction sequences, which member of the Tcf/Lef proteins is used and in particular on the promoter context within the target genes resulting in different binding partners. Thus, the question about the relevance of the interaction between Plakoglobin and the HMG domains of Lef1 and Tcf4 must be answered by the promoter context. However, a potential for Wnt target gene regulation exists. In case of certain Wnt target genes, this interaction could fulfill a more important role. The C-terminal transactivation domain of Plakoglobin is weak. However the improved DNA binding of Tcf/Lef proteins can lead to both activation and repression of these target genes. An interesting case represents the c-myc gene. An improved binding efficiency of Lef1, mediated by Plakoglobin, results in a downregulation because of the association of Plakoglobin with other corepressors. In contrary, an interaction with Tcf4 reinforces its DNA binding, which supports an upregulation (in combination with coactivators; Williamson et al., 2006).

7.7 The effects of the HMG box / β -catenin interaction on target genes

A general assumption for the Wnt signaling pathway is that Tcf/Lef transcription factors are already bound to their target sequences in the genome (e.g. Clevers et al., 2006). In the inactive state they are associated with Groucho repressors which are replaced by β -catenin upon activation of the pathway. This model indeed seems to be true for high affinity binding sites. However, my results suggest a different model for low affinity binding sites, often found in natural Wnt target genes.

This model is based on the observed effect of β -catenin on Lef1, Tcf3 or Tcf1 in combination with low affinity Tcf/Lef binding sites. Here β -catenin compensates the weaker Tcf/Lef DNA binding, leading to stronger expression levels of Wnt target genes. The β -catenin effect is mediated by the Tcf/Lef binding motifs, because in some experiments I varied the linker sequences between them, which did not lead to substantial differences in luciferase upregulation. In the enhancers of *Otx2*, *Axin2*, *c-myc* and *naked cuticle* several different Tcf/Lef binding motifs exist for upregulation, some of them considerably differ from the ideal Tcf/Lef consensus sequence. The question arises whether all weak binding sites can mediate the observed β -catenin effect?

In a first analysis of the two different Tcf/Lef binding sites in the *Otx2* enhancer electrophoretic mobility shift assay (EMSA) experiments were used to determine the relative affinity for Lef1 (Thomas Czerny, unpublished). The binding sites were compared to the consensus sequence CCTTTGATC. In the *Otxa* binding motif (CCTTTGAAC), the change of a thymine to an adenosine resulted in a Lef1 binding efficiency of 2% compared to the consensus sequence. In the case of *Otxb* (CCTTTGTTC), the difference was even higher, resulting in only 0.2% binding affinity compared to the ideal motifs. This reduction is due to an exchange of an adenosine with a thymine. Therefore, strong differences exist in the binding efficiency of natural Tcf/Lef binding sites.

Next I compared different Tcf/Lef binding sites, originating from the enhancers of the genes *c-myc* and *naked cuticle* (the sequences of the Tcf/Lef binding sites used are shown in the Chapter 6.1.11). The selected sites have in common that they get occupied by Tcf/Lef proteins upon Wnt pathway activation, possibly due to β -catenin interaction. However, this is in contradiction to the general view of the Wnt signaling pathway.

In the experiments using the *Axin2* or the *c-myc* regulatory regions, a two fold upregulation by β -catenin was observed. In addition reporters containing multimerized Tcf/Lef binding sites with deviations at several positions (pMlucF24Lefmyc and pMlucF24Lefnkd reporter) were used. The application of the artificial *c-myc* reporter led to a two fold and of the *nkd* reporter even to a four fold luciferase upregulation by β -catenin, again supporting the positive effect on Lef DNA binding. Summarizing up

all results, the β -catenin effect could be demonstrated for several natural Tcf/Lef binding sites derived from the *Otx2*, *Axin2*, *c-myc* and naked cuticle genes. All these Tcf/Lef binding sites differ from the consensus Tcf/Lef sequence. The highest luciferase inductions were reached with a Tcf/Lef binding site deriving from the naked cuticle gene and with one derived from the *Otx2* enhancer (*Otxb*). Probably the modification of the second nucleotide downstream of the three thymines has the strongest impact. However, all these findings perfectly fit into the hypothesis that β -catenin can improve the DNA binding of Tcf/Lef to particular target sequences. Further analysis with more candidate sequences will be necessary to qualify the exact sequence requirements of this effect. However, the data indicate that β -catenin has a profound effect on Tcf/Lef proteins not only by providing a transactivation domain but also by regulating their DNA binding efficiency.

7.8 A model for the improvement of Lef1, Tcf3, Tcf1-DNA binding through β -catenin

Combining all results, a model arises explaining the impact of β -catenin on Tcf/Lef DNA binding (see Figure 7.8).

As a first step, Wnt pathway activation takes place by binding of Wnt ligands to the Frizzled and LRP5/6 receptors, leading to the disassembly of the destruction complex in the cytoplasm. As a direct consequence high amounts of β -catenin accumulate in the cytoplasm and are shuttled into the nucleus. At particular target genes like *Otx2*, *c-myc*, *Axin2* or *naked cuticle*, Tcf/Lef proteins bind with a low occupation rate. With the help of β -catenin they become strongly attached to their target sequences. β -catenin efficiently binds to the N-terminal region of Tcf/Lef (β cat-BD). In addition it interacts with the HMG box. This interaction shifts the equilibrium of the HMG domain between the unfolded and the folded state to the latter, thereby stabilizing the DNA interaction. This process is essential for low affinity binding sites, where the interaction with β -catenin is needed for efficient binding of Tcf/Lef proteins to their targets. Subsequently, coactivators like Bcl-9/Legless, Pygopus and CBP/p300 are important for transcriptional upregulation. At the C-terminus of β -catenin chromatin remodeling factors like Brg-1 interact and further support the transcriptional initiation, including acetylation and demethylation processes. Finally a large complex forms centering

around the Tcf/Lef binding sites in the target genes leading to transcriptional activation or repression depending on the context.

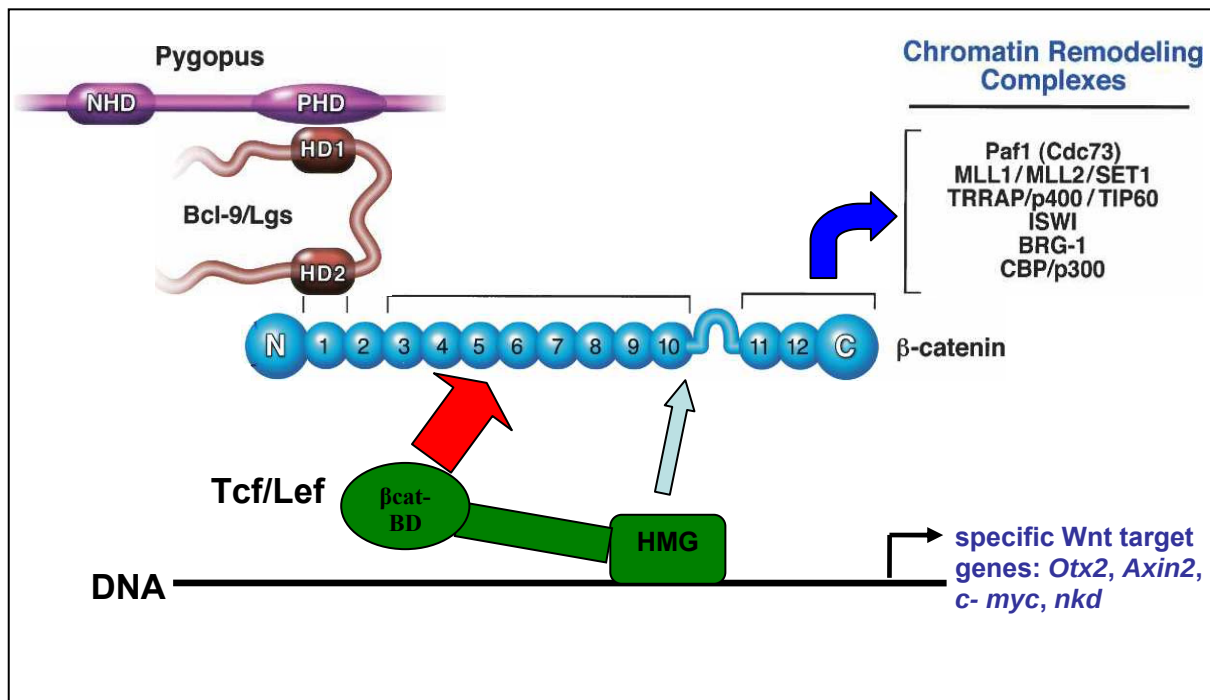


Figure 7.8. A model for Wnt target gene upregulation, taking into consideration the β -catenin effect on Tcf/Lef DNA binding. The result is an effective Wnt target gene activation. β cat-BD represents the β -catenin binding domain and HMG the high mobility group box for DNA interaction of Tcf/Lef. NHD indicates the N-terminal homology domain and PHD the C-terminal plant homeo domain of the Pygopus protein. The homeodomain HD1 of Bcl-9/Lgs (Legless) interacts with Pygopus and the homeodomain HD2 with β -catenin. The chromatin remodeling complexes Paf1, MLL1/MLL2/SET1, TRRAP/p400/TIP60, ISWI, BRG-1 and CBP/p300 cooperate C-terminally with β -catenin. The big red arrow represent a strong interaction and the small blue arrow a weak interaction of Tcf/Lef with β -catenin (see the text for more details).

7.9 Modifications of peptide nucleic acids lead to improved membrane permeability, optimal solubility and efficient DNA/RNA binding

The aim of these experiments in Medaka embryos was the application of peptide nucleic acids, which were chemically modified by the company Ugichem GmbH. As a first step, the membrane permeability of the PNAs into mammalian HEK 293 cells in cell culture experiments was examined. For this the PNA was labeled with fluorescent dyes and analysed for its intracellular distribution. After an incubation time of 24 hours, signals could be observed in the cells. The distribution of the PNAs was specific, the PNA appeared in granular structures. This means that these PNAs are able

to pass cell membranes in contrary to morpholino oligos. How they got into the cells, through diffusion or endocytosis, has to be evaluated in future experiments.

As a second step a stable HEK 293 cell line, including a reporter construct, was established, which expresses a GFP-luciferase fusion protein. The fusion protein comprises a fragment of the ODC protein, leading to an enhanced degradation and therefore a rapid turnover. This was done to detect the antisense function of the synthesized PNAs in mammalian cells. The PNAs were targeted to the *gfp* mRNAs. Although, two different PNAs with modified target regions were designed (differing concerning the translation start region) and different PNA incubation times were carried out, in no case a decrease of the GFP as well as the luciferase signal could be observed. The reason for this is probably an insufficient concentration in the cells, due to a low diffusion rate.

Subsequently, the same labelled PNAs, which were synthesized for the diffusion experiments in HEK 293 cells, were also used for diffusion experiments with Medaka embryos. Embryos shortly before hatching were dechorionized and incubated over night with the labelled PNAs. After washing, the uptake of the PNAs into the digestive tract could be observed, including the swim bladder. The PNAs were observed in the whole lumen and additionally within surrounding cells, which are part of the mucosa. The underlying mechanism is probably passive diffusion, indicating a membrane permeability of the PNAs also in vivo. The improved diffusion abilities of the PNAs represent a major progress in the development of functional PNAs. However additional experiments will be necessary to qualify the extent of this diffusion.

With the chemical modifications of the PNAs, the functional efficiency, the membrane permeability and solubility could be improved, leading to highly efficient antisense tools. In order to test their application in knockdown assays, experiments were done with coinjections of *gfp* mRNA with the specific PNAs, resulting in GFP signal reduction. The results were compared to those of morpholino oligos and unspecific PNAs. The strongest impact on the efficiency had the length of molecules. An optimal function was observed at a length of 16 units. The reasons that both shorter and longer PNAs were less efficient could be an increasing unspecificity in mRNA blocking. This

phenomenon could also be detected for morpholino oligos, reaching an optimal length at 25 to 26mers (Sumerton and Weller, 1997; Stein et al., 1997).

The functional efficiency of the synthesized Ugi-PNAs could be further improved by introducing additional side chains in combination with Nielsen-PNA monomers. The resulting mixed PNAs show better mRNA interaction and improved membrane permeability. In order to avoid aggregation of the PNAs in water, a trimethyl-lysine end group was added. The amino acid lysine possesses good solubility properties and with the addition of three methyl groups the overall charge is predominantly neutral. The trimethyl-lysine groups resulted in high solubility, but in the cells a stronger aggregation of the PNAs could be observed. In addition the synthesis of PNAs with dinitrophenyl acetic acid as another chemical end group did not lead to better results. As a consequence PNAs with one or two trimethyl-lysine groups were used for most experiments. In morpholino oligos the problem of low solubility of non-ionic compounds is solved by their linkage to phosphorodiamidate, leading to improved stacking interactions between the pairs of nucleic bases (Kang et al., 1992).

7.10 Effective knockdown of *Six3* and *Tcf3* by peptide nucleic acids in Medaka fish

The modifications of the PNAs which resulted in an optimal DNA binding, good membrane permeability and solubility were subsequently applied for synthesizing PNAs for endogenous target genes in Medaka. Firstly, the *Six3* gene, which plays an important role for eye development was chosen. As a direct control a morpholino oligo, which also targets the *Six3* gene, was used. The resulting phenotypes were compared to the experiments of Carl and colleagues (Carl et al., 2002), who did injection experiments with morpholino oligos for analysing the function of *Six3* in eye development in Medaka. The application of the PNA Six3L, targeting the translation start point, led to similar results like the morpholino. With lower concentrations of morpholino oligos a higher rate of phenotypes arose in comparison to Six3L injections, but in the first case the death rate of embryos was higher than for the PNA. The group of control PNAs (Ref16mix) did not show any *Six3* specific phenotypes, but nearly the same increase of dead embryos at high concentrations. This death rate can therefore be explained by unspecific toxicity processes. Especially by using high

concentrations, the p53 pathway can be activated, which results in increased cell death (Robu et al., 2007). The phenotypes of the specific *Six3* downregulation by the PNAs is in good agreement with data of *Six3* double knockout mice, which loose telencephalon, eyes and olfactory placodes and therefore the complete *Bf1* expression (Lagutin et al., 2003) (the *Bf1* gene is expressed in forebrain and anterior part of eyes). In *Six3* knockdown embryos, in strong phenotypes the complete forebrain was missing, including the *Bf1* expression (see manuscript, Chapter 9).

In comparison to *Six3L*, which includes one trimethyl-lysine end group, injections of the PNAs *Six3/1* (no specific end group) and *Six3/1N* (dinitrophenyl acetic acid end group) had a weaker impact on *Six3* blockage, shown by fewer *Six3* specific phenotypes. Probably these observations are based on a stronger mRNA binding efficiency of *Six3L* due to the trimethyl-lysine group. Thus, for the following experiments PNAs with at least one trimethyl-lysine group were used.

In addition, a second PNA, *Six3/2*, targeting an internal *Six3* sequence was synthesized for an internal block of the translation. For such a process the binding would have to be very strong, because after the translation machinery starts disturbing elements like secondary structures are normally removed. In this case the oligo would have to achieve a particularly strong interaction to block translation. Therefore oligos binding to an internal sequence are similarly removed. The application of *Six3/2* led to small, but convincing numbers of *Six3* phenotypes, indicating a stop of *Six3* expression. The basis for this might be the strong interaction of PNAs with RNA, whereas morpholino oligos are only efficient when they are considerably longer (25mers used for morpholino oligos, 16mers for PNAs). But the high death rate of the embryos at the effective concentrations indicates an unspecific toxicity, so that other variants must be developed for future applications. To demonstrate specificity, PNAs with the same sequence as *Six3L* were used as a basis for introducing one point mutation. This exchange resulted in a strongly reduced number of *Six3* phenotypes, because of the reduced binding efficiency. These results show that already the introduction of one mismatch has a dramatic effect on PNA binding, demonstrating the high efficiency of the PNAs. In general, the application of PNAs achieves a nearly equal rate of gene knockdown efficiency compared to morpholino oligos, only differing by

the concentration. Therefore the shorter length of the PNAs can be compensated by their higher binding strength.

As a second endogenous target gene for knockdown experiments *Tcf3* was used. *Tcf3* is involved in the establishment of anterior structures by antagonizing the Wnt gradient. In Zebrafish two *Tcf3* genes (*Tcf3a*, *Tcf3b*) were identified, which complement each other and reinforce their functions (Dorsky et al., 2003). Therefore, the existence of just one *Tcf3* gene in Medaka reflects an ancient situation, after which a gene duplication in Zebrafish followed (Ohta et al., 2000). In Zebrafish the specific *Tcf3* phenotypes of the *headless* mutant (point mutation of *Tcf3*, leading to loss of function; described in Kim et al., 2000) served as a basis for the classification of the phenotypes by the *Tcf3* PNAs. A direct comparison of the two antisense molecules, both targeting the translation start point of *Tcf3* mRNA, showed a higher efficiency of morpholino oligos compared to PNAs. An advanced PNA variant Tcf3K, including a lysine side chain, raised the levels of *Tcf3* blockage to those of morpholino injections. In addition the application of a mutated Tcf3K variant (two aberrant nucleic bases) led to low amounts of embryos with *Tcf3* phenotypes. Thus, the specific mRNA binding of PNAs could be further improved. The specificity of the *Tcf3* knockdown experiments was analyzed by determining the localization of *Wnt1* expression in injected embryos. *Wnt1* represents a marker for the mid-hindbrain boundary. The anterior shift of *Wnt1* expression in *Tcf3* impaired embryos documents the lack of *Tcf3* expression in the injected embryos. In Zebrafish impaired *Tcf3* function (point mutation in *Tcf3*) leads to an anterior shift of *Wnt1* (Kim et al., 2000) and upregulation of the Wnt pathway by lithium treatment or application of dominant negative *gsk3 β* mRNA results in a more anterior directed expression of *Wnt1* (van de Water et al., 2003).

Another variant of a *Tcf3* knockdown was done by using a PNA which targets a splice donor, hindering the correct splicing before translation. This leads to a nonfunctional Tcf3 protein. The application of Tcf3SPLL-PNA resulted in a higher number of *Tcf3* specific phenotypes. A combined injection of Tcf3LL in combination with Tcf3SPLL resulted in strong phenotypes at high numbers. Nearly the same number of *Tcf3* phenotypes could be obtained compared to morpholino oligos. These results again show the high binding efficiency of the PNAs, allowing many different applications.

Summarizing up the activity of the applied PNAs, they are almost as successful as morpholino oligos in blocking the function of endogenous genes. This fact is based on their high binding strength and efficiency. Their specificity is as good as that of morpholino oligos, which has been a problem of some antisense oligos or siRNAs. In Zebrafish (Urtishak et al., 2004) and *Xenopus* (Efimov et al., 2006) a similar efficiency for gene knockdown was reached by using variants of negatively charged HypNA-pPNAs, which consists of alternating trans-4-hydroxy-L-proline/phosphonate polyamides as backbone. The Ugi-PNAs show more specificity in gene knockdown, which is characterized by a lack of unspecific phenotypes, which were found with HypNA-pPNAs in comparable concentrations. In general, an advantage of the PNAs lies in the flexible application. Thus, a powerful tool for gene knockdown in *in vivo* systems could be developed.

8. Citations

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9. Manuscript

The following manuscript was submitted to “Nucleic Acid Research” at 8th of September 2011:

Side chain modified peptide nucleic acids (PNA) for knock-down of *six3* in medaka embryos

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Abstract

Synthetic antisense molecules have an enormous potential for therapeutic applications in humans. The major aim of such strategies is to specifically interfere with gene functions, modulating cellular pathways according to the therapeutic demands. Among the molecules which can block mRNA function in a sequence specific manner are peptide nucleic acids (PNA). They efficiently and selectively interact with RNA, however a drawback is their hydrophobic nature, strongly hampering *in vivo* applications. We generated new backbone modifications of PNAs, which exhibit amphipathic properties. When we examined the activity and specificity of these novel “Ugi-PNA” molecules in medaka (*Oryzias latipes*) embryos, high solubility and selective binding to mRNA was observed. Injection of Ugi-PNA molecules directed against the medaka *six3* gene, which is important for eye and brain development, resulted in a highly efficient gene specific knock down. When directly compared with morpholino antisense molecules Ugi-PNAs showed similar activity at higher concentrations, however they exhibited considerably less toxicity than morpholino oligomers.

Keywords

PNA, knock down, medaka, Six3

Introduction

More than 40 years ago, first attempts were made to block mRNA activity by antisense molecules (1). Generally, antisense oligonucleotides represent single-stranded molecules with nucleic acids for sequence specific binding of their complementary sequence in the target mRNA. The binding either results in mRNA cleavage, caused by the activation of endonucleases RNase H or L (2) or in the inhibition of translation (3). Over the time modifications of the nucleic bases, or the backbone were introduced to improve their activity and biological stability. In morpholino antisense molecules, the ribose is replaced by morpholino rings and non-ionic phosphorodiamidate is used instead of phosphodiester linkages (4). Their binding strength closely resembles that of RNA or DNA molecules, therefore molecules with a length of 25 nucleotides are used for efficient transcriptional blockage. For translational blocking 5' untranslated region or the region around the start codon of the target mRNA are selected (5). Morpholino oligomers can also be used for efficient blocking of the splice machinery (6). The highly specific effect of morpholino oligomers on gene silencing could be demonstrated in sea urchins (7), *Xenopus* (8), Zebrafish (9) and Medaka (10). Today they are widely used in many species for gene specific knock down.

In 1991 Nielsen and colleagues created peptide nucleic acids (PNA) which instead of the phosphate ribose ring of DNA contain a polyamide backbone (N-[2-aminoethyl]-glycine units) (11). PNAs bind to complementary RNA or DNA in a sequence-specific manner (12, 13). The chemical structure is responsible for a high stability against proteases and nucleases and thermal and pH fluctuations (14). The entire neutral charge of the molecule decreases the electrostatic repulsion, which results in high hybridization affinity with RNA and DNA. Consequently short probe lengths (13-18 bases) are sufficient for selective binding, thereby

reducing the probability for forming secondary structures (14, 15, 16). In addition the introduction of mismatches has a stronger effect on the stability of PNA/DNA interactions in comparison to DNA/DNA duplexes, demonstrating the high specificity of PNAs (17). Several *in vitro* techniques make use of the extraordinary affinity of PNAs (18, 19). *In vivo* techniques also strongly benefit from the highly specific binding of PNAs (20), however unmodified Nielsen-PNAs suffer from unfavourable properties making their application *in vivo* inefficient (21). One of the biggest problems of PNAs is the low solubility due to the absence of charges. Introduction of negative charges by forming hetero-oligomers of alternating trans-4-hydroxy-L-proline/phosphonate polyamides with DNA bases (HypNA-pPNA) led to considerable improvements and allowed specific down regulation of target genes in zebrafish embryos (22).

Here we tested a less dramatic modification of the PNA backbone in order to keep the conformation most similar to the well established original Nielsen-PNAs. The resulting Ugi-PNAs contain phosphonic ester side chains in an otherwise non-modified polyamide backbone. The neutral Ugi-PNAs show strongly improved solubility, but keep the affinity and specificity of Nielsen-PNAs. In particular mixed versions of Ugi- and Nielsen-PNA components show favourable properties. We demonstrate the efficiency of these new antisense molecules by blocking of gfp expression and *in vivo* downregulation of *Six3* gene function in medaka embryos.

Material and Methods

PNA synthesis

PNA monomer building blocks are commercially available. Optical pure (R-configuration according to CIP rules) Ugi-PNA monomer building blocks were synthesized according to a route reported previously (23, 24, 25). N²-Boc-N⁶, N⁶, N⁶-trimethyl-(L)-lysine iodide (TML)

as building block was prepared as published by Chen and Benoiton (26). A schematic presentation of the building blocks is shown in Supplementary Figure S1. All PNAs were synthesized on a fully automated solid phase synthesizer (Multisynthes Syro) according to a protocol developed by Koch (27).

The PNAs were dissolved in nuclease free water by repeatedly shaking and vortexing. Finally they were gently sonicated for 2 minutes with repeated pulses. Subsequently the PNAs were divided into aliquots of 100 μ l (2mM final concentration) and kept at -80°C.

Microinjection into medaka embryos

Embryos of the medaka Cab strain were used for all experiments. Stages were determined according to Iwamatsu (Iwamatsu, 2004). For the *gfp* experiments, first mRNA of *gfp* was *in vitro* transcribed using the T7 High Yield Message Marker Kit (Ambion) according to the manufacturer's instructions. The injection solution containing *gfp* mRNA (10 or 20ng/ μ l), PNAs (50-600 μ M) and RiboLock RNase inhibitor (2units/ μ l; Fermentas) were mixed on ice and then injected into embryos at the one-cell stage. Injection of *six3* antisense molecules was done at concentrations of 50 - 1200 μ M PNAs or morpholino oligos. After injection the embryos were incubated at normal conditions (at 27°C).

Quantification of the PNA concentration in the embryos

Sulforhodamine labelled PNAs (Gfp16mixRho) were injected at concentrations of 50 μ M, 100 μ M, 500 μ M and 900 μ M at the one-cell stage. At the four-cell stage pictures were made under the fluorescence microscope at standardised exposure times (1 ms, 10 ms, 100 ms and 1000 ms) and quantified by the ImageJ programme (background values were subtracted from all samples and only intensities within a linear range were considered for the quantification). For comparison cellulose sulphate beads of an average diameter of 730 μ m were soaked several days with the labelled PNA, shortly washed and then treated the same way under the

fluorescence microscope. The volume of the embryo at the four-cell stage was determined by measuring the average diameter of the blastomeres (250 μm) resulting in a volume of 33 nl for the embryo. Using the volumes of the beads and the embryos the internal concentration of the PNA in the embryos could be calculated and resulted in an average value of 25.7 μM for injections with 100 μM PNA (injections were performed by one person in a reproducible manner). The average injection volume therefore was 11.3 nl. All experiments were done in 5 fold repetitions.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed as described previously using DIG-labelled probes (28).

Results and Discussion

Synthesis of novel peptide nucleic acids

Nielsen-PNAs bind corresponding RNA sequences with high affinity and specificity however unfavourable properties like their low solubility hamper their application *in vivo*. By employing the building blocks shown in Supplementary Figure S1, we introduced phosphonic ester side chains into the otherwise non-modified backbone of Nielsen-PNAs. As a result novel PNA variants were obtained, which we call Ugi-PNAs (see Fig. 1).

First we investigated the properties of a 15mer Ugi-PNA. The oligomer exhibited good solubility in water and we were able to prepare highly concentrated stock solutions (10 mM). However, it turned out that Ugi-PNAs capped with acetyl at the 5' end tend to form stable foam after sonification at this concentration. A possible explanation might be the large number of phosphonic esters residues, which introduce a highly polar but not ionic amphiphilic character into the oligomer. The tendency to form foams was not observed when the Ugi-PNAs were permanently charged with trimethyl-lysine (TML) at the 5' end prior to capping with acetyl (see Supplementary Figure S1).

Ugi-PNAs efficiently block translation of *gfp* mRNA

In order to establish conditions for the application of Ugi-PNA molecules for antisense approaches we established an assay for translation blocking. Cell culture based assays include the problem of the transport of PNAs (or PNA/DNA duplexes) into the cells (29). To use an unbiased strategy we therefore injected PNAs into fish embryos. The Ugi-PNAs were co-injected together with *gfp* mRNA into medaka embryos at the one-cell stage. As target sequence within the *gfp* mRNA we selected a region directly after the AUG (see Fig. 2), which previously had been used for knock down strategies with morpholino oligos (10). The embryos continued development and 24 hours later the *gfp* signal was observed under the

fluorescence microscope. According to the *gfp* expression level, the embryos were categorized into 4 groups with strong, moderate, weak and no *gfp* signal, respectively (Fig. 3A-D). To consider the small variations of the injection procedure, we calculated an overall number of *gfp* signal intensity for all embryos, which is based on different weights for the individual groups of embryos. Strong embryos were counted as 100%, embryos of the moderate group as 30%, those of the weak group as 10% and those showing no *gfp* signal were counted as 0%. From this an average number for all embryos was calculated and then corrected by those for the injection of mRNA alone. The resulting value represents the average *gfp* intensity in % (mRNA alone results in 100%).

We first compared the efficiency of different lengths of the oligomers (12mer, 14mer, 16mer and 17mer; for sequence information see Fig. 2). The Ugi-PNAs (200 μ M) were co-injected with 10 ng/ μ l *gfp* mRNA. Although all PNA lengths were able to reduce the *gfp* signal in the embryos (Fig. 3E and Supplementary Table S1), the strongest effect was observed for the 16mer PNA (Gfp16; reduction to 28% average *gfp* intensity).

To investigate the influence of the steric hindrance of the phosphonic ester residues we synthesized a second set of 15 and 16mers. In this set an additional methylene group was introduced into the side chain (R-C3 instead of R-C2; see Fig. 1) to elongate the alkyl spacer between the phosphonic esters and the oligomer backbone. Furthermore, the number of side chains was reduced, forming a hybrid oligomer of Ugi- and Nielsen-PNAs components. The presence of Nielsen-PNA monomers in the molecules increased the binding affinity and consequently resulted in stronger reduction of the *gfp* signal compared to C2-Ugi-PNAs (data not shown). We therefore increased the amount of *gfp* mRNA for the co-injection assay to 20 ng/ μ l. This resulted in stronger *gfp* intensity, which allowed us to better evaluate the improved antisense effect of the mixed PNAs. At a concentration of 200 μ M, a 15mer PNA with a mixed backbone (Gfp15mix) showed a reduction to 59% of the *gfp* fluorescence

intensity. This result could be improved when a 16mer mixed PNA (Gfp16mix) was injected (41%), demonstrating a superior effect of 16mer PNAs also for mixed backbones. Similar effects were observed for a mixed PNA containing 4 TML residues (Gfp16mixL4). At higher concentrations of 400 μ M or 600 μ M the average gfp intensity was reduced to minimum levels of 12%. As a control for the specificity of the PNAs, we used a reference PNA with a completely unrelated sequence (Ref15mix). The gfp level in this control experiment was similar to the group of injected embryos without adding PNAs (Fig. 3F). Furthermore, we did not observe any toxicity of the PNAs in the fish embryos at higher concentrations (up to 600 μ M; see death rates in Supplementary Table S2).

Uniform distribution of PNA molecules in medaka embryos

To get more information about the fate of the PNAs in the embryos, we injected a sulforhodamine labelled mixed PNA variant (Gfp16mixRho; for sequence information see Fig. 2). After injection at the one-cell stage the PNA distributed equally into the dividing blastomeres (Supplementary Figure S2). A uniform distribution of the PNA in the embryo could also be observed at later stages, no signals were detected in the yolk. Using this labelled PNA we were able to exactly quantify the injection volume and the final concentration of the PNAs in the embryo. As a reference we used small beads (average 730 μ m), which were soaked for several days with a defined concentration of the PNA. A comparison of the fluorescence signals of these beads with the injected embryos allowed us to determine the injection volume to 11.3 nl on average (these reference injections and all following injections were done by the same person in a reproducible manner). Injection of antisense molecules at 100 μ M consequently resulted in a concentration of 25 μ M in the embryonic cells (the volume of the embryo was determined at the four-cell stage at 33 nl, for calculations see Material and Methods). Here uniform distribution within the cells was assumed.

Knock down of the *six3* gene in medaka embryos by Ugi-PNAs

The Gfp-PNA molecules were tested in an *in vivo* setting, but the mixing of gfp-antisense PNAs with the *gfp* mRNA in the injection solution could lead to PNA/RNA hybridization prior to injection into the embryos; to avoid this possibility, we decided to test the targeting specificity of the PNAs in a complex *in vivo* environment, with a large excess of non-target mRNA. We therefore selected the endogenous *six3* gene as a target for PNA knock down experiments.

Six3 is part of the Six family of proteins, whose members are characterized by the presence of an N-terminal Six domain and a homeodomain (30). *Six* genes are highly conserved across the animal kingdom. In *Drosophila* the *six3* homologue *optix* fulfills important functions in the development of the visual system (31). In vertebrates, *six3* and *six6* are expressed in developing areas of the lens, neural retina, retinal-pigmented epithelium, nasal placodes, optic chiasm and forebrain. They take over important tasks in forming the rostral brain and the eye, especially the retina and the lens (32, 33, 34). The critical function in eye development could also be demonstrated in medaka where inactivation of *six3* by morpholino knock-down resulted in lack of forebrain and eye structures (10).

Based on the results of the gfp experiments we synthesized a 16mer mixed C3-PNA for the knock down experiments (Six3L; for sequence information see Fig. 2). The chosen sequence is a subset of a published morpholino 25mer directed against the *six3* 5'-region, directly upstream of the start codon (10). After injection of the Six3L-PNA a variety of eye and forebrain phenotypes were observed, in good agreement with previous results obtained for morpholino oligos in medaka embryos (10). Based on the eye and forebrain phenotypes we divided these embryos into three groups. Embryos with a weak phenotype showed a size reduction of the eyes, which at the anterior part pointed towards the midline (Fig. 4A arrows). Embryos with a cyclopic eye phenotype were characterized as moderate phenotype and

embryos with almost no eye and forebrain formation were determined as strong phenotype (Fig. 4A). The eye and forebrain phenotypes in Six3L-PNA injected embryos were then confirmed by analyzing the expression of the winged-helix transcription factor *bfl*. It has been shown that *bfl* is essential for forebrain and eye development (35, 36) and is regulated by Six3 (37). As expected the *bfl* expression was strongly reduced in Six3L-PNA injected embryos, especially in those with strong eye and forebrain phenotypes (Fig. 4B).

The frequency of the obtained phenotypes was dependent on Six3L-PNA concentration. Whereas at lower concentrations (e.g. 50 μ M) only 10% of the surviving embryos showed a weak phenotype, at higher concentrations (e.g. 400 μ M) up to 95% of the surviving embryos exhibited eye and forebrain phenotypes (Fig. 4A,C). A further increase of the PNA concentration to 900 μ M only slightly improved the proportion of strong phenotypes, however the frequency of mortality increased dramatically (82%; 51 of 62 injected embryos) indicating toxic effects of PNAs at higher concentrations.

To examine the selectivity and the toxicity of the PNAs, we compared the frequency of the mortality and the obtained eye phenotypes in Six3L-PNA injected embryos with two other control groups. First we injected a mixed PNA of the same length, but with a completely unrelated sequence (Ref16mix-PNA). Although the frequency of dead embryos slightly increased up to 400 μ M PNA concentration, the surviving embryos did not show any malformations (Fig 4A; Ref16mix-PNA 400 μ M). As a second control experiment to evaluate the selectivity of the antisense function we synthesized a 16mer mixed PNA with a single mismatch (Six3Lmut-PNA; for sequence information see Fig. 2). Although the mutant PNA also resulted in *Six3* specific phenotypes, the frequency of embryos with eye and forebrain phenotype was strongly reduced (compare Figure 4C with 4D and see supplementary Table S3), suggesting that a single point mutation strongly affects the target selection of the mixed

PNAs. This property offers the possibility to design highly specific antisense molecules that are able to select between individual allelic sequences, differing by single point mutations.

We next compared the Six3L-PNAs directly with morpholino oligos, which represent the standard antisense molecules used for gene specific knock down in fish embryos (9). Compared to PNAs they show a less stringent binding to mRNA and therefore are used at lengths of 25 bases. We used a published target sequence for the medaka *six3* gene (10), which overlaps with our PNA sequences (for sequence information see Fig. 2). The phenotypes for this morpholino oligo were indistinguishable from those seen for the Six3L-PNAs, but they already appeared at lower concentrations compared to the PNAs (see Supplementary Table S3). At higher concentrations they however showed strong toxicity (for high mortality rate, see Fig. 4E). Therefore, morpholino oligos show comparable results at lower concentrations than PNAs, both for peak levels of knock down efficiency, as well as for toxic effects.

Conclusion

Unmodified Nielsen-PNAs do not work efficiently in injection experiments in fish (21), however, recently PNA derivatives have been applied successfully for this purpose. Urtishak and colleagues used negatively charged oligomers of alternating components (HypNA-pPNA; see Fig. 1) and could knock down selected targets (22). Nevertheless, considerably higher amounts of oligomers were used for injection (50–800 pg per embryo compared to 0.5–9 pg in our experiments). In this study the introduced backbone modifications strongly improved the properties of the PNAs. The increased hydrophilicity resulted in a largely enhanced solubility of the molecules and Ugi-PNAs worked efficiently in translational blocking of mRNA. The combination with Nielsen-PNAs in mixed molecules combined the favourable amphipathic properties of the Ugi-PNAs with the superior binding affinity of Nielsen-PNAs.

As a result, we could demonstrate a gene specific knock down of a single medaka gene *in vivo*.

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Figure legends

Figure 1. Chemical structure of PNAs.

Schematic structure of Nielsen-PNAs, Ugi-PNAs, mixed PNAs (containing both Nielsen- and Ugi-PNA monomers) and HypNA-pPNAs is shown. Ugi-PNAs contain C2-phosphonoester residues, mixed PNAs contain C3-residues.

Figure 2. Sequences of the antisense molecules and their targets.

The mRNA targets are shown in the 5'-3' orientation, capital letters indicate the coding region. The AUG start codon is marked by red overlay. A morpholino oligo is indicated by MO (Six3-MO). All other sequences represent PNAs, Ugi-PNA-C2 components are shown in black, Ugi-PNA-C3 components in red and Nielsen-PNAs components are marked by gray overlay. Underlined bases (black) represent mismatches. All PNA and morpholino oligos are shown in 3'-5' orientation. L means trimethyl-lysine and LLLL a combination of 4 such residues. SuRho indicates sulforhodamine B

Figure 3. Translational blocking of Ugi-PNAs after injection of gfp mRNA into medaka embryos.

The Ugi-PNAs were coinjected with gfp mRNA into medaka embryos at the one cell stage. 24 hours later the intensity of the gfp fluorescence was qualified as weak (B), moderate (C; mod) or strong (D). (A) Shows an uninjected embryo (no signal, corresponding to a complete knock down of gfp). The embryos are shown in a dorsal view, anterior to the top. A graph

summarizing the experiments for optimisation of the PNA length is shown in (E). The names of the PNAs are explained in Fig. 2. The embryos were injected with a mixture of 10 ng/μl gfp mRNA and 200 μM PNAs preincubated for 30 minutes on ice. After 24 hours the embryos were divided into groups according to their gfp signal intensity. Above the columns the calculated average gfp intensity in percent is indicated (see text for calculation). Similarly the results for mixed PNAs are shown in (F). Injections and evaluations were performed as in (E), except that 20 ng/μl mRNA were used. Note that the increased amount of mRNA results in higher numbers of average gfp intensity for comparable antisense function.

Figure 4. Injection of medaka embryos with Six3 PNAs.

A wild-type embryo (no PNA injection) at 3 days is shown in (A). Embryos injected with 400 μM Ref16mix-PNA are indistinguishable from wild-type embryos; at 900 μM unspecific phenotypes were detected (both in A). The Six3L-PNA injected embryos were evaluated 3 days after injection at stage 29. The phenotypes were divided into weak, moderate and strong (for criteria see text). Arrows indicate the anterior parts of the eyes pointing to the midline in weak embryos. The black arrowhead indicates remnants of the eye structures. All embryos in (A) are at stage 29 (34 somites). In (B) the expression of the *bf1* gene in wild-type and strongly affected embryos injected with Six3L-PNAs is shown (both at stage 22; 9 somites). Note the presence of *bf1* expression in the otic vesicles (38), both in wild-type as well as in Six3L-PNA injected embryos (marked by red arrowheads). Dorsal views of the anterior part of the embryos are shown. The quantitative evaluation of the Six3 knock down experiments is shown in (C) and (D) for Six3L-PNA and Six3Lmut-PNA, respectively. Calculated were the percentages of phenotypes in the surviving embryos. (E) Shows the mortality rate of embryos injected at the indicated concentration (survival was examined 24 hrs after injection, see also Supplementary Table S3).

Fig1

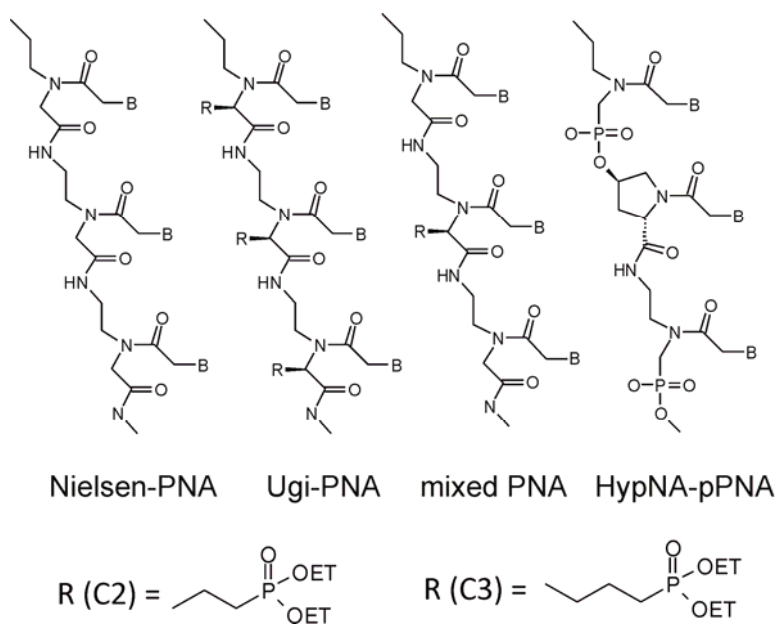


Fig 2

Gfp mRNA	5'-ccggucgccacc	AUG	GUGAGCAAGGGCGAGGAGCUGUUCAC-3'
Gfp12			3'-CGTTCCCGCTCC-L-5'
Gfp14			3'-CGTTCCCGCTCCTC-L-5'
Gfp15			3'-CGTTCCCGCTCCTCG-L-5'
Gfp16			3'-CGTTCCCGCTCCTCGA-L-5'
Gfp17			3'-CGTTCCCGCTCCTCGAC-L-5'
Gfp15mix			3'-CGTTCCCGCTCCTCG-L-5'
Gfp16mix			3'-CGTTCCCGCTCCTCGA-L-5'
Gfp16mixL4			3'-CGTTCCCGCTCCTCGA-LLLL-5'
Gfp16mixRho			3'-CGTTCCCGCTCCTCGA-L-SuRho-5'
Ref15mix			3'-TGAGAAACCGTCGCT-L-5'
Ref16mix			3'-TTCGCTCTGTGCTG-L-5'
Six3 mRNA	5'-guccgccgccugccuucccacacgcgcucag	AUG	GUUUUCA-3'
Six3L			3'-GACGGAAGGGTGTGCG-L-5'
Six3Lmut			3'-GACGGAAGGTTGTGCG-L-5'
Six3-MO			3'-CAGGCGGCGGACGGAAGGGTGTGCG-5'

Fig 3

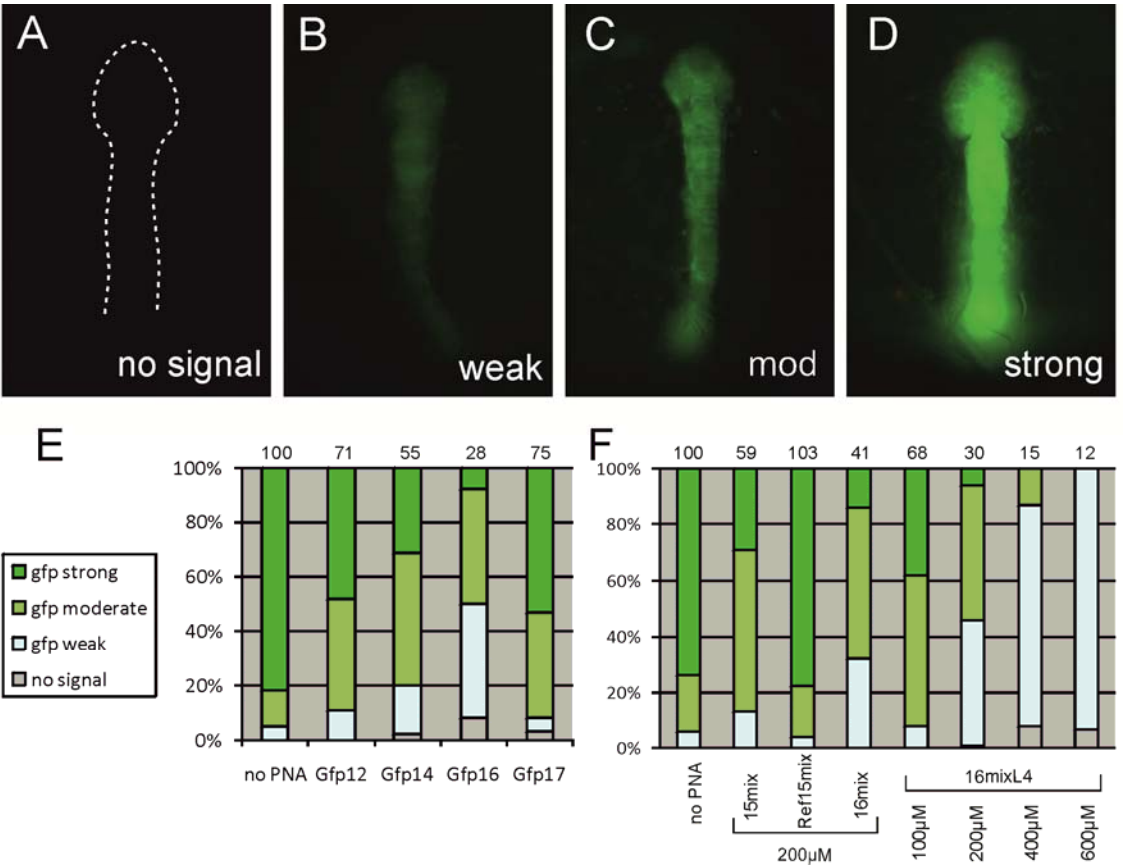


Fig 4

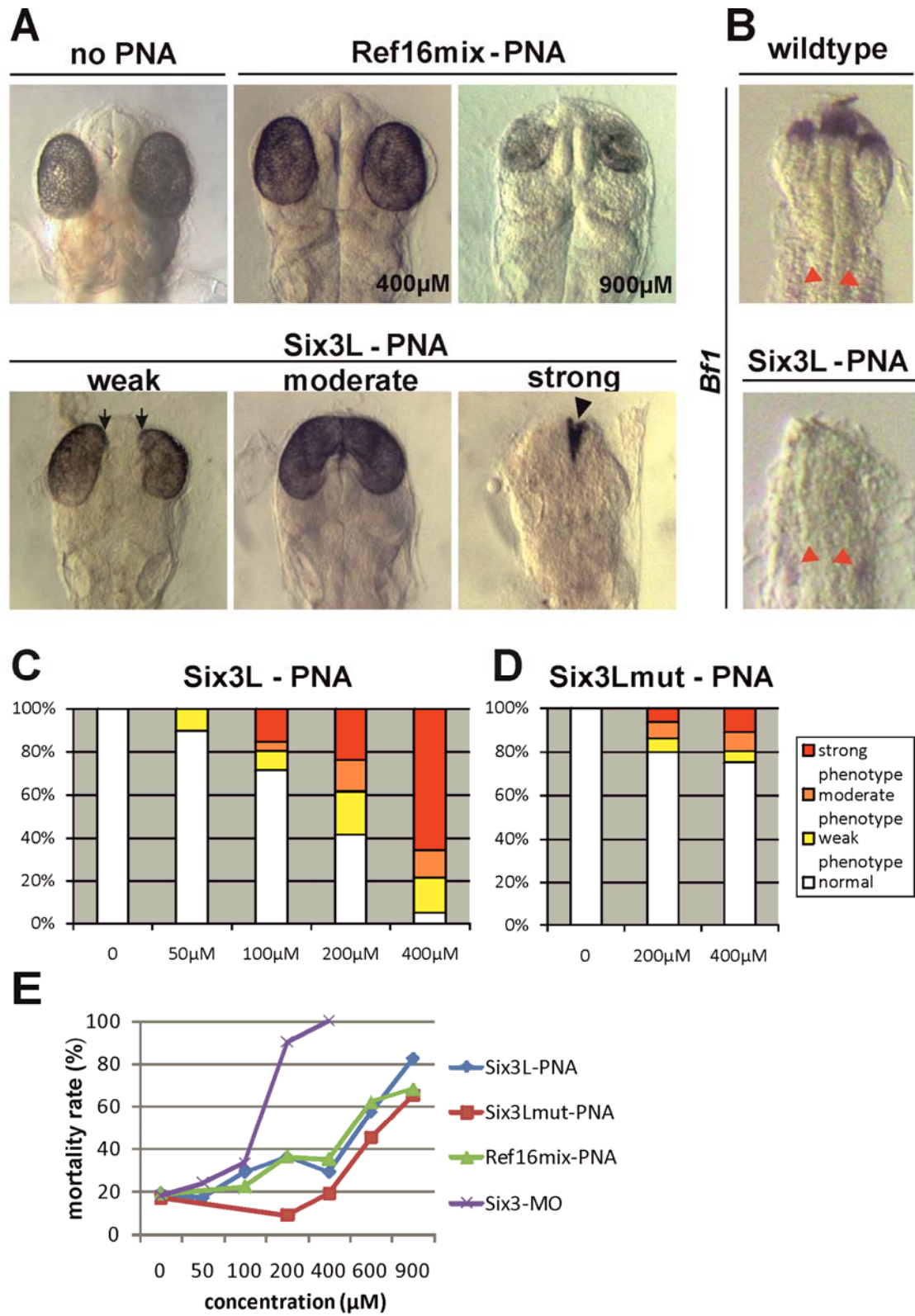


Fig S1

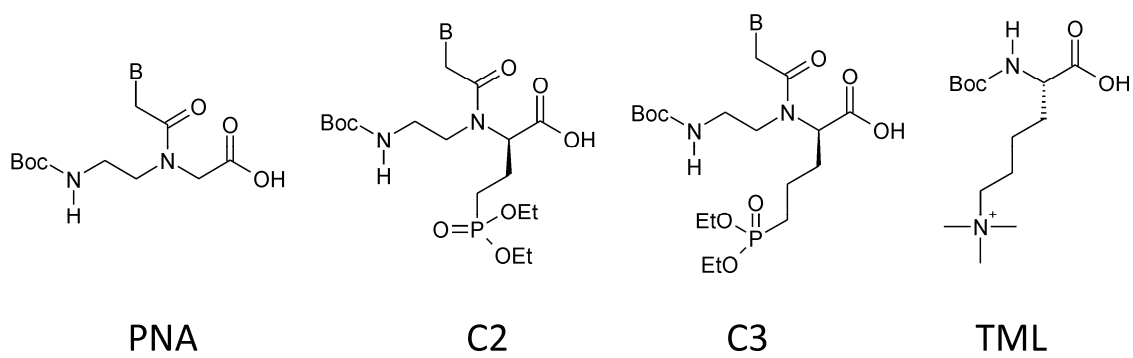


Fig S2

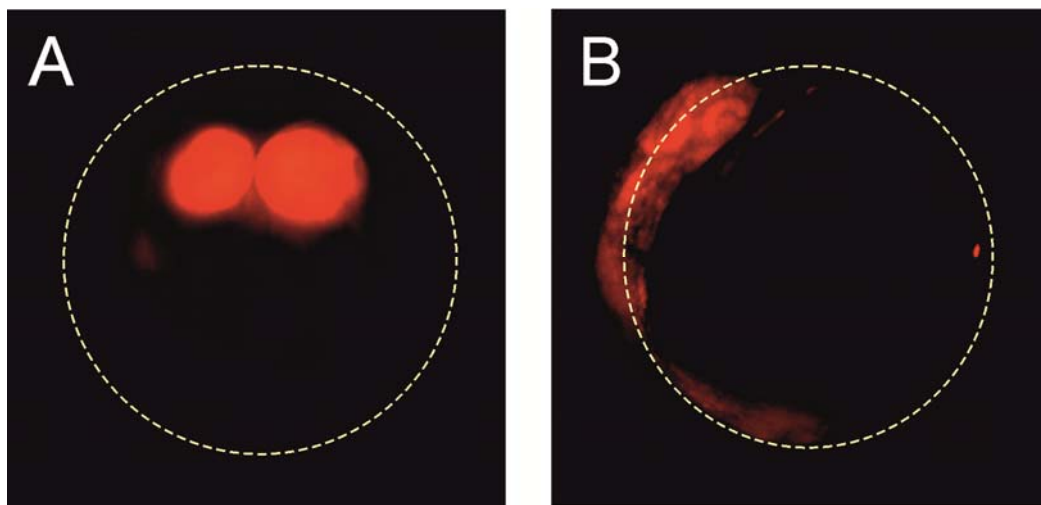


Table S1

coinjected PNA	no PNA	Gfp12	Gfp14	Gfp16	Gfp17
injected embryos	72	71	71	71	67
dead	11	7	10	5	6
death rate	15%	10%	14%	7%	9%
gfp signal strong	50	31	19	5	32
gfp signal moderate	8	26	30	28	24
gfp signal weak	3	7	11	28	3
no gfp signal	0	0	1	5	2
average gfp intensity	100%	71%	55%	28%	75%

Table S2

coinjected PNA	no PNA	Gfp15 mix	Ref15 mix	Gfp16 mix	Gfp16mixL4			
concentration		200 μ M	200 μ M	200 μ M	100 μ M	200 μ M	400 μ M	600 μ M
injected embryos	195	47	138	48	29	157	44	32
dead	22	2	15	4	3	18	6	2
death rate	11%	4%	11%	8%	10%	11%	14%	6%
gfp signal strong	129	13	96	6	10	8	0	0
gfp signal moderate	34	26	22	24	14	66	5	0
gfp signal weak	10	6	5	14	2	63	30	28
no gfp signal	0	0	0	0	0	2	3	2
average gfp intensity*	100%	59%	103%	41%	68%	30%	15%	12%

Table S3

concentration	0	50μM	100μM	200μM	400μM	600μM	900μM
Six3L-PNA							
number of embryos	83	36	65	86	142	67	62
dead	16	6	19	31	41	38	51
death rate	19%	17%	29%	36%	29%	57%	82%
strong phenotype	0	0	7	13	66	12	8
moderate phenotype	0	0	2	8	13	7	2
weak phenotype	0	3	4	11	17	4	0
normal	67	27	33	23	5	6	1
phenotypes in surviv.	0%	10%	28%	58%	95%	79%	91%
Six3Lmut-PNA							
number of embryos	96			104	124	105	112
dead	16			9	23	47	73
death rate	17%			9%	19%	45%	65%
strong phenotype	0			6	11	10	14
moderate phenotype	0			7	9	5	1
weak phenotype	0			6	5	5	2
normal	80			76	76	38	22
phenotypes in surviv.	0%			20%	25%	34%	44%
Ref16mix-PNA							
number of embryos	83		27	25	26	39	19
dead	16		6	9	9	24	13
death rate	19%		22%	36%	35%	62%	68%
strong phenotype	0		0	0	0	0	0
moderate phenotype	0		0	0	0	0	0
weak phenotype	0		0	0	0	0	0
normal	67		21	16	17	15	6
phenotypes in surviv.	0%		0%	0%	0%	0%	0%
Six3-MO							
number of embryos	66	29	27	59	17		
dead	12	7	9	53	17		
death rate	18%	24%	33%	90%	100%		
strong phenotype	0	12	11	6	0		
moderate phenotype	0	6	7	0	0		
weak phenotype	0	2	0	0	0		
normal	54	2	0	0	0		
phenotypes in surviv.	0%	91%	100%	100%			

10. Danksagung

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11. Curriculum vitae:

Personal data:

Name: Sebastian Dorn

Date of birth: 22. July 1978

Place of birth: Vienna (Austria)

Nationality: Austrian

Marital status: married (with Ruth Dorn)
one daughter (Elisa Maarja, born in January 2010)

Parents: Josef Dorn, certified engineer for machine building
Monika Dorn, teacher and head of kindergarten

Wife: Ruth Dorn, kindergarten and religious education teacher

School education:

1984- 1988 Volksschule Lacknergasse (catholic boarding school)

1988-1996 Realgymnasium Haizingergasse (focus on natural sciences and
mathematics)
Leaving exam: Matura

Education (University), research and professional experiences:

1996- 2009 Biology (MSc: 2004)

1996- 2004 Focus on Zoology (elective subject: Genetics and
Microbiology)

2003- 2004 Civilian service at St. John`s Ambulance

2003- 2009 Focus on Genetics and Microbiology

11/2002-2003 Diploma thesis at the Konrad-Lorenz Research Station in
Grünau (Upper Austria) under the supervision of Prof. Kurt
Kotrschal
Title of thesis: “Correlation of air pressure and temperature
with Corticosterone, Testosterone and behaviour in greylag
geese (*Anser anser*) and Northern Bald Ibis (*Geronticus
eremita*)”
Diploma exam: 15. 10. 2004

2006-2008 attending three practical courses as a tutor at the Veterinary
Medicine University of Vienna in the lab of Dr. Thomas
Czerny

02/2006-2011 PhD thesis at the Veterinary Medicine University of Vienna
in the lab of Dr. Thomas Czerny under the supervision of
Prof. Erwin Heberle-Bors: ”Interactions between LEF/TCF
and β -catenin for transcriptional activation of Wnt
signalling and antisense function of modified peptide
nucleic acids in Medaka fish”

Publications: “Side chain modified peptide nucleic acids (PNA) for knock-
down of *six3* in medaka embryos”, submitted to Nucleic
Acid Research at 8th of September 2011

two further publications about the PhD thesis are currently
under progress