universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

Target genes of β-catenin in early mouse limb development

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Magister der Naturwissenschaften (Mag. rer.nat)

Verfasserin / Verfasser:	Alexander Apschner
Matrikel-Nummer:	0340419
Studienrichtung (lt. Studienblatt):	Molekulare Biologie
Betreuerin / Betreuer:	Dr. Christine Hartmann

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Meinen Eltern und Großeltern

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Abstract

In recent years Wnt-Signaling has been implicated to play an important role in the control of differentiation of mesenchymal cells to chondrocytes. A key observation has been that stabilization of β-catenin in the early limb bud mesenchyme represses differentiation of mesenchymal cells into skeletal precursors. Probably this is mediated by transcriptional repression of the transcription factor *Sox9*, which is a master regulator in chondrocyte differentiation.

The object of this thesis was to identify putative mediators of this *Sox9* repression. Target genes of canonical Wnt signaling in limb bud development were identified and tested for their potential to repress *Sox9*.

To do so I used gene expression data already available. I validated 48 candidate genes suggested by the data analysis using *in situ* hybridisation and semi-quantitative RT-PCR, respectively. 10 genes could be confirmed as being positively regulated in the limbs of β-catenin gain of function mice. For three of them, the transcription factors Tcf4 (Itf2), Irx5 and Fosl2 (FRA2), I cloned parts of their promoters into luciferase reporter vectors and tested their response to β-catenin/Wnt-signaling. Furthermore I cloned their cDNAs and tested their effect on part of the *Sox9* promoter in luciferase reporter assays. For Tcf4 and Irx5 I could confirm their positive response to canonical Wnt-signaling, as well as their potential to repress *Sox9 in vitro*.

To gain insight into the *in vivo* function of these genes, constructs for specific overexpression in the early limb bud mesenchyme have been generated. By the end of the thesis work one round of pronucleus injections has been carried out for the gene *Tcf4,* which yielded in one transgenic embryo with a strong limb phenotype. Unfortunately further analysis was not carried out due to the fact of the diploma work had ended.

In conclusion this work suggests a number of new genes as targets of Wnt/β-catenin signaling in limb development. For two of them *Irx5* and *Tcf4* I established a possible direct regulation by the β-catenin/Wnt-pathway and that they have the potential to inhibit *Sox9* at least *in vitro*. Further studies would now be necessary to examine whether they also excert a negative effect on *Sox9 in vivo* and to establish their potential functions during limb development.

Zusammenfassung

Untersuchungen der letzten Jahre führten zum β-catenin/Wnt-Signalweg als möglichen Schlüssel zur Kontrolle der Differenzierung von mesenchymalen Zellen zu Knorpelzellen. Stabilisierung von β-catenin in der frühen Gliedmaßenanlage scheint die Expression des Transkriptionsfaktors Sox9 und damit die Differenzierung in Knorpelzellen zu inhibieren. Es ist jedoch unklar wie diese Kontrolle von Sox9 durch β-catenin/Wnt erfolgt. Diese Diplomarbeit ist Teil eines Projektes das zum Ziel hat den Mechanismus dieser Kontrolle aufzuklären.

Dazu sollen β-catenin/Wnt-regulierte Gene der frühen Gliedmaßenentwicklung identifiziert und auf einen möglichen Einfluss auf die Sox9-Expression getestet werden. Am Beginn dieser Diplomarbeit standen bereits Daten einer Genexpressionsstudie zur Verfügung. Nach Analyse dieser Daten wurden 48 mögliche Kanditatengene mittels In-situ-Hybridisierung bzw. RT-PCR validiert. Es konnten so 10 Gene identifiziert werden, welche in der Gliedmaßenanlage von Embryonen, die konditionell stabilisiertes β-catenin expremieren, hochreguliert sind. Darunter befanden sich die Transkriptionsfaktoren Tcf4 (Itf2), Irx5 und Fra2 (Fosl2). Die Promotoren dieser Gene wurden in Luciferase Reporter Assays auf ihre β-catenin/Wnt Abhängigkeit untersucht. Weiteres wurden diese Gene in Luciferase Assays mit einem Sox9-Promoter-Reporterkonstrukt getestet. Um Hinweise auf die Funktion der Gene *in vivo* zu erhalten wurden die kodierenenden Regionen der Gene in einen Vektor kloniert, welcher eine transiente Überexpression in transgenen Embryos erlauben sollte. Für das Gen Tcf4 wurde eine erste Runde von Injektionen durchgeführt. Diese lieferten jedoch nur einen transgenen Embryo, welcher jedoch einen Phenotyp in der Gliedmaßenanlage zeigte. Durch das zeitlich bedingte Ende der Arbeit waren leider keine weiteren Analysen möglich. Zusammenfassend wurden in dieser Arbeit eine Anzahl möglicher Zielgene des β-catenin/Wnt-Signalweges identifiziert. Für zwei davon Irx5 und Tcf4 ist eine direkte Abhängigkeit vom β-catenin/Wnt Signalweg sehr wahrscheinlich. Weiters hatten beide Gene das Potential die Sox9-Expression zumindest *in vitro* zu inhibieren. Jedoch wären weitere Untersuchungen notwendig um eine mögliche Funktion dieser Gene in der Knorpelzellendifferenzierung bzw. Gliedmaßenentwicklung aufzudecken.

1. Introduction

1.1 Relevance of the Research

The endoskeleton is one of the key features of vertebrates in comparison to non-vertebrates and a detailed understanding of its formation and maintenance is highly desirable as diseases of the musculoskeletal system as osteoporosis or osteoarthritis present a major burden to the health systems of industrialized countries.

By studying the developmental processes forming the skeleton (skeletogenesis) its major cell types, osteoblasts and chondroblasts and the signaling pathways controlling their formation, differentiation and maturation researchers hope to find new approaches in cures for these diseases. In recent years major advances have been made in the characterization of molecular properties of these cells through the identification of key transcription factors and markers required for the formation and differentiation of these cells. For example Sox9 has been identified as a key factor in chondrocytes, while Runx2 and Osterix have been shown to be essential in osteoblasts, however the molecular mechanisms underlying the transcriptional regulation of these factors are still not well understood (Wagner and Karsenty 2001).

1.2 Skeletogenesis

Skeletogenesis can happen via to two distinct processes intramembranous ossification and endochondral ossification. The latter is the predominant process of bone formation in mammals as all bones of the axial and appendicular skeleton are formed by this process, while only the flat bones of the skull and lateral halves of the clavicles are entirely formed by a membranous ossification process.

In intramembranous ossification condensed mesenchymal cells directly differentiate into osteoblasts, whereas in endochondral ossification mesenchymal cells first differentiate into chondrocytes that form a cartilage scaffold at the location of the future bone and prefiguring it in size and shape. Endochondral ossification is a step-

wise process (Fig. 1) by which mesenchymal cells become committed to enter the chondrogenic lineage. First they condense into compact nodules a process dependent on the cell-cell adhesion molecules N-cadherin and N-CAM (Oberlender and Tuan 1994; Hall and Miyake 1995). In the next step chondrocytes proliferate rapidly and form the cartilage scaffold for the future bone, in parallel they secrete a cartilage specific extracellular matrix with Collagen type II (encoded by the *Col2aI* gene) and Aggrecan as the most prominent components. After the cartilage scaffold reaches a certain size cells in the centre stop to proliferate and undergo maturation to become first prehypertrophic and then hypertrophic chondrocytes (Horton, Machado et al. 1993). These cell types can be distinguished by their matrix as prehypertrophic chondrocytes still produce Collagen type II, whereas hypertrophic chondrocytes produce Collagen type X and Fibronectin instead (Linsenmayer, Chen et al. 1991; Poole, Matsuoka et al. 1991). The extracellular matrix secreted by the hypertrophic chondrocytes eventually becomes calcified and hypertrophic chondrocytes themselves undergo apoptosis.

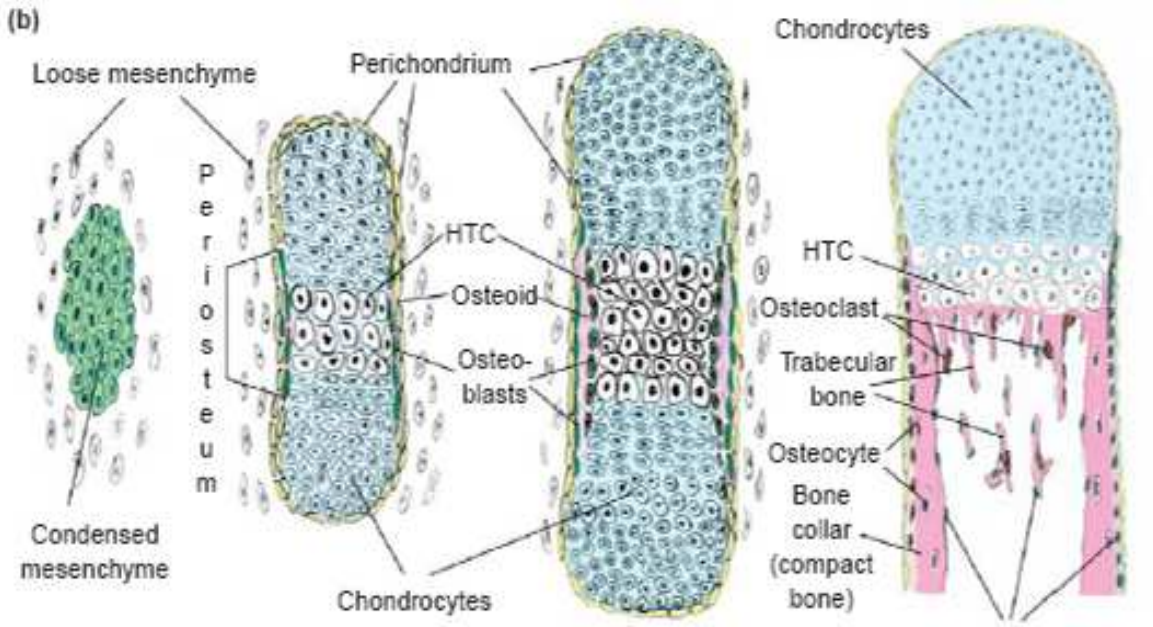


Fig.1: Steps of endochondral ossification (Hartmann 2006)

During endochondral ossification the first osteoblasts are formed within the condensed layer of perichondrial cells, which surround the centre of the cartilage scaffold. This process is requires a signal from the prehypertrophic chondrocytes Indian hedgehog (IHH). Thereby the osteoblastogenesis is temporally coupled to

chondrogenesis (Kronenberg 2003). Hypertrophic chondrocytes also produce angiogenic factors attracting blood vessels, which eventually invade the calcified chondrocytic scaffold and bringing along osteoblasts, which start to form trabecular bone using the mineralized matrix scaffold of the hypertrophic chondrocytes as a template. In addition osteoclasts invade, whose function is to absorb bone and thereby help to maintain bone and calcium homeostasis during adult life.

The maturation process of chondrocytes is controlled by a feedback loop consisting of parathyroid hormone related peptide (Pthrp) produced by proliferating chondrocytes at the articular ends of the forming skeletal elements and Ihh, which is produced by prehypertrophic chondrocytes (Fig. 2) (Kronenberg 2003). High levels of Pthrp signaling are thought to keep cells in the proliferative state thereby repressing maturation. Only cells that are far enough away can escape this repressive effect and mature into prehypertrophic Ihh producing cells. Ihh on the other hand is required to maintain high levels of Pthrp.

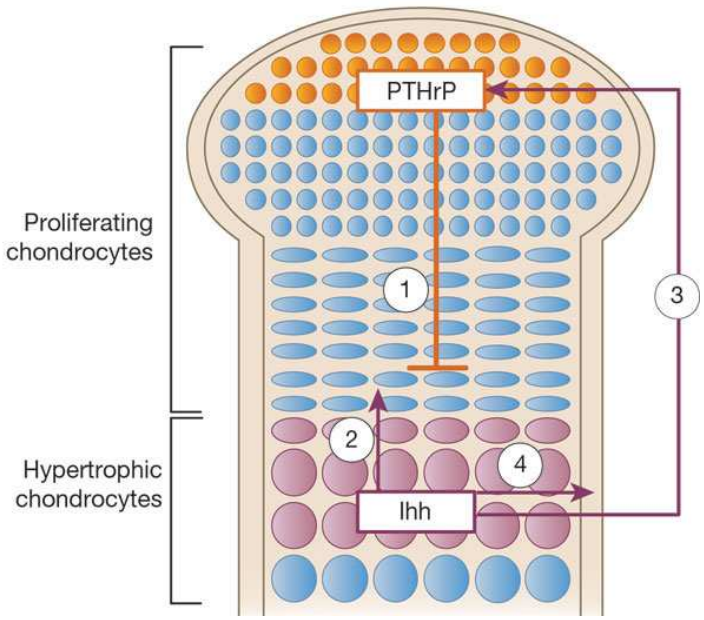


Fig. 2: The PTHrP/Ihh negative feedback loop: PTHrP keeps cells proliferative and prevents differentiation (1). Ihh stimulates proliferation of columnar chondrocytes (2). Ihh stimulates osteoblastogenesis in the perichondrium (4) and proliferation (Kaiser Permanente (2009) in chondrocytes. Ihh stimulates PTHrP expression (3) (Kronenberg 2003)

1.3 The limb as a model for skeletogenesis

A widely used model to study skeletogenesis is the limb of mouse and chicken, as the limbs of these organisms comprise major features of the human limb: They are formed by endochondral ossification, have synovial joints and a similar skeletal pattern. Furthermore, alterations in patterning can be easily identified. In addition the chicken limb is accessible to manipulation during embryogenesis.

In embryonic development the vertebrate limb originates from the lateral plate mesoderm. Presumably Hox-Genes place the limb fields at the right places along the longitudinal axis of the embryo. Another important advantage of the limb is that the signaling centres determining the proximal-distal (P-D), anterior-posterior (A-P) and dorsal-ventral (D-V) pattering are relatively well understood (Fig. 3).
In mice limb bud outgrowth starts at about E9,5 at the level of the forelimb and around E10 at the level of the hindlimb. A mesenchymal-epithelial feedback loop consisting of Fibroblast growth factor (Fgf) family members is responsible for the proximodistal growth and patterning by maintaining a population of multipotent progenitor cells in the most distal part of the limb bud (Yu and Ornitz 2008). The epithelial centre for this signaling system is localized in a thickening of the ectoderm, the so-called apical ectodermal ridge. It comprises one of the important pattering systems in the limb. Another well established patterning system is the zone of polarizing activity, which later on in development determines A-P patterning and thereby digit identity. The key to its function is a group of cells at the posterior margin producing the morphogen Sonic hedgehog, which forms a posterior to anterior gradient in the distal limb field (Riddle, Johnson et al. 1993).

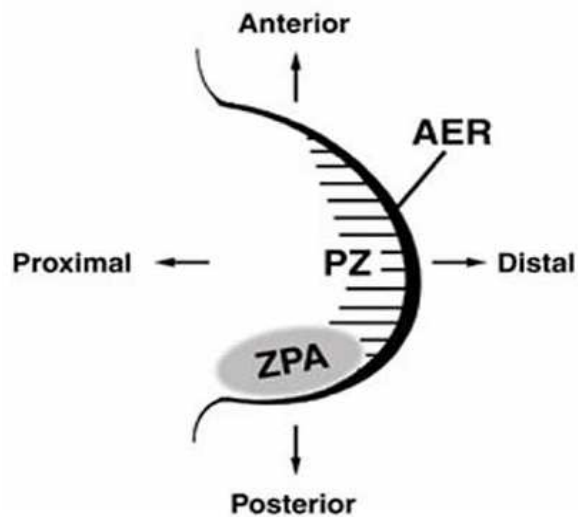


Fig. 3: Signaling centers of the limb. AER – Apical Ectodermal Ridge, ZPA – Zone of polarizing activity, PZ progress zone.

Source: http://www.cmrb.eu/centre-investigacio/en_desarrollo_miembros.html

The third signaling centre determining the D-V identity (back vs. palms) is comprised out of non-AER dorsal ectoderm, which expresses as a key factor Wnt7a. Wnt7a is required to repress ventral ectodermal fate and to induce the expression o the transcription factor Lmx1b in the dorsal mesenchyme thus establishing dorsal (Parr, Shea et al. 1993). Whereas expression of the transcription factor engrailed establishes the ventral fate within the ectoderm (Cygan, Johnson et al. 1997).

However, one important question has not yet been entirely solved: How is it achieved that the skeletal anlage is situated in the centre of the developing limb bud? Since this requires spatial and temporal regulation of cell fate determination this question goes hand in hand with the question: How is the expression of Sox9 the master-regulator of chondrogenesis regulated?

First insights into a possible mechanism came from the observation that the ectoderm can inhibit chondrogenesis (Solursh, Singley et al. 1981). Thus signals from the ectoderm might be involved in restricting chondrogenesis to the core of the limb bud. In particular Wnt-ligands, which are expressed in the limb ectoderm (Roelink and Nusse 1991; Parr, Shea et al. 1993; Barrow, Thomas et al. 2003), have been implicated as candidate signaling molecules as their overexpression can inhibit chondrogenesis *in vitro* (Rudnicki and Brown 1997; Hartmann and Tabin 2001). Thus Wnt-signaling is probably involved in this chondro-inhibitory effect.

1.4 WNT-signaling

Wnt signaling pathways have been shown to participate in a number of biological processes eg embryonic development, tissue morphogenesis, body patterning, and tumorigenesis (Clevers 2006). The name Wnt is derived from the founding members encoded by the *Drosophila wingless* (*Wg*) gene and *int-1*, a viral inserted mutation causing mammary tumor in mice. Wnt genes encode secreted glycoproteins, which in addition are palmitoylated. A total of 19 *Wnt* genes have been identified in human and mouse. According to their activity in two assays, the *Xenopus* axis induction (Du, Purcell et al. 1995) and the transformation of mammary epithelial cell assays (Wong, Gavin et al. 1994) they have been subdivided in former times into two groups: The Wnt-1 class (eg Wnt-1, -3a, -7a, and 8) that activates the so called canonical Wnt pathway and the Wnt5-class (eg 4, 5a, and -11) that signals presumably via one of the noncanonical pathways. However, this subdivision is probably not really accurate as it has been shown that the signaling pathway utilized depends primarily on which receptors are present (He, Saint-Jeannet et al. 1997).

The main Wnt-receptor a seven transmembrane receptor is encoded by the Frizzled (Fzd) gene family. It is proposed that activation of the canonical pathway requires in addition as coreceptors, either lipoprotein-related protein 5 or 6 (LRP-5, LRP-6), which are not required in non-canonical signaling (Clevers 2006). Extracellularly Wnt signaling is modulated by a number of secreted antagonists: Dickkopf (Dkk), which binds to LRP-5/6 and Kremen leading to an internalization of the complex thereby antagonizing canonical Wnt-signaling, secreted frizzled related protein (sFRP), which acts as a decoy receptor for the Wnt ligands, and Wnt inhibitory Factor (Wif), which also binds directly to Wnt ligands. Thus the latter two are thought to antagonize canonical as well as non-canonical Wnt-signaling. Recently a study has been published that argues that sFRPs not only inhibit Wnts but also can function to increase their range of diffusion within a tissue (Mii and Taira 2009).

Activation of the canonical pathway leads to inhibition of the phosphorylation and degradation of the transcriptional cofactor β-catenin, which is mediated in the absence of a suitable Wnt-ligand by the destruction complex consisting of the glycogen synthase kinase GSK-3β, Axin and the scaffold protein Adenomatous

Polyposis Coli (APC). This inhibition is mediated by Dishevelled a factor that is phosphorylated by Casein Kinases, which themselves are activated by binding of Wnts to the LRP co-receptor (Clevers 2006). As a consequence the cytoplasmic level of unphosphorylated β-catenin increases and β-catenin is translocated into the nucleus where it acts as a coactivator for T-cell factors (*Tcf7*/*Tcf7l1*/*Tcf7l2*) and Lympymphoid enhancer binding factor 1 (*Lef1*) transcription factors (Clevers 2006). This activatory complex then turns on canonical Wnt-target genes as *c-jun*, *c-myc*, or *E-cadherin* (Willert, Epping et al.).

Non-canonical Wnt signaling pathways are more divers and not yet completely understood, but have in common that they are β-catenin-independent. Two of the non-canonical pathways described are the Wnt/Ca^{2+}-pathway, which is mediated by increase of intracellular Ca^{2+} concentrations and the planar cell polarity (PCP) pathway, which involves activation of Rho-GTPases (Clevers 2006). As non-canonical pathways are not of interest for this thesis no further details are discussed. An overview of Wnt signaling pathways implicated in cartilage development is shown in Fig. 4.

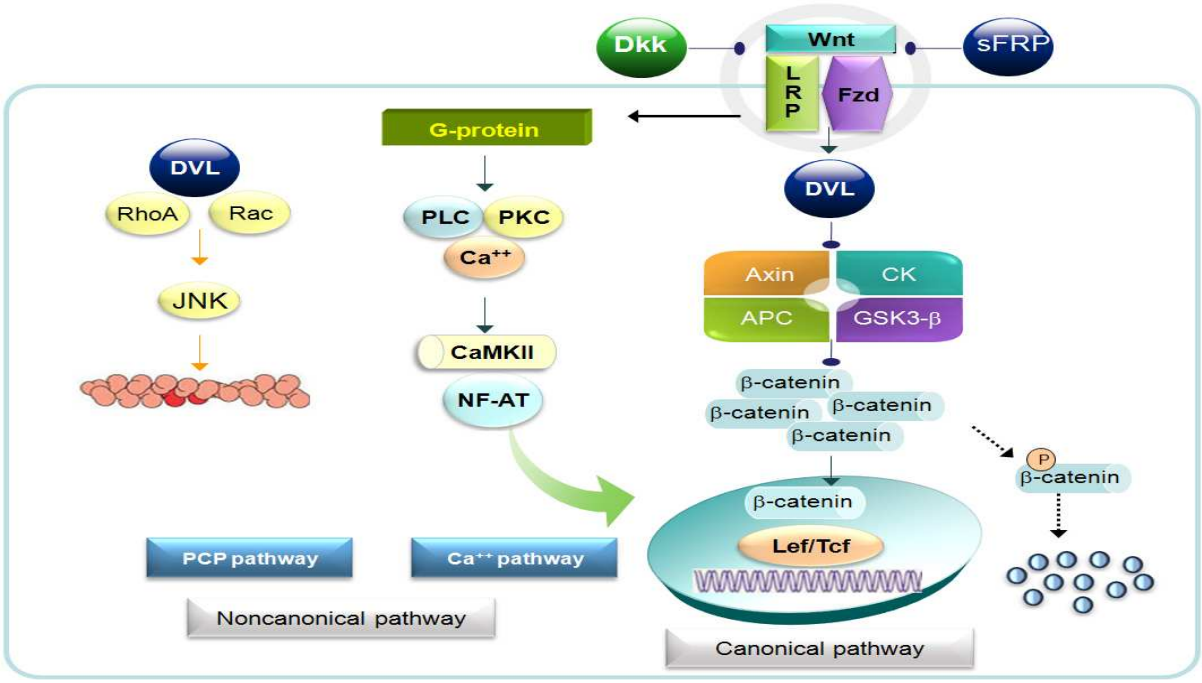


Fig. 4: Overview of the noncanonical and canonical Wnt-signaling pathways (Chun, Oh et al. 2008).

1.5 Sox9 – a master regulator of chondrogenesis

Sox9 is a member of the family of Sox transcription factors that were initially identified as being related to Sry, the male Sex determination transcription factor localised on the Y-chromosom. They have a high mobility-group box (HMG) domain, which mediates DNA binding, DNA bending and interaction with other transcription factors. The Sry-related high-mobility-group box (Sox) family has 20 members in mouse and humans. Sox factors have been shown to act in organogenesis in different organs as testis, bone, or oligodentrocytes (Akiyama 2008). Some years ago mutations in *Sox9* were found to be the cause of Campomelic dysplasia a skeletal disease leading to a variety of symptoms including short stature and bowing of the limbs (Foster, Dominguez-Steglich et al. 1994; Wagner, Wirth et al. 1994). Analysis of the role of Sox9 in mice lead to the discovery that it controls amongst other genes the expression of *Col2a1*, which is highly expressed by chondrocytes (Ng, Wheatley et al. 1997; Zhao, Eberspaecher et al. 1997). Furthermore using conditional mouse models it was shown that Sox9 is essential for the condensation of the mesenchyme during limb skeletogenesis (Akiyama, Chaboissier et al. 2002), the commitment of osteochondroprogenitors, proliferation, differentiation, and maturation of chondrocytes and as a negative regulator of hypertrophic conversion (Akiyama 2008). The latter is thought to be in part mediated by its interaction with β-catenin on the protein level (Akiyama, Lyons et al. 2004), whereby Sox9 can interfere with canonical Wnt-signaling (Topol, Chen et al. 2009).

In essence Sox9 is thought to be the master regulatory gene for the chondrocytic lineage and as such the control of *Sox9* expression is the key regulatory mechanism during chondrogenesis.

1.6 Wnt Signaling in Skeletal development

In the past our lab has investigated the role of Wnt/β-catenin signaling in limb development with a particular focus on skeletogenesis (Hill, Spater et al. 2005; Hill, Taketo et al. 2006; Spater, Hill et al. 2006). In these studies different β-catenin alleles were used: for the loss-of function (LOF) studies performed we used a mouse line carrying a β-catenin null allele (Huelsken, Vogel et al. 2000) in combination with a

conditional β-catenin loss of function allele (Huelsken, Vogel et al. 2001). For the gain-of function studies, in which constitutive activation of canonical signaling is achieved, a mouse line carrying the conditional β-catenin exon 3 floxed allele was used, which upon Cre-deletion leads to the formation of a truncated, stabilized form of β-catenin (Harada, Tamai et al. 1999).

One of the key findings in our previous studies was that the *Sox9* expression was expanded in β-catenin LOF limb buds and abolished in β-catenin GOF-limbs (Hill, Spater et al. 2005). This has led to our working-hypothesis that canonical Wnt-signaling can repress *Sox9* expression and thereby controls chondrogenesis and skeletal patterning (Fig. 5 A)

Recent work by ten Berge and colleagues confirmed the chondro-inhibitory effect of Wnt-signaling and identified N-myc as a Wnt-target gene that promotes proliferation in subectodermal regions (ten Berge, Brugmann et al.). Furthermore they showed that concentration and duration of Wnt signaling is an important factor in cell fate determination and patterning for soft connective tissue in the limb. Their model integrating FGF and Wnt signaling is shown in Fig. 5 B.

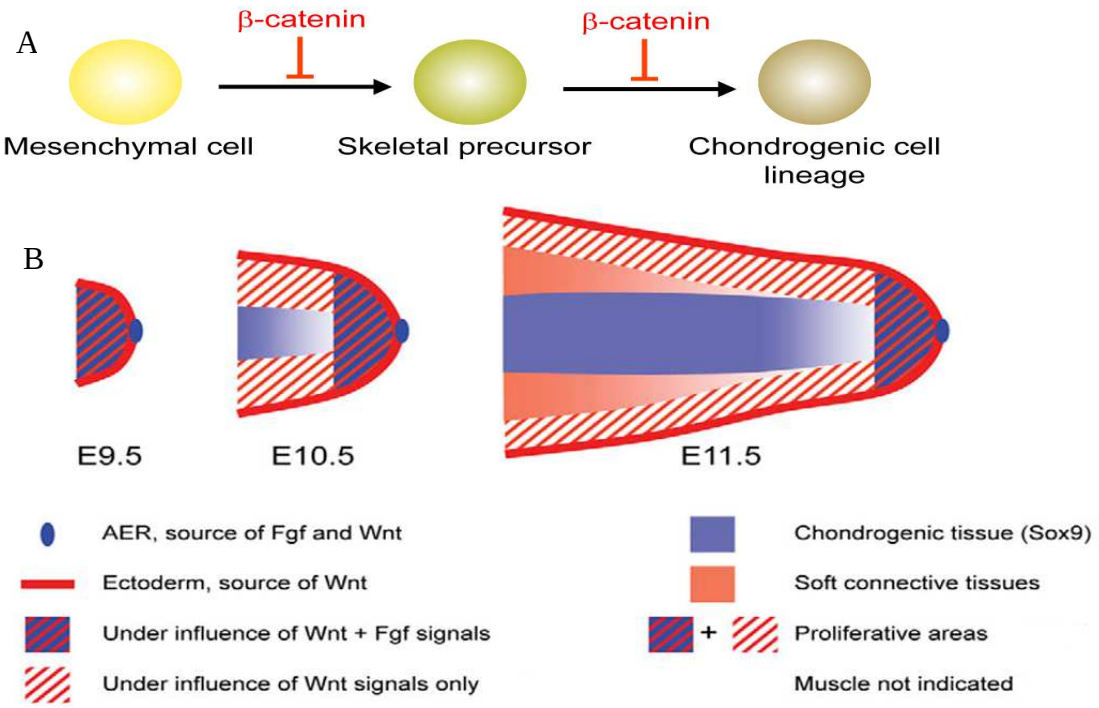


Fig. 5: Model of β-catenin/Wnt signaling in chondrocyte differentiation (A). Model of FGF/Wnt signaling in limb patterning (ten Berge, Brugmann et al. 2008) (B)

However, the question that remains is by which means represses Wnt-signaling *Sox9* and thereby chondrogenesis.

As proliferation and differentiation often are mutual exclusive processes it could be that simply maintaining proliferation is sufficient to repress *Sox9*. However there are many factors known to prevent differentiation by inhibiting the genetic programmes directly. Therefore it could well be that canonical Wnt-signaling is either directly involved in the repression of *Sox9* or indirectly through the upregulation of other transcription factors that then inhibit *Sox9* expression.

1.7 Aim of the thesis

The main project of this thesis was part of an endeavour to uncover the potential mechanism by which canonical Wnt/ β-catenin signaling exhibits its chondroinhibitory effect and transcriptionally represses Sox9.

Three different mechanisms would be feasible (see also Fig. 6)

a) direct – (Tcf/Lef)/β-catenin act negatively on the *Sox9* promoter
b) Indirect – Wnt/β-catenin activates target genes (factor X) that repress *Sox9*
c) combinatory – Factor X is activated by Wnt-signaling and acts in concert with components of the Wnt-pathway to repress *Sox9*

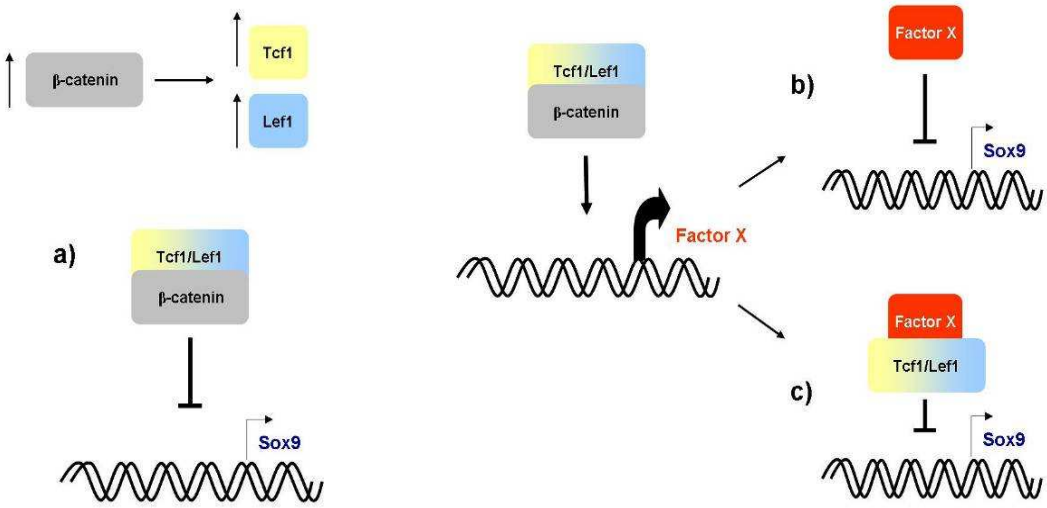


Fig. 6: Potential mechanisms for *Sox9* repression by canonical Wnt/β-catenin signaling.

Currently we have a team effort in our lab investigating the several different possibilities for *Sox9* regulation by Wnt/β-catenin signaling (see Fig. 6). The work of this thesis was dedicated to identify transcription factors and other potential factors, which might act as the proposed factor X, and to investigate their role in *Sox9* regulation and limb development.

1.8 Target genes of canonical Wnt-signaling in limb development

As described above the possible factor X should be regulated by β-catenin/Wnt-signaling. To identify such genes two members of the lab, Natalia Lyashenko and Daniela Kostanova-Poliakova, had carried out gene expression arrays using material from the conditional β-catenin mouse strains described above.

These screens were carried out using mesenchymal cells, which have the potential to differentiate into chondrocyte precursors (E11.5) and Col2a1-expressing chondrocytes (E15.5). In detail E11.5 limb buds were isolated from mice homozygous for either the floxed lof allele or the floxed exon3 gof allele, single cell suspensions were generated from them, and cells were infected with adeno-gfp (control) or adeno-cre and further cultured as high-density micromass cultures before isolation of the RNA at defined time points.

For the isolation of chondrocytes, mice carrying the conditional β-catenin alleles were crossed with Col2-Gfp mice (Grant, Cho et al. 2000) in order to use fluorescence activated cell sorting (FACS) to isolate chondrocytes as the cells within the limbs at this stage of development are already very inhomogeneous. Isolated cells were then infected with the respective adeno-viruses and cultured under chondrogenic conditions. RNA was isolated at defined time points and either sent to Affymetrix for analysis on mouse gene expression array chips or analyzed on in house microarray chips. The process preceding the gene expression approach is outlined in Fig. 7.

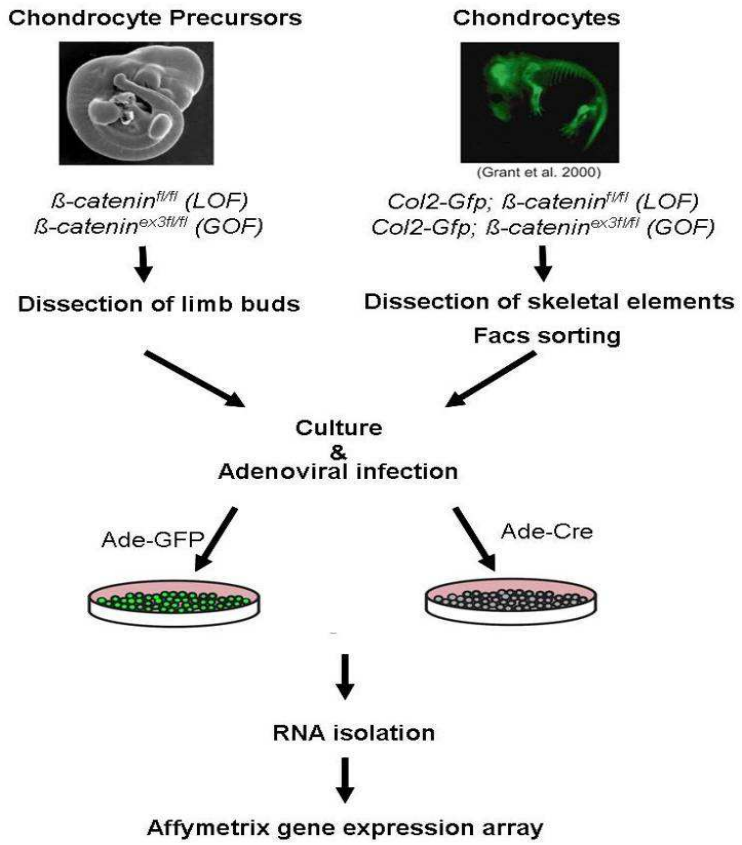


Fig. 7: Summary of the gene expression study approach to identify β-catenin target genes in early limb development

At the beginning of the thesis work the results from this gene expression profiling approach were already available in the lab. In more detail the aim of the thesis was to use the available expression data to identify potential Wnt-target genes in the limb, confirm their up-regulation *in vivo* (using in-situ hybridisation and RT-PCR) and select interesting target genes (candidates for factor X) for further analysis. This included cloning of their promoters to carry out Luciferase-reporter assays to confirm their regulation by a Lef/TCF-β-catenin complex. Furthermore it involved cloning of their full-length open-reading frames (ORFs) into expression vectors in order to test whether their expression had an effect on a *Sox9*-luciferase-reporter construct. Finally we wanted to look at the activity of potentially interesting factors with regard to chondrogenesis *in vitro*, using micromass culture systems and *in vivo* by generating transient transgenic mice overexpressing the factor of interest under the limb mesenchyme specific *Prx1* promoter. An overview of the work is given in the flow chart shown in Fig. 8.

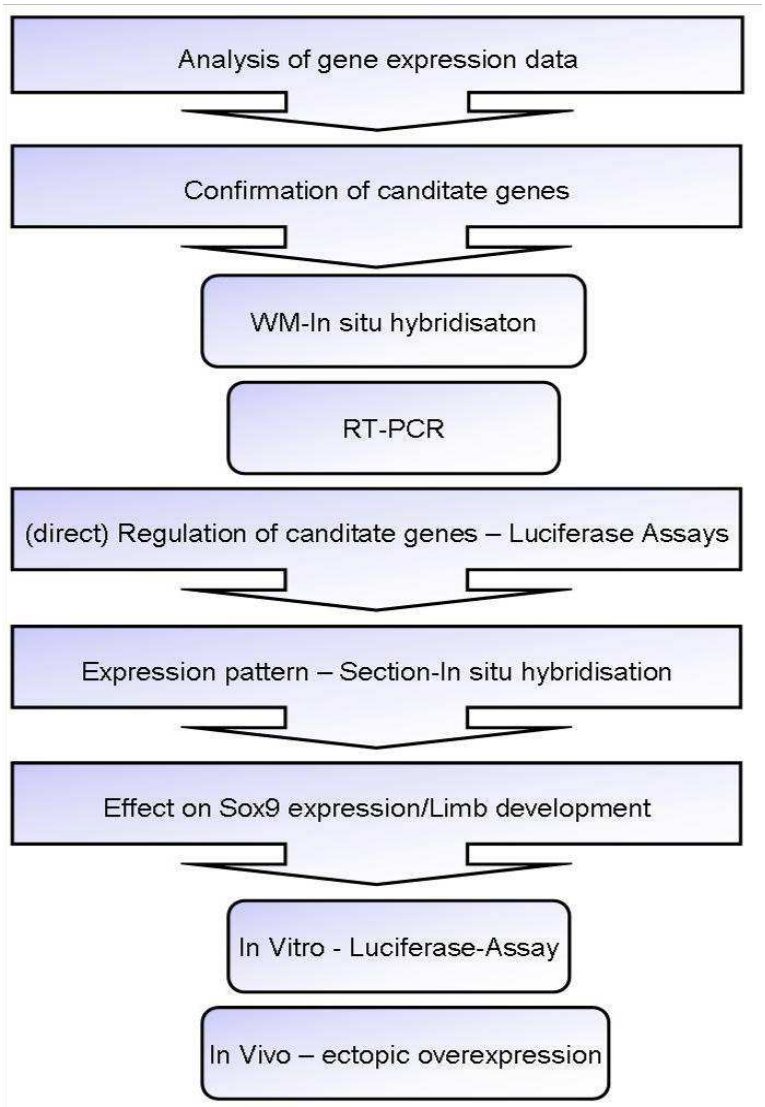


Fig. 8: Work flow overview of the thesis work

In addition to the approach outlined above using the results from the gene expression analysis as a starting point, the promoters of two other genes: *CyclinD1* a target of canonical Wnt-signaling (Shtutman, Zhurinsky et al. 1999; Tetsu and McCormick 1999) and a member of the Tcf-family *Tcf7l2* (Clevers 2006) were analyzed in luciferase reporter assays.

This work was a follow-up based on the results gained by previous students in the lab, which had observed using in-situ hybridisations that both genes were down-regulated in β-catenin GOF mice and up-regulated in β-catenin LOF mice. Their Promoter-studies were carried out test whether they are *in vitro* regulated by a Tcf/Lef-β-catenin complex according to the *in vivo* observations.

2. Results

2.1. Candidate gene approach

2.1.1 Tcf7l2

Previously it has been shown that the nuclear Wnt-signaling components *Lef1* and *Tcf1* (*Tcf7*) are up-regulated in limbs upon conditional stabilization of β-catenin (Hill, Taketo et al. 2006). This indicates a positive auto-regulation of the pathway. Consequently, in-situ hybridisations have been carried out for *Tcf7l2* (also known as *Tcf4*) another member of the Tcf/Lef family. For this factor, however the opposite was observed, a down-regulation in β-Catenin GOF and an up-regulation in β-catenin LOF limbs.

In order to analyse whether *Tcf7l2* is indeed negatively regulated by activation of the pathway I had isolated and cloned a 2,3 kb genomic region upstream of the *Tcf7l2* transcriptional start into the luciferase reporter vector pGL4 and carried out luciferase assays. Cotransfections were performed for the reporter vector and expression vectors for *Lef1* and *β-catenin*, or *Tcf1* and *β-catenin* into 293T cells in order to measure the effect on the luciferase activity. In addition a *renilla* plasmid was co-transfected for normalisation purposes.

In order to identify potential Tcf/Lef binding sites the up-stream region of Tcf7l2 had been subjected to a Transcription Element Search (Schug 2008) using the TESS system provided by the University of Pennsylvania (http://www.cbil.upenn.edu/cgi-bin/tess/tess). This analysis resulted in the identification of 32 potential binding sites for Lef1/Tcf1 within the 2,4kb promoter region (Fig. 9 A).

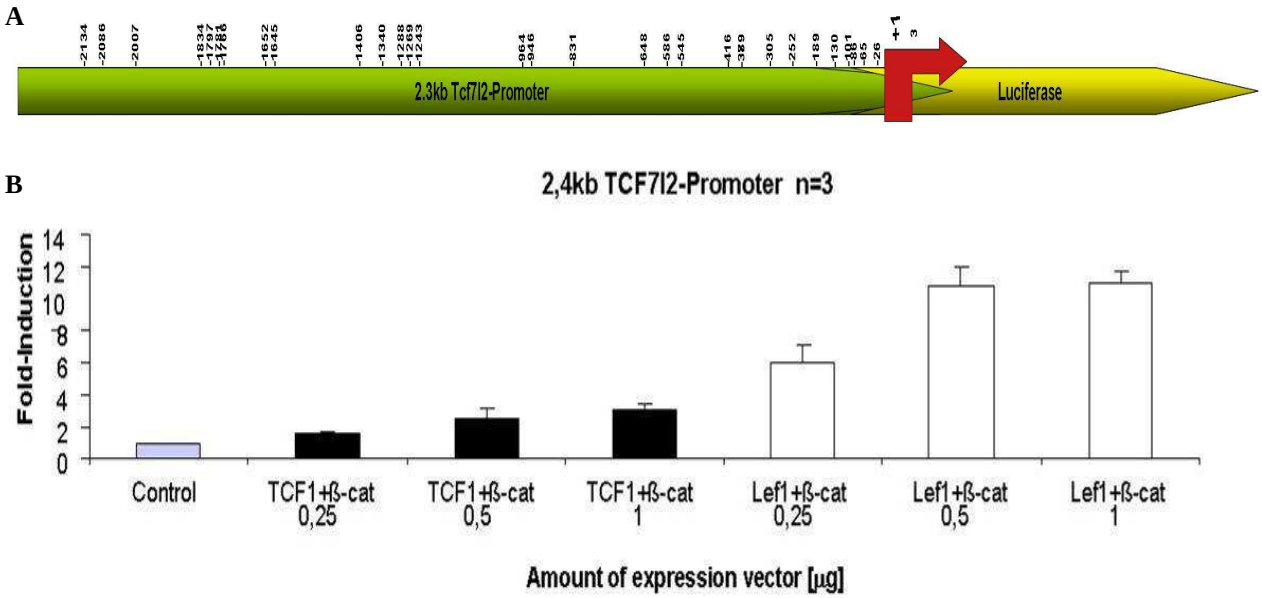


Fig. 9: Predicted Tcf1/Lef1 binding sites on the *Tcf7l2* promoter (A). Luciferase-assay with the reporter-construct shown in (A) and increasing amounts of Tcf1/β-catenin or Lef1/β-catenin (B).

The luciferase results shown in Fig. 9 B clearly do not support the observations made *in vivo*. Opposite to the down-regulation that had been observed upon stabilization of β-catenin, *in vitro* an upregulation of up to 3-fold in the case of Tcf1/β-catenin co-transfection and up to 12-fold upon Lef1/β-catenin expression. A similar difference in the efficiency of the two transcription factor complexes was observed throughout most of the experiments using different reporter constructs (also see below); where with few exceptions Lef1 always gave a stronger effect than Tcf1 in combination with β-catenin.

From the above result it can be concluded, that the *in vivo* down-regulation may be due to secondary effects and is not directly mediated by Wnt/β-catenin signaling.

2.1.2 CyclinD1

The second candidate gene that I looked at was *cyclinD1*. Its regulation by Wnt-signaling is particularly interesting as it is part of the cell-cycle regulation system and

as described in the introduction Wnt-signaling can promote proliferation in sub-ectodermal limb mesenchyme.

In other tissues/cells such as colon carcinoma cells CyclinD1 has been shown to be positively regulated by β-catenin (Shtutman, Zhurinsky et al. 1999; Tetsu and McCormick 1999). Previous members of the lab had carried out section in-situ hybridisations on β-catenin GOF and LOF limbs to check for a similar regulation in limb-development but they found instead of the expected up-regulation a down-regulation of CyclinD1 in the β-catenin GOF limbs.

Again in order to get an idea if this down-regulation of *cyclin D1* observed in the limb bud mesenchyme may be mediated directly by canonical Wnt-signaling a part of the promoter was cloned into a *luciferase* reporter vector and luciferase assays were carried out. In addition, like in the case for all other potential target genes the promoter region was subjected to an in silico analysis for putative Tcf/Lef binding sites using TESS. This revealed the presence of 20 potential binding sites within the genomic 2,4 kb up-stream region of the *cyclin D1* gene (Fig. 10 A).

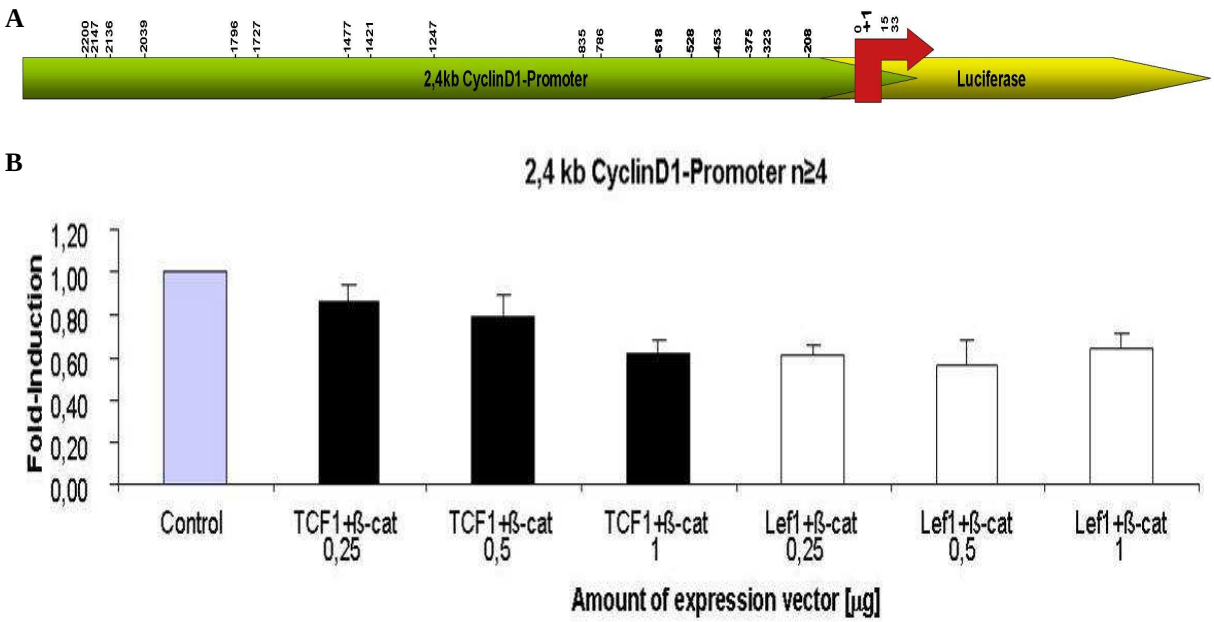


Fig. 10: Predicted Tcf1/Lef1 binding sites on the *cyclinD1* promoter (A). Luciferase-assay with the reporter-construct shown in (A) and increasing amounts of Tcf1/β-catenin or Lef1/β-catenin (B).

In the luciferase-assays (Fig. 10 B) a slight down-regulation of luciferase activity was observed upon co-transfection of Tcf1 and β-catenin or Lef1 and β-catenin. A dose

dependent effect was observed for Tcf1/β-catenin. For Lef1/β-catenin expression was decreased to about 0,6-fold with all concentrations. This suggests that *cyclinD1* might indeed be negatively regulated by Wnt/β-catenin signaling; however, the repressive effect was not very strong and further investigations would be necessary to further confirm that *cyclinD1* is repressed by the canonical Wnt-signaling in the limb mesenchyme. Due to emphasis on the main project described below no further experiments were carried out.

2.2. Identification and characterization of β-catenin deregulated genes.

2.2.1 Primary Screen

The data for the primary screen had been generated in the lab prior to my arrival as described in the introduction (Fig. 7). To identify potential candidate genes two different setups for the data analysis were used, which are summarized in Fig.11.
In the first setup we selected for genes that were up-regulated in chondrocyte precursors expressing stabilized β-catenin (GOF) in comparison with the adeno-GFP infected controls and were down-regulated in cells in which β-catenin activity had been lost (LOF) in comparison with the control.

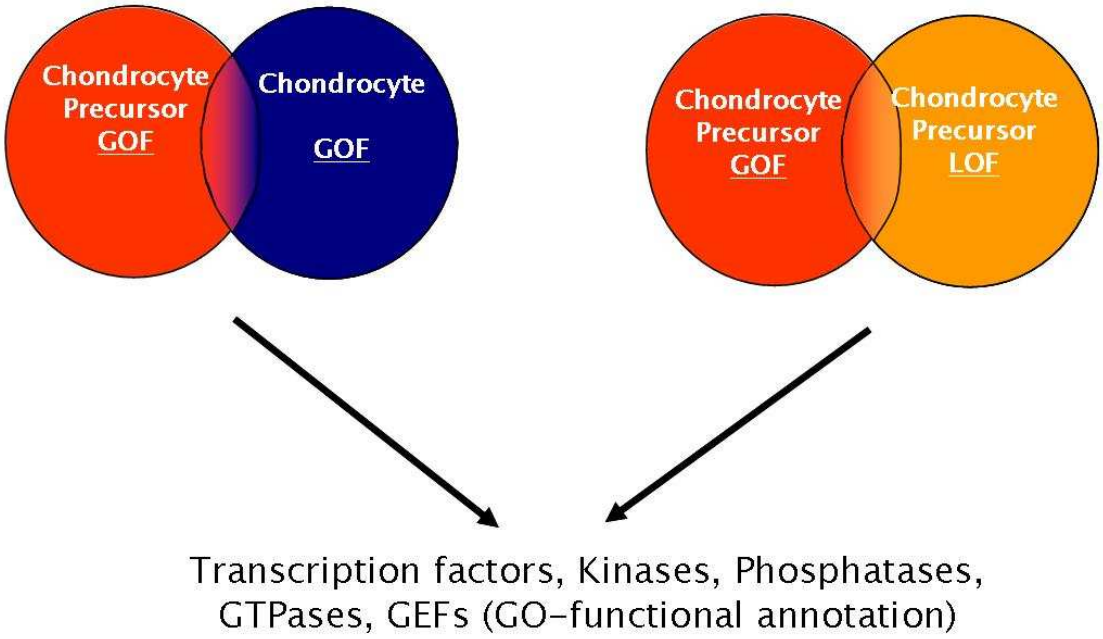


Fig. 11: Overview of the gene expression array data analysis

In the second setup, genes were selected that were up-regulated in chondrocyte precursors expressing stabilized β-catenin (GOF) as well as in chondrocytes, which expressed stabilized β-catenin (GOF). Both setups thereby should identify genes that are positively regulated by canonical Wnt-signaling and could play a role in transcriptional regulation or cell signaling. It is important to mention that the up-

regulation in both cell types, precursors and chondrocytes, led to a repression of *Sox9* expression.

Next we sorted the set of genes selecting for functions in signaling and transcriptional regulation. Gene Ontology (GO) functional annotations were used to filter for genes associated with the following functions: effector of transcription, kinase or phosphatase function, GTP exchange factor (GEF) or GTPase activating protein (GAP). Details of the analysis are described in material & methods.

The results from this analysis are shown in Table 1 and Table 2. The first setup yielded 16 genes shown in table 1.

Table 1: Chondrocyte Precursors GOF vs. LOF

Affymetrix ID	Gene	Description	GOF (log fold-change)	LOF (log fold-change)
1440370_at	Abca13	ATP-binding cassette, sub-family A (ABC1), member 13	1.31	-0.70
1458929_at	Abca9	ATP-binding cassette, sub-family A (ABC1), member 9	0.67	-0.52
1443870_at	Abcc4	ATP-binding cassette, sub-family C (CFTR/MRP), member 4	0.70	-0.73
1447301_at	Akap5	A kinase (PRKA) anchor protein 5	1.37	-1.92
1439892_at	Brsk1	BR serine/threonine kinase 1	0.94	-0.37
1440572_at	Dusp19	dual specificity phosphatase 19	1.00	-1.25
1422318_at	Foxd4	forkhead box D4	0.98	-1.11
1427494_at	Hoxb7	homeo box B7	1.10	-1.07
1454734_at	Lef1	lymphoid enhancer binding factor 1	0.57	-0.53
1422262_a_at	Lhx6	LIM homeobox protein 6	0.56	-1.27
1429833_at	Ly6g6e	lymphocyte antigen 6 complex, locus G6E	0.44	-1.56
1439585_at	Mdn1	midasin homolog (yeast)	2.41	-1.79
1445134_at	Mkl2	MKL/myocardin-like 2	0.42	-1.67
1421704_a_at	Pik3c2g	phosphatidylinositol 3-kinase, C2 domain containing, gamma polypeptide	1.87	-0.75
1420637_at	Prps2	phosphoribosyl pyrophosphate synthetase 2	0.57	-0.45
1443786_at	Utf1	undifferentiated embryonic cell transcription factor 1	0.5	-3.18

The second analysis shown in table 2 yielded 42 genes. Some of the factors identified like Lef1, Tcf7, Twist1, En1, or Msx2 were already known targets of canonical Wnt signaling (Hooper 1994; Roose, Huls et al. 1999; Filali, Cheng et al. 2002; Willert, Epping et al. 2002; Howe, Watanabe et al. 2003). Furthermore these genes had been characterized in the context of limb development in our lab and other labs as been regulated by Wnt/β-catenin signaling, which was reassuring that the experimental set-up, data-quality and analysis might be sufficient to identify genes in limb development that are regulated by canonical Wnt-signaling.

Table 2: Chondrocyte Precursor GOF vs. Chondrocytes GOF

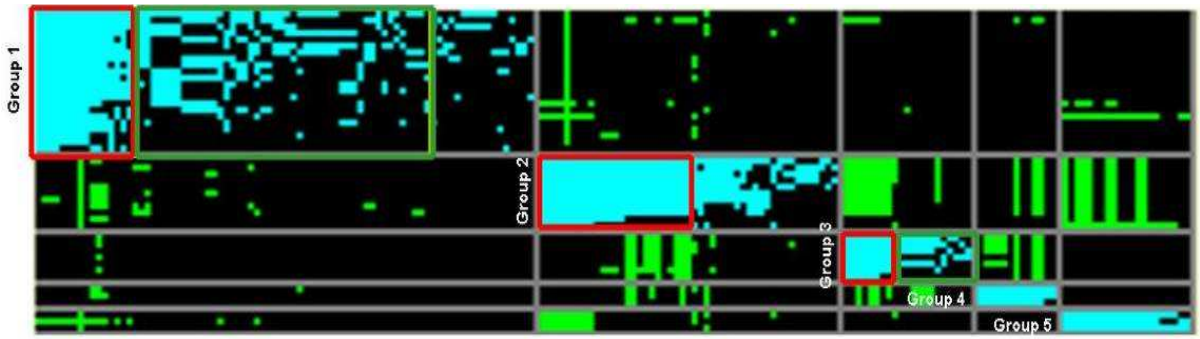
Affymetrix ID	Gene	Description	GOF chondrocyte precursors (log fold-change)	GOF chondrocytes (log fold-change)
1427490_at	Abcb7	ATP-binding cassette, sub-family B (MDR/TAP), member 7	0.52	0.72
1458455_at	Abra	actin-binding Rho activating protein	2.44	1.58
1451867_x_at	Arhgap6	Rho GTPase activating protein 6	0.61	1.04
1436512_at	Arl4c	ADP-ribosylation factor-like 4C	0.60	0.96
1426167_a_at	Camk4	calcium/calmodulin-dependent protein kinase IV	1.21	2.12
1449152_at	Cdkn2b	cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	1.15	0.79
1436569_at	Depdc2	DEP domain containing 2	0.49	0.75
1418618_at	En1	engrailed 1	0.95	1.15
1455426_at	Epha3	Eph receptor A3	0.61	0.75
1427783_at	Erbb4	v-erb-a erythroblastic leukemia viral oncogene homolog 4 (avian)	1.33	1.12
1423232_at	Etv4	ets variant gene 4	1.01	1.59
1458961_at	Eya3	eyes absent 3 homolog (Drosophila)	0.45	0.58
1421442_at	Flt4	FMS-like tyrosine kinase 4	0.58	1.14
1437247_at	Fosl2	fos-like antigen 2	0.84	0.54
1444226_at	Foxo3a	forkhead box O3a	0.59	0.57
1435283_s_at	Gm967	gene model 967	0.51	0.45
1454959_s_at	Gnai1	guanine nucleotide binding protein (G protein), alpha inhibiting 1	0.44	0.58
1422704_at	Gyk	glycerol kinase	0.65	0.50
1421072_at	Irx5	Iroquois related homeobox 5 (Drosophila)	0.87	0.41
1454734_at	Lef1	lymphoid enhancer binding factor 1	0.57	1.31
1426516_a_at	Lpin1	lipin 1	0.50	0.47
1430564_at	Mobkl1a	MOB1, Mps One Binder kinase activator-like 1A (yeast)	0.73	0.59
1449559_at	Msx2	homeobox, msh-like 2	0.53	0.51
1421867_at	Nr3c1	nuclear receptor subfamily 3, group C, member 1	0.51	0.41
1450821_at	Pcaf	p300/CBP-associated factor	0.47	1.16
1451737_at	Pik3r1	phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)	0.77	2.72
1443144_at	Prkcb1	protein kinase C, beta 1	0.57	2.66
1416588_at	Ptprn	protein tyrosine phosphatase, receptor type, N	0.76	1.87
1427448_at	Rabep1	rabaptin, RAB GTPase binding effector protein 1	0.44	0.72
1451236_at	Rerg	RAS-like, estrogen-regulated, growth-inhibitor	0.83	2.64
1436470_at	Rims2	regulating synaptic membrane exocytosis 2	0.96	1.15
1440878_at	Runx1	runt related transcription factor 1	0.58	0.67
1436499_at	Sgms1	sphingomyelin synthase 1	0.41	0.52
1432139_at	Srpk3	serine/arginine-rich protein specific kinase 3	2.87	1.45
1450997_at	Stk17b	serine/threonine kinase 17b (apoptosis-inducing)	0.72	0.42
1421078_at	Tcf23	transcription factor 23	2.84	1.23
1433471_at	Tcf7	transcription factor 7, T-cell specific	0.54	2.06
1444730_at	Tlk1	tousled-like kinase 1	1.36	0.82
1418733_at	Twist1	twist gene homolog 1 (Drosophila)	0.50	1.47
1460104_at	Vps4b	vacuolar protein sorting 4b (yeast)	0.60	0.63
1425425_a_at	Wif1	Wnt inhibitory factor 1	0.51	3.14
1434149_at	Tcf4*	Transcription factor 4*		1,78

* This factor did not turn up in the data analysis but was confirmed before as upregulated in chondrocytes by D. Kostanova.

As an additional validation of the data the results were clustered by use of the **D**atabase for **A**nnotation, **V**isualization and **I**ntegrated **D**iscovery = DAVID (Dennis, Sherman et al. 2003; Huang da, Sherman et al. 2009). This tool is available online (http://david.abcc.ncifcrf.gov/), it can be used to classify large gene list into functional

related gene groups and rank the importance of the discovered gene groups. To carry out this analysis the genes from both set-ups were merged.

The results are shown in Fig. 12. As expected two major clusters appeared in the gene enrichment analysis. One cluster of 22 genes for transcription factors (Group1), many of them known to be involved in developmental processes or cell differentiation and a second cluster of 11 genes with putative functions in intracellular signaling (Group2). 15 genes were not accounted for in this analysis.



Group 1 Transcription Factors

1 ACTIN-BINDING RHO ACTIVATING PROTEIN **2** FORKHEAD BOX O3A **3** LYMPHOID ENHANCER BINDING FACTOR 1 **4** CYCLIN-DEPENDENT KINASE INHIBITOR 2B (P15, INHIBITS CDK4) **5** TWIST GENE HOMOLOG 1 (DROSOPHILA) **6** ENGRAILED 1 **7** HOMEO BOX, MSH-LIKE 2 **8** RUNT RELATED TRANSCRIPTION FACTOR 1 **9** MKL/MYOCARDIN-LIKE 2 **10** HOMEO BOX B7 **11** TRANSCRIPTION FACTOR 7, T-CELL SPECIFIC **12** UNDIFFERENTIATED EMBRYONIC CELL TRANSCRIPTION FACTOR 1 **13** TRANSCRIPTION FACTOR 23 **14** FORKHEAD BOX D4 **15** FOS-LIKE ANTIGEN 2 **16** GENE MODEL 967, (NCBI) **17** LIM HOMEOBOX PROTEIN 6 **18** P300/CBP-ASSOCIATED FACTOR **19** TRANSCRIPTION FACTOR 4 **20** IROQUOIS RELATED HOMEOBOX 5 (DROSOPHILA) **21** ETS VARIANT GENE 4 (E1A ENHANCER BINDING PROTEIN, E1AF) **22** EYES ABSENT 3 HOMOLOG (DROSOPHILA)

__Transcriptional Activity, Nuclear localisation

__Transcriptional Activity, Nuclear localisation, Developmental process

Group 2 Cell-signalling

1 CALCIUM/CALMODULIN-DEPENDENT PROTEIN KINASE IV **2** PROTEIN KINASE C, BETA 1 **3** TOUSLED-LIKE KINASE 1 **4** SERINE/THREONINE KINASE 17B (APOPTOSIS-INDUCING) **5** BR SERINE/THREONINE KINASE 1 **6** SERINE/THREONINE KINASE 23 **7** EPH RECEPTOR A3 **8** V-ERB-A ERYTHROBLASTIC LEUKEMIA VIRAL ONCOGENE HOMOLOG 4 (AVIAN) **9** FMS-LIKE TYROSINE KINASE 4 **10** PROTEIN TYROSINE PHOSPHATASE, RECEPTOR TYPE, N **11** MOB1, MPS ONE BINDER KINASE ACTIVATOR-LIKE 1A (YEAST)

__posttranslational protein modification, phosphate metabolic process, kinase activity, etc.

Group 3

1 VACUOLAR PROTEIN SORTING 4B (YEAST) **2** ATP-BINDING CASSETTE TRANSPORTER SUB-FAMILY A MEMBER 9 **3** ATP-BINDING CASSETTE, SUB-FAMILY A (ABC1), MEMBER 13 **4** GLYCEROL KINASE **5** ATP-BINDING CASSETTE, SUB-FAMILY C (CFTR/MRP), MEMBER 4 **6** ATP-BINDING CASSETTE, SUB-FAMILY B (MDR/TAP), MEMBER 7 **7** MIDASIN HOMOLOG (YEAST)

__ATP-binding

__Transport

Group 4

1 RAS-LIKE, ESTROGEN-REGULATED, GROWTH-INHIBITOR **2** ADP-RIBOSYLATION FACTOR-LIKE 4C **3** GUANINE NUCLEOTIDE BINDING PROTEIN, ALPHA INHIBITING 1

GTP/GDP-Binding

Group 5

1 PROTEIN TYROSINE PHOSPHATASE, RECEPTOR TYPE, N **2** DUAL SPECIFICITY PHOSPHATASE 19 **3** EYES ABSENT 3 HOMOLOG (DROSOPHILA)

Phophatase activity

Fig. 12: Gene functional enrichment analysis by DAVID

2.2.2 Secondary Screen

To validate the 58 genes suggested by the analysis of gene expression data, whole mount *in-situ* hybridisations were performed for the majority of the genes using *β-cat*$^{\Delta ex3Prx1/+}$ (GOF) mice and their wild-type (WT)-littermates. For the five genes mentioned above that had previously been investigated in the context of Wnt-signaling and limb development no *in-situ* hybridisations (ISH) were performed. For 48 of the remaining 53 genes I generated *in-situ* probes. No probes were created for the genes: Tlk1, Vps4b, Arl4c, Abcc4, and Abca9 as suitable Riken clones were not available and Pcr-amplification did not succeed. The results from the whole mount *in-situ* hybridisations (WM-ISH) are summarized the Table 3.

Differential expression observed in wt versus gof	**Specific pattern but no differential expression observed**	**Probe unspecific/expression pattern not detectable**
Fosl2 (Fra2), Irx5, Prkcb1, Runx1, Tcf4, Wif1	Abcb7, Akap5, Brsk1*, Camk4, Cdkn2b, Depdc2, Epha3, Erbb4, Etv4, Eya3, Foxo3a*, Glyk, Gm967, Gnai1*, Hoxb7, Lhx6, Lipn1, Ly6g6e, Mdn1, Mkl2, Nr3c1, Pik3r1*Prps2, Rapeb1, Rims, Srpk3, Stk17b	Abca13, Abra, Arhgap, Flt4, Fox4d, Pcaf, Pi3c2g, Rerg, Sgms1, Tcf23, Utf1

*for this genes differential expression was observed in Section ISH and/or RT-PCR

Table3: Summary of the whole-mount In-situ hybridisation results

Whole mount stainings in which a differential expression was visible are shown in Fig. 13. Examples for some of the other genes are shown in Figure 14. For most genes the sample size was n=1, for genes where a differential expression was observed or suspected after the initial first round staining a second sample was analyzed (n=2).

In addition to the whole mount *in-situ* hybridizations I tried to confirm the differential gene expression using semi-quantitative RT-PCR on RNA isolated from the limb buds of E11,5 *β-cat*$^{\Delta ex3Prx1/+}$ (GOF) mice and their WT-littermates. For a selected number of genes I finally performed section *in-situ* hybridisations.

The RT-PCRs were carried out to get an independent confirmation for the results from the WM-ISH and to detect differences in expression levels, which might have been not easily visually detectable on the whole-mount staining (Fig. 15). Section *in-situ* hybridisations were performed to analyze the respective expression patterns inside the limb bud. Results from those are shown in Fig. 16.

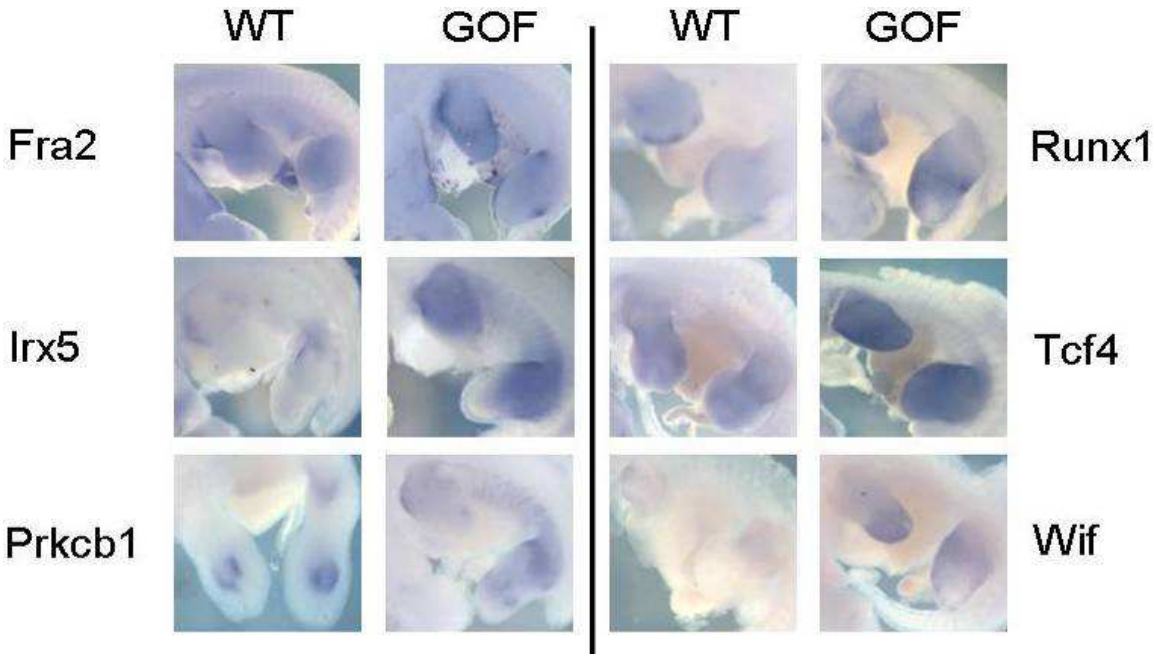


Fig. 13: Whole mount *In-situ* hybridisations. Genes with stronger expression in the β-catenin GOF embryos

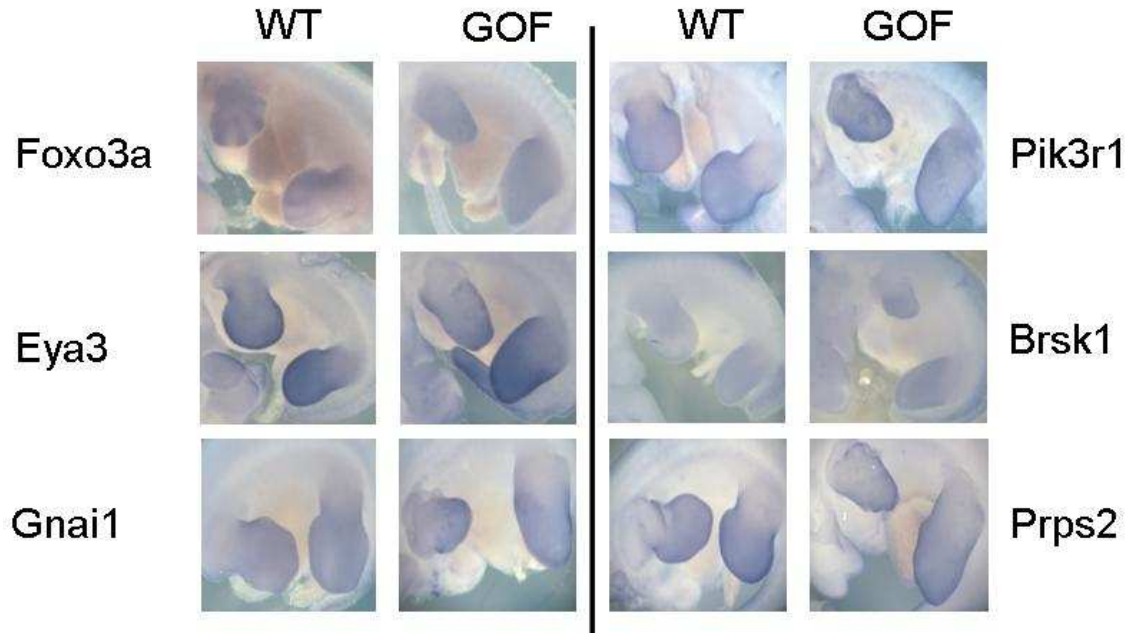


Fig. 14: Examples of genes with limb specific expression, but no clearly detectable difference in the expression between WT and β-catenin GOF embryos

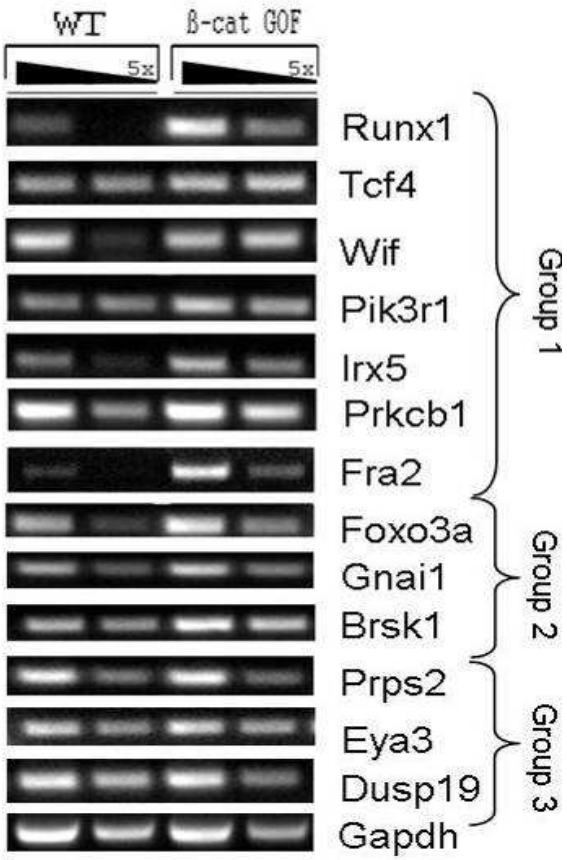


Fig. 15: Semi-Quantitative PCR
Group 1 - genes that are upregulated in this assay as well as the WM-ISH.
Group 2 - genes that have not been detected as upregulated in the WM-ISH but show differential expression in the SQ-RT-PCR.
Group 3 - examples of genes that were not differentially expressed in both assays. Gapdh: loading control, -RT not shown.

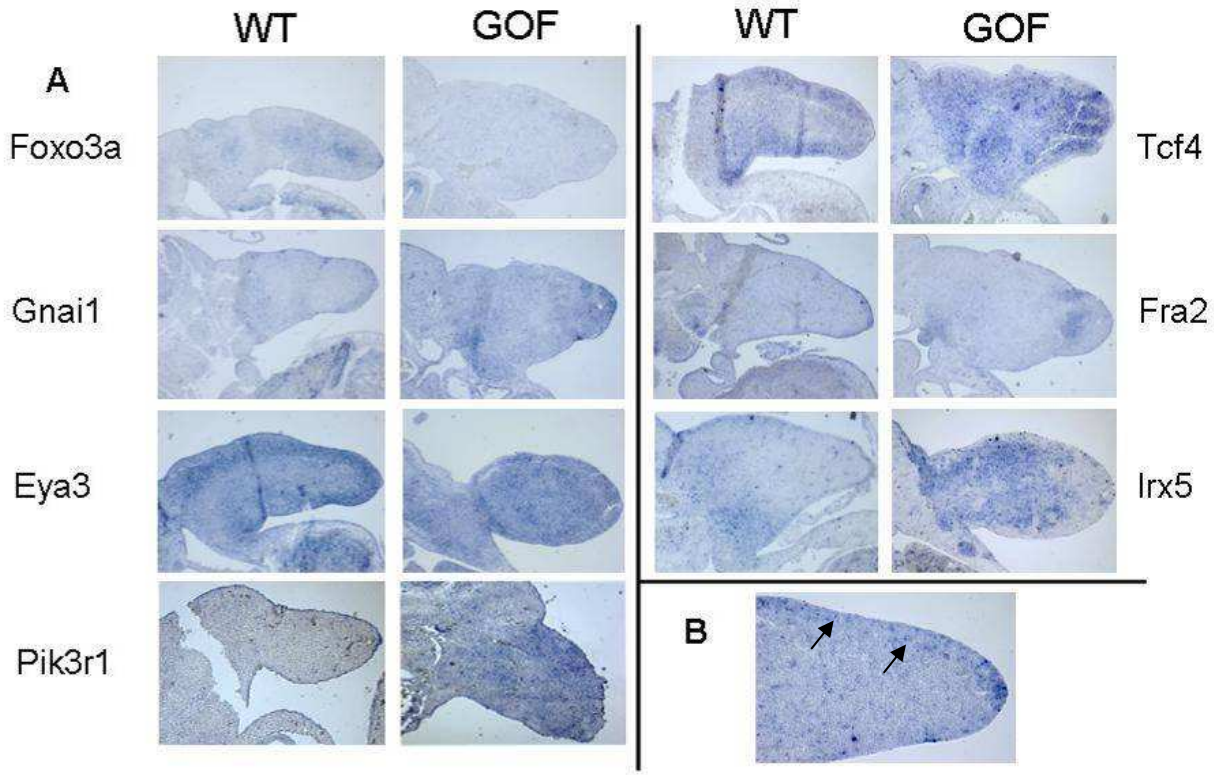


Fig. 16: Section ISH for E11,5 embryos (A). Alternative WT-section for Irx5; arrows indicate subectodermal expression (B).

Based on the results from the secondary screens the following genes were considered to be positively regulated by Wnt/β-catenin signaling: *Brsk1*, *Foxo3a*, *Fra2*, *Gnai1*, *Irx5*, *Pik3r1*, *Prkcb1*, *Runx1*, *Tcf4*, and *Wif1*. For *Brsk1* and *Foxo3a* this is based solely on the results from the semi-quantitative RT-PCR. For all other genes differential expression had been detected in WM-ISH, RT-PCR and Section-ISH.

For our initial question concerning factors, which might be involved in the transcriptional regulation of *Sox9,* the genes *Tcf4*, *Irx5* that encoded transcription factors and showed an interesting wild type expression patterns were of particular interest to us. For example, *Tcf4* showed an expression pattern somewhat complementary to that of Sox9 and congruent to the localisation of *Tcf1*, *Lef1* transcripts and endogenous high levels of β-catenin protein (Hill, Spater et al. 2005; Hill, Taketo et al. 2006).
For *Irx5* we could also detect such a subectodermal expression pattern in the central region of the limb (Fig. 16 B) in addition to the strong proximal expression domain that has been documented previously (Houweling, Dildrop et al. 2001) (Fig. 16). Interestingly *Irx5* upregulation in the β-catenin GOF limbs seems to be excluded from the most distal part of the limb (Fig. 13/Fig. 16).
Eya3 was also interesting to us as it showed an expression pattern similar to that of *Tcf4* in the wild-type; however β-catenin stabilization did not cause a detectable upregulation in the secondary screen assays conducted.
Brsk1, *Gnai1*, *Prkcb1* and *Runx1* were confirmed relatively late during the screening process therefore it was not possible for time reasons to carry out any additional investigations for these genes. *Wif1* was also not further investigated as it is known to be a regulator of Wnt-signaling and has already been investigated for its potential role in skeletogenesis (Hsieh, Kodjabachian et al. 1999; Surmann-Schmitt, Widmann et al. 2009).

2.2.3 Confirmation of Wnt/β-catenin target genes by promoter studies

Promoter studies in the response to β-catenin/Wnt signaling were carried out for *Pik3r1* by the summer student Franz Gruber under my supervision, however as his results could not be reproduced by myself no further results are shown for this gene.

Furthermore promoter studies were carried out on the upstream regulatory regions of the transcription factors Tcf4, *Fra2* (*Fosl2*) and *Irx5*. The expression of *Tcf4* and *Irx5* had been up-regulated in GOF mutants as confirmed in my secondary screens and both genes showed interesting expression patterns in the wild-type. *Fra2* was included because it was up-regulated and has already been shown to play a role in chondrocytes maturation (Karreth, Hoebertz et al. 2004).

2.2.3.1 Fra2 shows no response to canonical Wnt signaling and only a neglectabe effect on Sox9 expression *in vitro*

Fra2 is a member of the AP1 (activating protein 1) family and is encoded by the *Fosl2* gene. The AP1 transcription factor consists of dimers of members formed by either heterodimers of Fos (Fos, Fra1, Fra2 and FosB) and Jun (Jun, JunB and JunD) members, which encode basic leucine zipper domain proteins, or homodimers of Jun family members. AP1 has been shown to be involved into developmental processes as cell differentiation or apoptosis as well as in oncogenic transformation (Jochum, Passegue et al. 2001). Fra2 has previously been shown to have an effect on chondrocyte maturation (Karreth, Hoebertz et al. 2004). However this study did not investigate a potential influence of Fra2 on early differentiation events as for example an influence on *Sox9* expression. A potential regulation of *Fra2* expression by the canonical Wnt-signaling pathway was also not known. In contrast to other AP1 components, such as Fra1 and c-Jun, which have been described as direct targets of Wnt-signaling in human colonic adenocarcinomas (Mann, Gelos et al. 1999).
To address whether *Fra2* is regulated by the canonical Wnt-pathway I cloned a 1,9 kb genomic up-stream region of its promoter into a luciferase reporter vector. Bioinformatic analysis of the *Fra2* promoter revealed 24 potential binding sites for Lef1/TCF1 within the 1,9 kb up-stream region. However, when the promoter was tested for its responsiveness to Lef1 and TCF1 in combination with their cofactor β-catenin in 293T cells, no upregulation was detectible (Fig. 17). This result suggests that *Fra2* is not a direct target of the canonical Wnt-pathway.

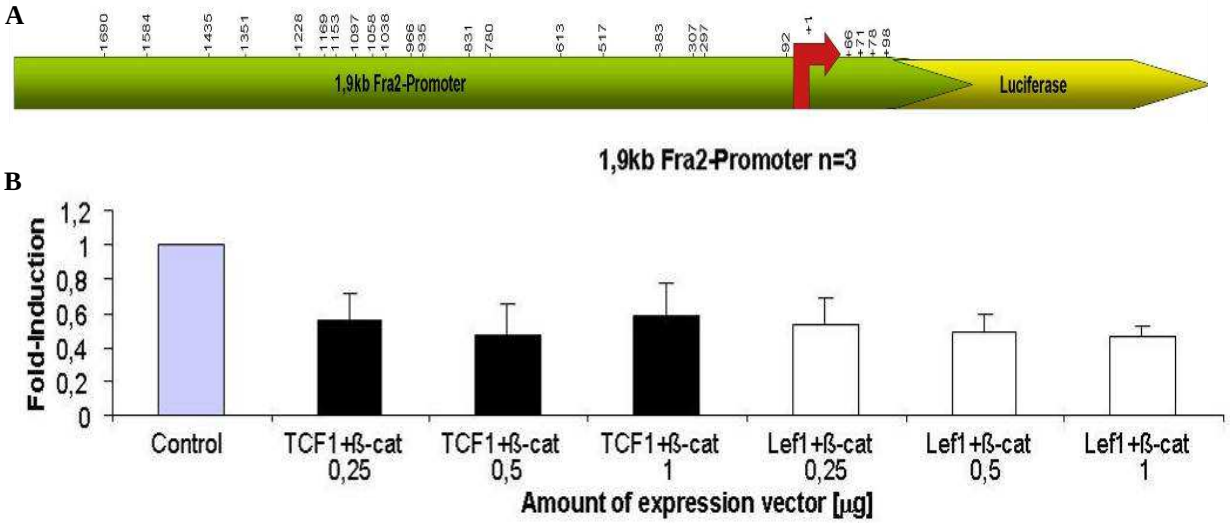


Fig. 17: Predicted Tcf1/Lef1 binding sites on the *Fra2* promoter (A). Luciferase-assay with the reporter-construct shown in (A) and increasing amounts of Tcf1/β-catenin or Lef1/β-catenin (B).

To investigate if Fra2 could have an effect on *Sox9* I had cloned the Fra2 ORF into an expression vector and co-transfected the Fra2 expression construct with a 2.9 kb *Sox9* luciferase reporter, which had previously been generated in our lab by others. My bioinformatical analysis of the *Sox9* reporter indicated the presence of 25 putative AP1 binding sites (Fig. 18).

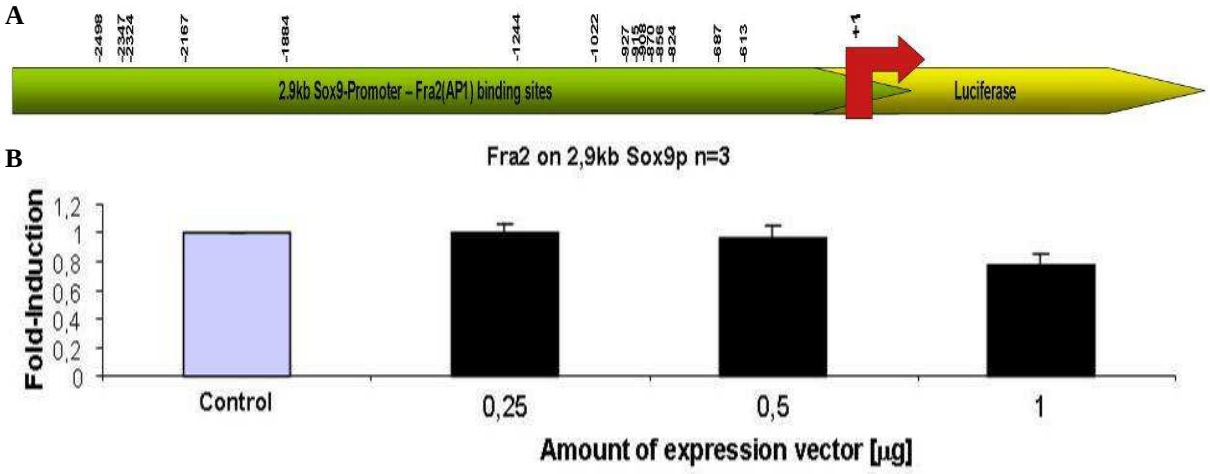


Fig. 18: Predicted AP1 binding sites on the *Sox9* promoter (A). Luciferase-assay with the reporter-construct shown in (A) and increasing amounts of Fra2 expression vector (B).

The luciferase assay revealed only a slight down-regulation to a maximum of 0,8-fold with the highest concentration of expression vector used (Fig. 18). This result suggests that Fra2 may not have an effect on *Sox9* expression.

2.2.3.2 Irx5 can be induced by canonical Wnt signaling and represses Sox9 *in vitro*

Iroquois genes have first been identified in the IRX-C complex of *Drosophila melanogaster,* which contains the three highly similar homebox genes *araucan* (orthologue to IRX1,3), *caupolican* (orthologue to IRX2,5), and *mirror* (orthologue to IRX4,6). These genes have a conserved homeodomain of the three-amino acid length extension (TALE) superclass and a familiy specific conserved 13 amino acid-residue motif, the so-called Irobox (Burglin 1997).

Vertebrates have six Irx-gene homologues, which are arranged in two genomic clusters - one consisting of *Irx1*, *Irx2*, and *Irx4* and the second of *Irx3*, *Irx5* and *Irx6* and probably arose by duplication of the IRX-C complex (Peters, Dildrop et al. 2000). It is has been shown in various species that *Irx* genes play a role in specifying tissue patterns during development (Cavodeassi, Modolell et al. 2001).

In the vertebrate limbs *Irx* genes are expressed in well defined patterns with *Irx1* and *Irx2* being expressed in the digit condensations at E13.5 and the future joint regions at E14.5, while *Irx3* and *Irx5* are mainly found in the proximal region of the limb at E11.5 and in the interdigital mesenchyme at E13.5 (Houweling, Dildrop et al. 2001). So far there have been no published studies reporting a function of *IRX* genes in limb development.

In order to address whether *Irx5* is regulated by a β-catenin/Tcf transcriptional complex, we searched for the presence of potential Tcf/Lef binding sites in the up-stream regulatory region. This revealed the presence of 24 potential sites within a 1,9 kb region (Fig. 19A). Next I isolated and cloned this 1,9kb region of the putative *Irx5*-promoter region into a pGL4 vector and tested for its responsiveness to the canonical Wnt transcriptional mediators Tcf1/β-catenin and Lef1/β-catenin, respectively. For both combinations a dose dependent up-regulation could be observed. Tcf1/β-catenin could induce a 2-fold upregulation, when 1μg of both expression vectors was used. A similar level of activation could already be observed at 0,25 μg and 0,5 μg of Lef1/β-catenin expression vectors. At the concentration of 1μg of Lef1/β-catenin a

very significant increase in activity was observed in measurements conducted (Fig. 19).

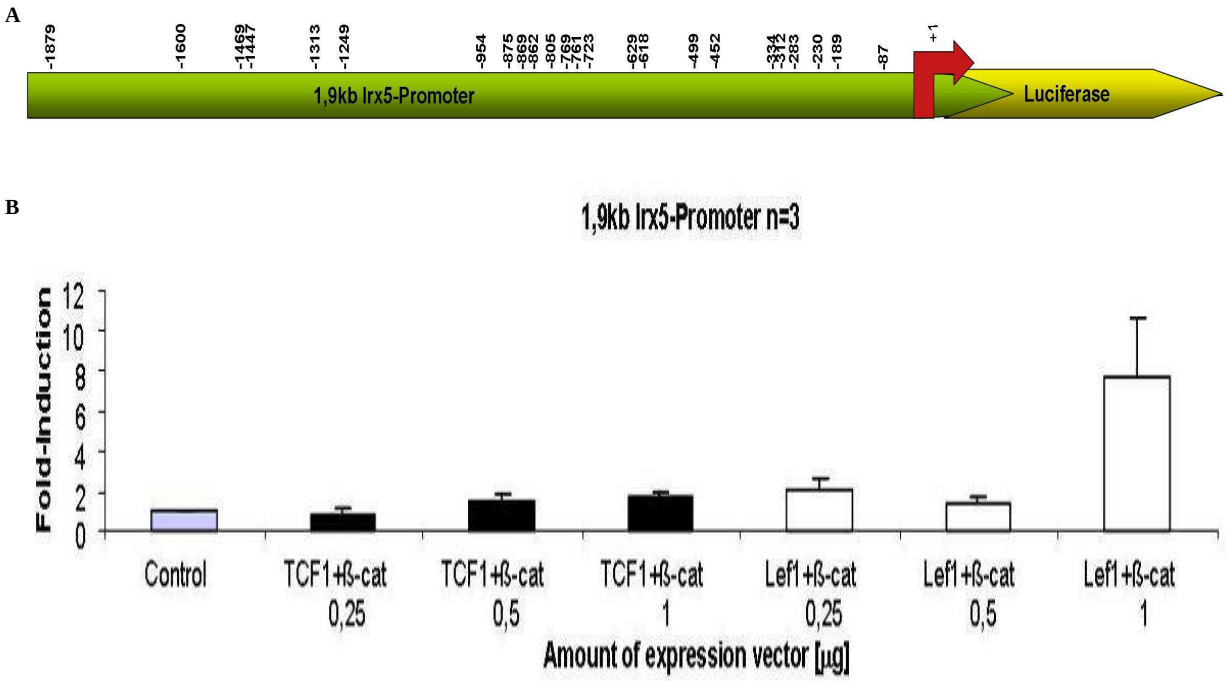


Fig. 19: Predicted Tcf1/Lef1 binding sites on the *Irx5* promoter (A). Luciferase-assay with the reporter-construct shown in (A) and increasing amounts of Tcf1/β-catenin or Lef1/β-catenin (B).

To test if Irx5 could be the factor X influencing *Sox9* expression I cloned the coding region into an expression vector and co-transfected it with the 2,9kb *Sox9*-luciferase reporter construct into 293T cells. Up to now no canonical binding site for the IRX transcription factors has been published, however using Uniprobe a data collection based on protein/DNA binding arrays suggests the presence of three potential sites within the 2,9kb *Sox9* promoter region (Newburger and Bulyk 2009) (see Fig. 20A).

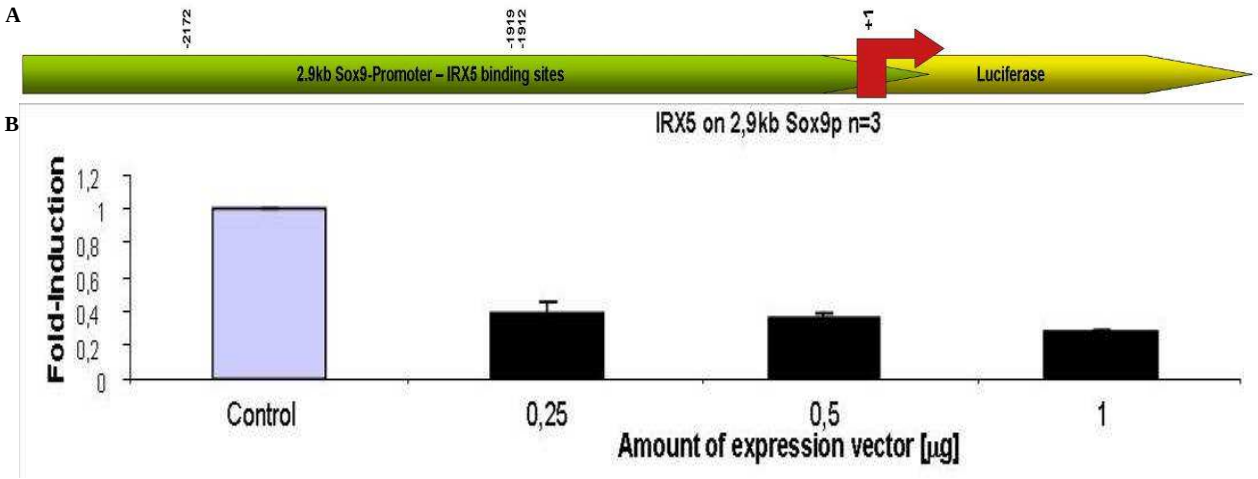


Fig. 20: Predicted Irx5 binding sites on the *Sox9* promoter (A). Luciferase-assay with the reporter-construct shown in (A) and increasing amounts of *Irx5* expression vector (B).

Co-transfection of the *Irx5* expression plasmid resulted in a strong dose dependent repression of the luciferase signal with a reduction down to 0,3-fold of the signal with the highest concentration of *Irx5* expression vector (Fig. 20B).
Given the strong repressive *in vitro* activity of Irx5 on the *Sox9* promoter I constructed a *Prx1-Irx5pA* transgenic construct to study the *in vivo* function of Irx5 upon overexpression in the limb-mesenchyme. Unfortunately the pro-nucleus injections could not been carried out within the time frame of my thesis work.

2.2.3.3 Tcf4 is up-regulated by canonical Wnt signaling and represses Sox9 *in vitro*

The third factor that was investigated in more detail during the work for this thesis was the basic helix-loop-helix (bHLH) transcription factor 4 (TCF4, also called: ASP-I2, bHLHb19, E2-2, E2.2, ITF-2, ME2, MITF-2, SEF-2, and TFE). The *Tcf4* gene encodes two major protein isoforms: a short one with a length of 511 amino acids (TCF4b) and a long one with a length of 670 amino acids (TCF4a). These isoforms result from alternative promoter usage. Both of them share the bHLH domain at the carboxy-terminus, however the shorter isoform, TCF4b, is lacking a domain located within the amino-teminal region, which seems to be important for the inhibitory transcriptional activity reported for TCF4 (Skerjanc, Truong et al. 1996).

Furthermore, this gene was of interest to us as it has been reported previously that expression of the longer isoform, TCF4a, responds to β-catenin signaling (Kolligs, Nieman et al. 2002). The original *in-situ* probe as well as the RT-PCR primers used in my secondary screens were however not isoform specific. Given the previous observations by Kolligs and colleagues on the human and rat *Tcf4* promoters, we concentrated for our promoter studies on the putative promoter region of the longer *Tcf4a* transcript. Within the 2,3 kb up-stream region of the transcriptional start of mouse *Tcf4a* a total of 35 putative Tcf/Lef binding sites were identified using bioinformatic analysis.

The luciferase assay showed no significant up-regulation upon co-transfection of TCF1/β-catenin. In contrast an up to 2,5-fold increase in luciferase activity was observed upon co-transfection of increasing amounts of Lef1/β-catenin expression vectors. These results suggest that the longer isoform of *Tcf4a* is also inducible by canonical Wnt-signaling in the mouse (Fig. 21).

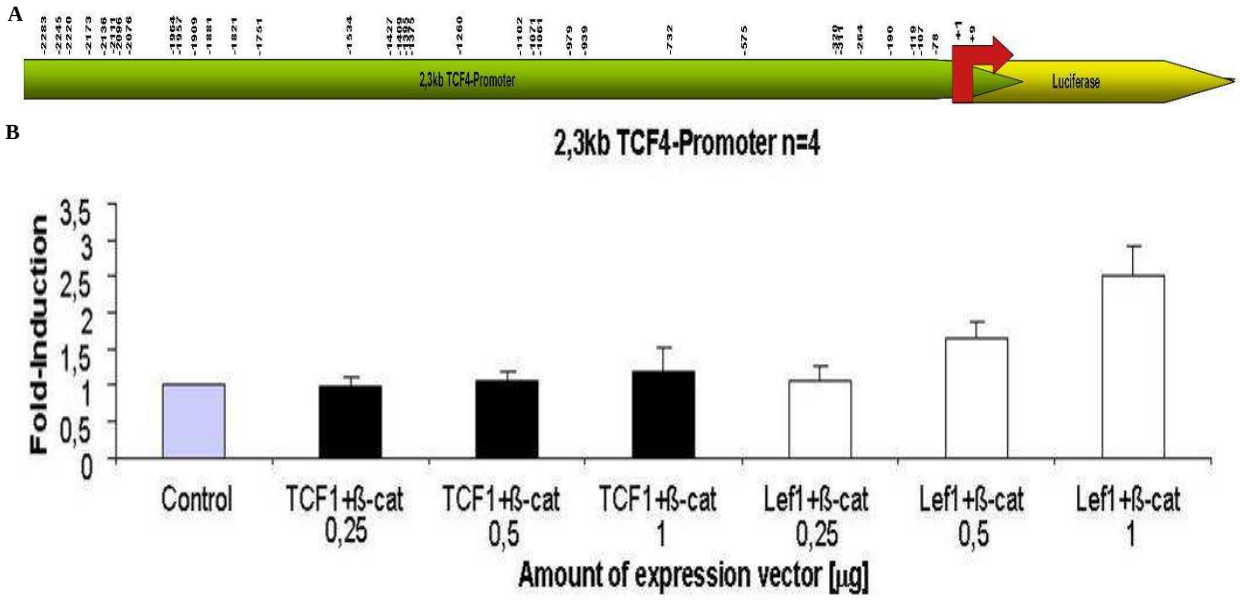


Fig. 21: Predicted Tcf1/Lef1 binding sites on the *Tcf4a* promoter (A). Luciferase-assay with the reporter-construct shown in (A) and increasing amounts of Tcf1/β-catenin or Lef1/β-catenin (B).

In order to address the question whether Tcf4 might serve as factor X in the regulation of *Sox9* I performed luciferase assays co-transfecting an expression plasmid carrying the ORF of the long Tcf4 isoform (TCF4a) and the *Sox9*-luciferase reporter construct. Again based on my bioinformatical analysis using the Uniprobe

database 7 putative binding sites for TCF4, which has been shown to bind to the E-box motive CANNTG via its bHLH domain, were predicted to be present in the 2,9 kb *Sox9* promoter region. bHLH-proteins have been reported to mediate transcriptional activation as well as repression and as these proteins are known to act as dimers it is thought that this differential activity depends on the combination of binding partners. A dose dependent decrease of the luciferase activity was observed in these co-transfection assays (Fig. 22). Thus implicating the β-catenin regulated TCF4a protein as a potential negative regulator of *Sox9* at least *in vitro*.

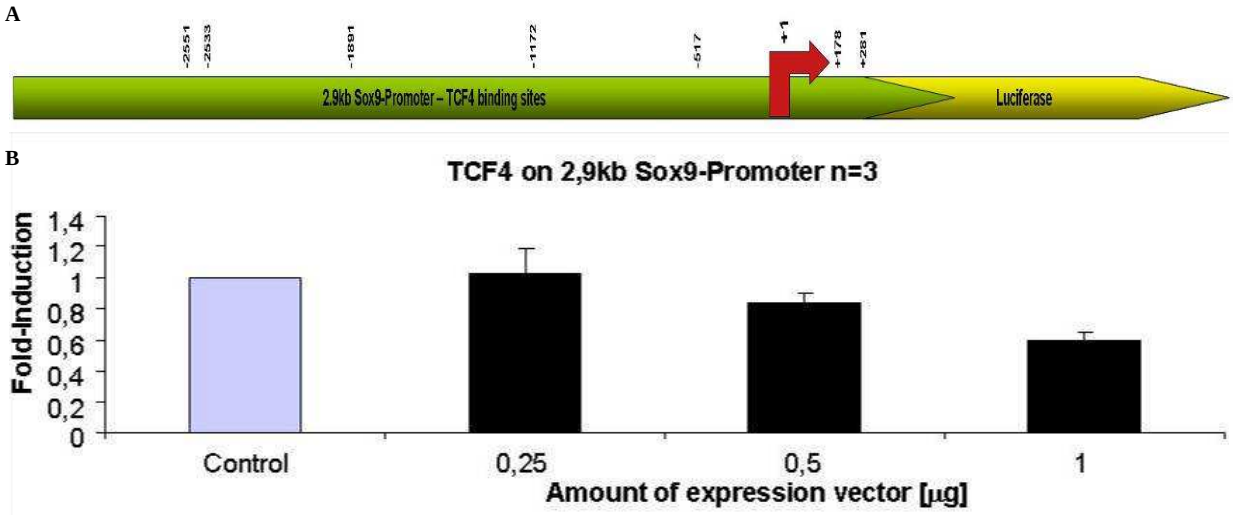


Fig. 22: Predicted Tcf4 binding sites on the *Sox9* promoter (A). Luciferase-assay with the reporter-construct shown in (A) and increasing amounts of *Tcf4a* expression vector (B).

2.2.3.4 Tcf1 but not Lef1 has an additive effect on Tcf4a mediated *Sox9* repression *in vitro*

Based on the previous observations that activation of β-catenin results in repression of *Sox9* expression, but up-regulation of *Tcf1* and *Lef-1* in the limb mesenchyme (Hill, Spater et al. 2005; Hill, Taketo et al. 2006) the possibility that a Tcf/β-catenin complex could directly mediate repression on the *Sox9* promoter had also been investigated in our lab. Ongoing work by other lab members revealed that the canonical pathway, in particular acting through Tcf1 might have the potential to repress *Sox9* at least *in vitro*.

To investigate a potential additive effect between the transcription factors acting down-stream of canonical Wnt-signaling and being positive targets thereof and the bHLH factor TCF4 on the *Sox9* promoter, I carried out luciferase reporter assays co-expressing a constant amount of either Lef1 or Tcf1 in addition to increasing amounts of TCF4a.

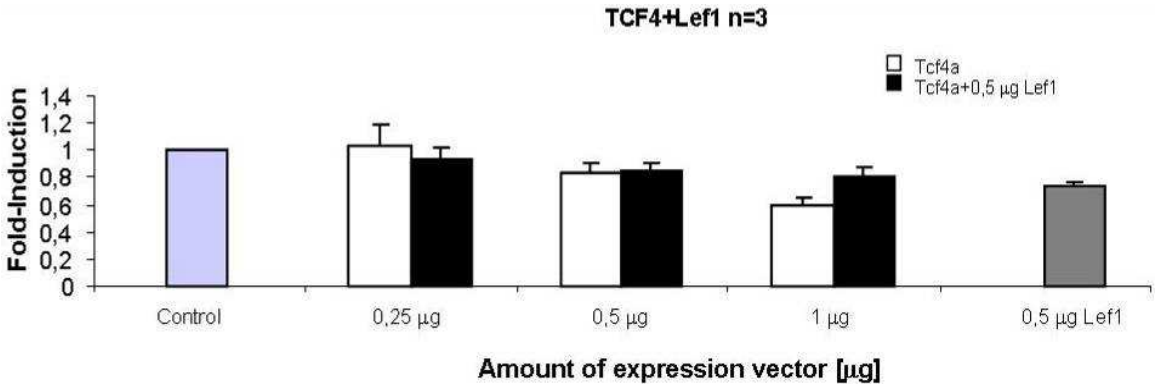


Fig. 23: Luciferase Assay with stable amount of TCF4a and increasing amounts of Lef1.

No statistically significant additional repressive effect was observed upon co-treatment with increasing amounts of TCF4a together with constant levels of Lef1 (two-tailed t-test) (Fig. 23).

In contrast addition of increasing amounts of TCF4a together with constant amounts of Tcf1 resulted in a statistically significant difference in the repressive effect for the highest concentration of Tcf4a (1µg), when compared to either Tcf4a or Tcf1 alone (Fig 24). This suggests an additive repressive effect of these two factors on the *Sox9*-promoter.

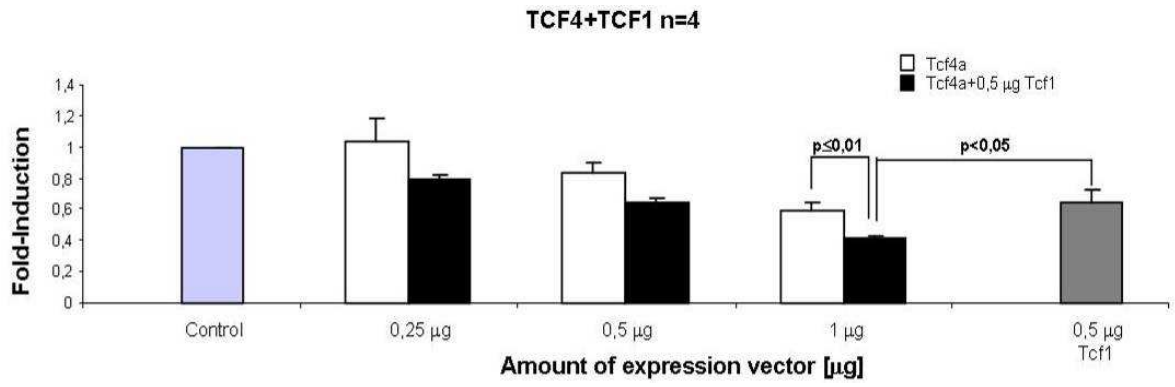


Fig. 24: Luciferase Assay with stable amount of Tcf4a and increasing amounts of Tcf1 on the *Sox9*-promoter.

It would be interesting to perform similar combinatorial experiments with these factors using a lower concentration for Tcf1, as 0,5 µg already resulted in a repression that was stronger than the combination of the factors at concentrations of 0,25µg and 0,5µg for Tcf4a, as such potentially masking a combinatorial effect at lower concentrations.

To investigate if addition of β-catenin has a positive effect on this repression the same experiment was carried out using a Tcf1/β-catenin fusion protein, as using this fusion protein a more pronounced repressive effect compared to Tcf1 or Tcf1 in combination with β-catenin had been observed. Unfortunately this experiment could not be interpreted (Fig. 25) as the fusion protein alone resulted in a strong repression at the concentration of 0.0625 µg. Thus in future experiments even lower concentration for the Tcf1/β-catenin fusion protein should be used.

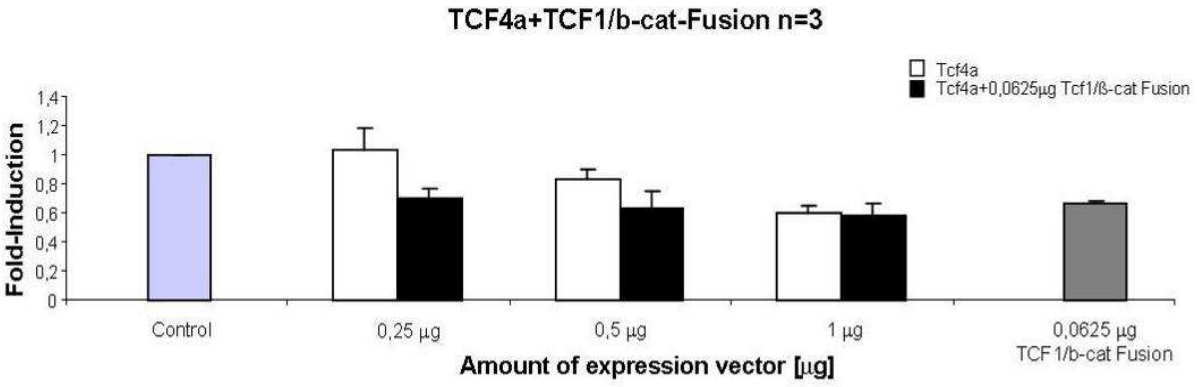


Fig. 25: Luciferase Assay with stable amount of Tcf4a and increasing amounts of Tcf1/β-catenin fusion protein on the *Sox9*-promoter.

2.2.4 Overexpression of Tcf4a results in a limb phenotype

A transient transgenic approach was used to investigate the function of Tcf4a in limb development with a particular focus on chondrogenesis *in vivo.* For this I had cloned the cDNA of *Tcf4a* into a backbone vector containing as upstream elements a chicken insulator, a 3 kb *Prx1* promoter region and as down-stream elements an SV40 poly A (Logan, Martin et al. 2002). The construct is shown in Fig. 26.

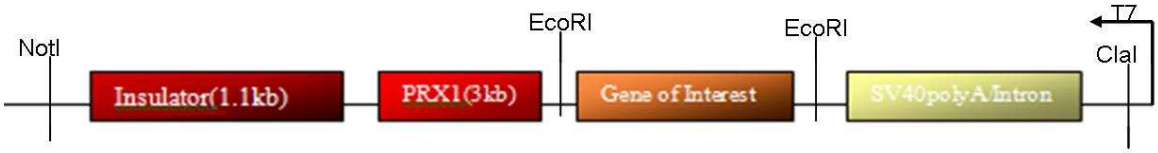


Fig. 26: Construct used for limb mesenchyme specific overexpression.

Similar constructs have also been generated for the other two genes, *Irx5* and *Fra2*, which I had more closely examined during the course of this thesis in order to produce transient transgenic embryos to examine the *in vivo* function upon overexpression.

Unfortunately, it had only been possible to do one round of pro-nucleus injections for the factor Tcf4a before the end of my thesis. From this one round I was able to retrieve 2 litters of 2 embryos each at E12,5 of embryonic development. Of the four embryos one showed an obvious morphological difference. By genotyping PCR I could confirm that this particular embryo was carrying the transgene (Fig. 27).

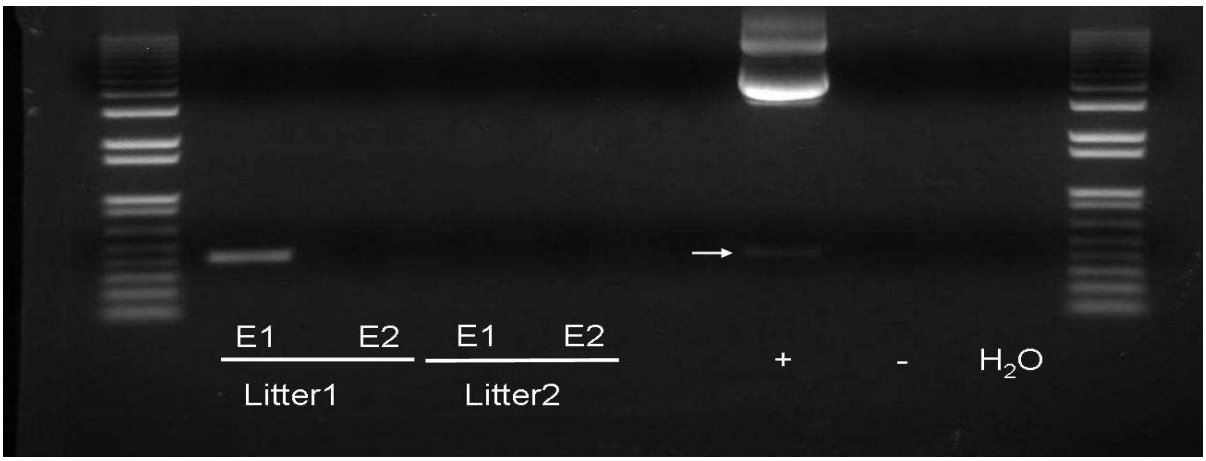


Fig. 27: Genotyping of the embryos received upon pronucleus injections.

This result I got at the very end of my diploma thesis work. Therefore I was not able to carry out a detailed analysis of the transgenic phenotype. However, morphologically this embryo was reduced in its overall size and showed a limb phenotype (Fig. 28). The observed phenotype in the limb resembled grossly the limb abnormalities seen in the limb-specific *Sox9* knock-out (Akiyama, Chaboissier et al. 2002). This is what we would have expected from a factor, which is capable to repress *Sox9* in the limb bud.

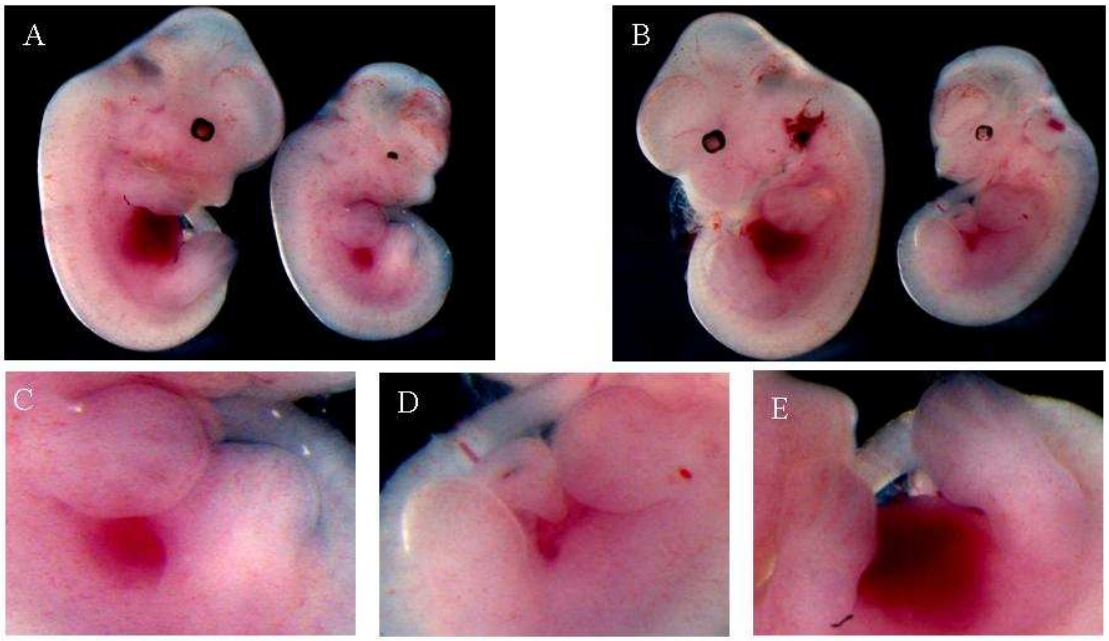


Fig. 28: Right (A) and left (B) view of the transgenic embryo and its WT-littermate. Magnifications of the limb region of the transgenic embryo (C=right, D=left) and WT-litterate (E).

In addition, I noticed upon further inspection of the transgenic embryo that there were phenotypic differences with respect to the left and right site of the body (Fig 28 A, B, C, D). This may be due to differential expression of the transgene, which is known to occur in founder mice. The most interesting phenotypic feature was that digit condensations were strongly reduced. Condensations were visible in the right forelimb (Fig. 28C) but almost no condensations were visible in the left fore- and hindlimbs (Fig. 28D). In contrast in the limbs of the wild-type embryo condensations were clearly developed at E12,5. Additionally, as the *Prx1* promoter also drives expression in the head mesenchyme a phenotypic difference was visible in the shape of the head. However, since the sample size was only n=1 this observations have to

be taken with caution and would have to be confirmed through the generation of additional transgenic animals showing a similar phenotype. This would also allow to establish whether overexpression of *Tcf4a* negatively affects *Sox9* expression and to examine its potential role limb development.

3 Discussion

3.1 CyclinD1 but not Tcf7l2 is negatively regulated by Wnt/β-catenin-signaling in vitro and in vivo

Previously it has been observed by performing *in-situ* hybridizations on sections through the limb buds of *Prx1-Cre* conditional *β-catenin* LOF or GOF embryos that *cyclinD1* and *Tcf7l2* expression was altered. Particularly the results from the GOF limbs suggested that both genes might be negatively regulated by stabilization of β-catenin. However, a negative regulation could only be confirmed in the case of *cyclinD1* using luciferase-promoter studies. This is particularly interesting as *cyclinD1* has been described to be a direct, positively regulated target of Wnt/β-catenin signaling in human colon cancers (Tetsu and McCormick 1999); (Shtutman, Zhurinsky et al. 1999). However, the notion that *cyclinD1* is a direct target has been challenged recently (Terauchi, Tsuji et al. 1999). My *in vitro* promoter studies would suggest that its expression can even be repressed by a β-catenin/Tcf complex, which is in agreement with the *in vivo* observations by previous lab members. Thus in order to show that *cyclinD1* is indeed a direct target of the β-catenin pathway in the limb mesenchyme, ChIP-experiments using Lef1-, Tcf1-, and β-catenin antibodies with primary material from mouse limbs would have to be performed in order to provide evidence that these factors bind to the *cyclinD1* promoter in the limb. Such experiments are currently on their way in our lab.

As the canonical Wnt-signaling so far has been primarily described as being an activatory pathway of gene expression additional experiments would have to be carried out in order to understand the mechanism how a repressive effect is achieved.

In the case of *Tcf7l2* the *in vitro* promoter studies clearly show that *Tcf7l2* is positively regulated by a β-catenin/Tcf or Lef complex. This is in contrast to what has been observed *in vivo*. However, the discrepancy might be due to a suppression of cell-fate in the limbs upon stabilization of β-catenin, as a number of other genes have been shown to be deregulated in these limbs (Hill, Taketo et al. 2006).

3.2 Target Genes of Wnt-signaling in limb bud development

From the transcriptome analysis screens carried out using limb bud cells and chondrocytes I could confirm a total of 10 genes out of the 53 genes, which I had analyzed further. So together with the 5 genes previously confirmed in our lab in total 15 out of 58 genes, which were deregulated based on the microarray analysis, could be confirmed by *in-situ* hybridization and/or RT-PCR to have a higher expression level in β-catenin GOF limbs than in limbs of wild-type embryos. The ones identified by me were components of intracellular pathways, such as the BR serine/threonine kinase 1 (Brsk1), the Protein kinase C beta 1 (Prkcb1), the Phosphatidylinositol 3-kinase regulatory subunit 1 (Pik3r1), the Adenylate cyclase inhibiting G-alpha protein (Gnai1), transcription factors such as Irx5, Runx1, Tcf4, Foxo3a, Fra2, and components of the Wnt-pathway, such as the Wnt-inhibitory factor 1 (Wif1). With the exception of Wif, none of the other genes had been described as a target of Wnt-signaling up to now (http://www.stanford.edu/~rnusse/pathways/targets.html). For the the kinase or kinase-related genes *Brsk1*. *Prkcb1* and *Pik3r1* knock-out mice are described in the literature, but no skeletal abnormalities have been reported in these mice (Leitges et al., 1996; Kishi, Pan et al. 2005, Fruman, Snapper et al. 1999; Terauchi, Tsuji et al. 1999; Chen, Mauvais-Jarvis et al. 2004). The *Prkcb1* expression pattern is very interesting, as it appears to be restricted at least at early stages of limb development to the future carpal region (see Fig. 13). If it is indeed the carpal region would have to be further investigated and it would be interesting to see what the expression looks like at later stages. Nevertheless its early expression pattern excludes it as a potential factor X, since this factor should be expressed similarly to the transcription factors Tcf1 and Lef1 in the sub-ectodermal mesenchyme of the early limb bud. Pik3r1 is a modulator of the phosphatidylinositol 3-kinase (Pik3), which is a central intracellular signaling component. It has so far been implicated to play a functional role in osteoclast maturation, but not in chondrogenesis/ostegenesis (Munugalavadla, Vemula et al. 2008). Knock out mice were not documented to show a skeletal phenotype (Fruman, Snapper et al. 1999; Terauchi, Tsuji et al. 1999; Luo, McMullen et al. 2005); however the PIK3 pathway has been shown to be linked to Wnt-signaling via activation of GSK3 (Naito, Akazawa et al. 2005). Of interest is also the observation that Pik3r1 can interact with β-catenin and increase its activity as a transcriptional cofactor (Espada, Peinado et al. 2005).

For the inhibitory G alpha subunit Gnai1 only few studies have been published yet, primarily focusing on a neurobiological context and for the targeted knock-out no skeletal phenotype has been described (Pineda, Athos et al. 2004; Kishi, Pan et al. 2005). The same is true for Brsk1 which also was investigated in a neurobiological context and where for targeted knock outs no skeletal phenotype was described (Kishi, Pan et al. 2005).

Of the transcription factors Foxo3a can probably be ruled out as acting as factor X since its expressed in the wild-type limb in a pattern congruent to Sox9 (Fig 16). Furthermore, I could not detect a broad upregulation by *in-situ* hybridization only by RT-PCR. But Foxo3a could in principle still be involved in the modulation of Sox9 expression in a positive or negative fashion or play a role in skeletogenesis in a different way. In support of the latter a recent publication using chondrocyte specific expressed constituvely active or dominant negative Akt has implicated a role for Foxos in skeletogenesis acting downstream of Akt signaling to promote chondrocytes proliferaton (Rokutanda, Fujita et al. 2009). However such a role in skeletogenesis is not supported the *Foxo3a* knock-outs, where no skeletal phenotype has been reported (Castrillon, Miao et al. 2003; Hosaka, Biggs et al. 2004). This could be due to redundancy between Foxo1, Foxo3a and Foxo4 as they show overlapping expression (Furuyama, Nakazawa et al. 2000) and are regulated by Akt (Brunet, Bonni et al. 1999).

Another factor implicated by the transcriptome analysis and confirmed in *in-situ* hybridisation and RT-PCR was Runx1. It has been shown to promote chondrocytic differentiation (Wang, Belflower et al. 2005) and was shown to have similar expression pattern as Runx2 in chondroprogenitors of the axial skeleton (Smith, Dong et al. 2005). Interestingly, *Runx2* has already been shown to be a target of Wnt-signaling in chondrocytes (Dong, Soung do et al. 2006). Further investigations as Luciferase assays and ChIP experiments could be used to establish if *Runx1* is also regulated by Wnt-signaling.

Eya3 was another transcription factor implicated by the primary screen; however the secondary screens did not show a differential expression. What makes this factor interesting is that section *in-situ* hybridisations revealed strong subectodermal expression in the wild-type limb (see Fig. 16). This makes it a candidate to be regulated by Wnt/Fgf as proposed in the model by ten Berge and colleagues (ten Berge, Brugmann et al. 2008). Real-time PCR would be sufficiently sensitive to

exclude a regulation by canonical Wnt signaling. Interestingly the Eya3 knock-out mice showed a phenotype with decreased bone mineral content and shorter body length (Soker, Dalke et al. 2008), but so far no further studies are published investigating the underlying mechanisms for this effects.
As the remaining transcription factors Fra2, Irx5 and Tcf4 have been investigated in further detail they are discussed more extensively in the following.

3.3 Fra2 is probably not directly regulated by canonical Wnt signaling

Fra2 in the wild-type was not significantly expressed in the limb bud mesenchyme and was only up-regulated in some distal patches upon stabilization of β-catenin. The up-regulation was further confirmed by RT-PCR. However, on the basis of the *Fra2* luciferase reporter assay I would conclude that it is probably not a direct target of the canonical Wnt-pathway, although I can not completely rule it out, given that the promoter we tested is only a 1,9 kb region and additional TCF/LEF binding site were present in the up-stream region of this construct. Despite the local restricted up-regulation we had decided to have a closer look at Fra2, particularly as other members of the AP1 complex have been indicated as target gene of Wnt-signaling (Mann, Gelos et al. 1999). Furthermore Fra2 was of interest as it already has been shown as a factor in chondrogenesis (Karreth, Hoebertz et al. 2004).

3.4 Irx5 is upregulated by canonical Wnt-signaling and can repress Sox9 in vitro

In *in-situ* hybridizations the Iroqouis box transcription factor, *Irx5*, showed a clear upregulation in β-catenin GOF limbs (Fig. 14). This was furthermore confirmed by luciferase reporter assays that showed Lef1/Tcf1 plus β-catenin to be sufficient to induce transcription from a 1,9kb part of the *Irx5* promoter. Section *in-situ* hybridizations of wild-type limb showed a pattern of subectodermal expression (Fig. 16 B) in addition to the proximal expression domain (Fig. 16 A) that was already documented by Houweling and colleagues (Houweling, Dildrop et al. 2001). As Wnts are thought to be secreted from the ectoderm – this subectodermal expression together with the upregulation upon β-catenin stabilization and luciferase assays

presents good evidence that *Irx5* may be activated by β-catenin/Wnt signaling during limb development.

Yet among the mammalian Irx genes *Irx3* has been shown to be inducible by Wnt3a in neural tissue (Braun, Etheridge et al. 2003). Interestingly Irx3 has an overlapping expression pattern to Irx5 in the developing brain as well as in limb buds (Houweling, Dildrop et al. 2001). Further support for the observation that Wnt-signaling could activate Irx transcription factors comes from Gomez-Skarmeta and colleagues who observed an upregulation of the *Xenopus* Irx homolog *Xirx1* when they injected a dominant negative form of Gsk-3 or wild-type β-catenin into the animal pole of Xenopus embryos (Gomez-Skarmeta, de La Calle-Mustienes et al. 2001).

In addition we tested the effect of Irx5 on *Sox9* expression. These luciferase assays showed a strong repressive effect of Irx5 on the 2,9kb *Sox9* promoter construct. The repressive effect is not entirely surprising as Irx proteins have been described to act primarily as transcriptional repressors (Gomez-Skarmeta, de La Calle-Mustienes et al. 2001; Kudoh and Dawid 2001; Itoh, Kudoh et al. 2002; Matsumoto, Nishihara et al. 2004) and only rarely as activators (Bao, Bruneau et al. 1999; Matsumoto, Nishihara et al. 2004).

Irx3 and *Irx5* overlap in their expression pattern in the limb. A similar situation was observed in different tissues also for the other paralogue gene pairs of the cluster *Irx1/Irx2* and *Irx4/Irx6* (Houweling, Dildrop et al. 2001). This indicates that they share enhancers for the same tissues and due to their high conservation could functionally replace each other (Cavodeassi, Modolell et al. 2001; Houweling, Dildrop et al. 2001).

So far only *Irx2*, *Irx4* and *Irx5* targeted knock outs have been published. *Irx5* knock-out mice showed aberrances in the nervous system, eyes and reduced size but a skeletal patterning defect has not been documented (Cheng, Chow et al. 2005). Due to the overlapping expression patterns of *Irx3* and *Irx5* a study of their function may require an *Irx3/Irx5* double knock-out. In general the study of Irx genes in the limb would be very interesting as they show a very well defined pattern during limb development (Houweling, Dildrop et al. 2001) and are proposed to be important for the determination of developmental territories (Cavodeassi, Modolell et al. 2001). To our knowledge no comprehensive studies of *Irx* gene functions in limb development are published.

3.5.1 Tcf4 is positively regulated by Wnt/β-catenin and represses Sox9 in vitro

As described above Tcf4 encodes for 2 major isoforms Tcf4a and Tcf4b. An isoform unspecific *in-situ* probe indicated a clear upregulation upon β-catenin stabilization (Fig. 13). Section *in-situ* hybridization revealed that the wild-type expression is restricted to subectodermal regions of the developing limb (Fig. 16 A). This pattern is to some extent congruent to the region under influence of subectodermal Wnts as proposed in the model by tenBerge et al (see Fig. 5).

As in the literature Tcf4a had already been suggested to be a direct target gene of Wnt-signaling (Kolligs, Nieman et al. 2002) all further investigations were focused on this longer isoform. In luciferase assays using a 2,3kb part of the *Tcf4a* promoter we could confirm *Tcf4a* as a potential direct target gene of Wnt signaling.

More interestingly, overexpression of Tcf4a was able to repress luciferase expression driven by the 2,9kb *Sox9* promoter, showing that Tcf4a has the potential to repress *Sox9 in vitro*; however this effect was less significant then for Irx5. This repressive effect is in concordance with previous studies that described Tcf4a as a factor involved in gene repression depent on its binding partner (Skerjanc, Truong et al. 1996; Petropoulos and Skerjanc 2000). As a slight additive repressive effect of Tcf4a in combination with Tcf1 was observed it is possible that in the limb Tcf4a in combination with Tcf1 might act in the repression of *Sox9*. This would have to be established in further in vitro and in vivo studies in order to assess the biological relevance of this finding.

3.5.2 Tcf4a overexpression leads to a limb phenotype

To investigate the *in vivo* function of Tcf4a the generation of transient transgenic embryos was planned. As described one transgenic embryo was obtained, which showed a limb phenotype but due to end of the diploma work I was not able to carry out any further analysis of the transgenic embryo or to reproduce the result. However, *in-situ* hybridizations that were carried out by Hermann Novak in the mean-time on limb sections of this embryo revealed that *Sox9* and *Col2aI* levels did not show a significant downregulation, which one would have expected if Tcf4a is a negative regulator of *Sox9*. Therefore these preliminary results suggest that Tcf4a

can most likely be excluded to act as an inhibitor of *Sox9* on its one. However, there is still the possibility that Tcf4a together with another transcription factor, which is up-regulated upon stabilization of β-catenin, represses *Sox9*.

A possible explanation for the observed phenotype could be the inhibition of cell proliferation. Support for this hypothesis comes from a recent study by Herbst and colleagues, which found that Tcf4a and Tcf4b can act as p53 independent positive regulators of the cell cycle inhibitor p21 in colon cancer cell lines (Herbst, Bommer et al. 2009; Herbst, Helferich et al. 2009). This would stand in contrast to the model by tenBerge et al. as Tcf4a is expressed in the proliferative region of the wild-type limb bud (Fig. 3). However; the growth retardation also could be due to secondary effects of the overexpression and would need to be reproduced.

Targeted knock outs of *Tcf4* have so far not revealed skeletal patterning defects (Zhuang, Cheng et al. 1996; Bergqvist, Eriksson et al. 2000). Nevertheless Tcf4 is potentially involved in skeletogenesis, as in the human Pitt-Hopkins Syndrome, which is caused by mutations in the *Tcf4* gene, aberrant thumb morphology has been observed in some cases. Additionally many patients suffer from a small stature (de Pontual, Mathieu et al. 2009).

In summary it can be concluded from the work presented that Tcf4 most likley is a target gene of Wnt-signaling in limb development and that its overexpression leads to a strong phenotype. Further studies will be necessary to reveal the functional details. In future work it will be important to carry out assays discriminating between the two isoforms. Another potential challenge in revealing the TCF4 function in limb development is that bHLH-factors often act as heterodimers and that the identification of its putative limb mesenchyme specific binding partner(s) is all but a trivial task.

4 Material and Methods

4.1 Solutions and Buffers

BM Purple AP substrate (Roche)

50x Denhardt's: 1% (w/v) Ficoll 400
1% (w/v) Polyvinilpyrrolidone
1% (w/v) BSA
in MPW
Filter and store at –20 ℃.

Glycerol Mounting Medium (Dako)

2X HBS: 8g NaCl
0.2g Na2HPO4-7H2O (0.14g if using dihydrate; [phosphate] must be 1.5 mM)
6.5 g HEPES
pH to 7.05 and bring up to 500 ml with distilled water.
Aliquot and store at –20℃.

HISS Sheep Serum, heat inactivated at 56C for 30'.
store at –20 ℃

Hybridization solutions

Section In-situ:	10mM Tris pH7.5
	600mM NaCl
	1mM EDTA
	0.25% SDS
	10% Dextran Sulfate
	1x Denhardt's
	200ug/ml yeast tRNA(Gibco)
	50% formamide
	Store at –20℃
Whole mount ISH:	50% Formamide
	5x SSC, pH 4.5 (stock: 20x)
	50 µg/ml tRNA (stock: 10mg/ml)
	1% SDS (stock 10%)
	50 µg/ml heparin (stock: 100mg/ml)
	store at -20℃
2x MABT:	200mM Maleic Acid
	300mM NaCl
	add 10M NaOH to adjust pH to 7.5
	0.2% Tween-20
MPW-DEPC:	Add 1ml DEPC/1l MPW, incubate oN and autoclave
NTMT pH9.5:	100mM NaCl
	100mM Tris pH9.5
	50mM MgCl2
	0.1% Tween-20
10 x PBS:	1.37M NaCl
	27mM KCl
	100mM Na2HPO4
	20mM KH2PO4 in MPW

PBS-DEPC:	100ml 10x PBS + 900ml MPW-DEPC
PBT:	1X PBS + 0.1% (section) or 1% (whole mount) Tween
10xSSC:	3M NaCl 0.3M sodium acetate pH to 7.0 (NaOH)
20x SSC pH 4.5:	6M NaCl 0.6M sodium acetate adjust pH 4.5 with citric acid
Solution I:	*50%* Formamide 5x SSC, pH 4.5 1% SDS
Solution III:	50% Formamide 2x SSC, pH 4.5
10x TAE:	4mM Tris-acetate 1mM EDTA pH 8.0
10x TBE:	1M Tris base 880mM Boric acid 40ml 0.5M EDTA (pH 8)
1X TBS:	50 mM Tris.HCl, pH 7.4 150 mM NaCl
TBST:	1X TBS 1% Tween
1x TE:	Tris-EDTA-Buffer, pH 8.0
10xTEA:	1M Triethanolamine pH 8.0

10X TNE Buffer: 100mM Tris
2.0M NaCl
10mM EDTA; pH 7.4

Yolk Sac Buffer: 50 mM Tris (pH 8.0)
100 mM EDTA
0.5% SDS
1.6 μl of fresh 10 mg/ml Proteinase K

Media

DMEM High Glucose (in house) + L-Glutamine, Penicillin/Streptavidin, 10% Foetal Bovine Serum, + 1% non-essential Amino-Acids

LB: Add the following to 800ml H_2O:
10g Bacto-tryptone.
5g yeast extract.
10g NaCl.
Adjust pH to 7.5 with NaOH.
Adjust volume to 1L with dH_2O
Sterilize by autoclaving

Mouse Strains

To obtain conditional β-catenin gain-of function mice (β-cat$^{\Delta ex3Prx1/+}$) Prx1-Cre females (Logan, Martin et al. 2002) were crossed with homozygous exon3 floxed β-catenin females (Harada, Tamai et al. 1999). Embryos were identified by visible changes in the limb morphology.

293T Cell Line:

293T is a modified human embryonic kidney cell line that contains the SV40 large T-Antigen, which allows replication of plasmids containing the SV40 origin. (Graham,

Smiley et al. 1977). Cells are cultured in the high glucose DMEM media as described above at 37 ℃, 5% CO_2. Cells were split 1:20 when confluent (about every 4 days).

4.2 Analysis of Affymetrix Gene Expression Data

Data Analysis was carried out by use of Microsoft Access. P-values of 0,01-0,05 which are usually used for this kind of analysis only gave very few candidate genes therefore the threshold for the p-value was set to <0,2. The threshold for the logarithmic fold change was set to >0,4 to include also genes that are close 0,5. These parameters were applied to the combined datasets of Affymetrix Lyashenko GOF14h/Kostanova GOF24h and Lyashenko GOF14h/LOF36h, respectively. These results were then filtered for genes with a putative function in cell-signaling or transcriptional control by connecting the Affymetrix IDs to the Gene Ontology Molecular Function Database (Ashburner, Ball et al. 2000) and using a query for the terms: transcript* (*=transcriptional activator, transcriptional inhibitor, transcriptional regulator etc), Kinase, Phosphatase, GTP, GEF.

4.3 In-situ Hybridizations

Template Generation

Most *In-situ* probes used in this work could be created by using the corresponding clones from the Riken library provided by the IMP-Biooptics department. The cDNA-sequences were obtained from ENSEMBL.org (Hubbard, Aken et al. 2009), and suitable Riken clones were identified by Blast-search in the Fantom Riken online database (Maeda, Kasukawa et al. 2006). Importantly, it was avoided to include conserved domains for all the probes generated. Those were identified using the conserved domain database from NCBI (Marchler-Bauer, Anderson et al. 2009).

A total of 14 probes with no corresponding clones in the Riken library, or for which Riken clone for other reasons could not be used were generated by PCR. For this purpose, cDNA from wild-type E15,5 whole embryo was used. Primers were designed to give products of 400-500bp using the Primer 3.0 program (Rozen and

Skaletsky 2000). Again the regions were checked for conserved domains. PCR-conditions were equal to Semi-Quantitative PCR described below. Primer sequences are shown below.

Stock #	Gene	Sequence
HAR2066	Mobkl1a fw	ATGAGCTTCTTGTTTGGTAGTCG
HAR2067	Mobkl1a rev	GTTTGTTCCATCTGCCCAGT
HAR2068	Rims2 fw	TCCTCCCTAGTAGATCCAACC
HAR2069	Rims2 rev	CTTTGCTAGGGCACAATGTG
HAR2070	Abca9 fw	GGAAGAGACACTTGAGACTGG
HAR2071	Abca9 rev	CCAGAAAGCCGACTCTCGA
HAR2146	Gm967 fw	ACCAGGAGCAGGAGTCTTCA
HAR2147	Gm967 rev	TTCATGAATGTCCGGAGTCA
HAR2148	Flt4 fw	CTGTACCTGCAGTGCGAGAC
HAR2149	Flt4 rev	CAGAGCTAACCCAGCAAAGG
HAR2150	Abcb7 fw	GGTGCCCTTACTGTTGGAGA
HAR2151	Abcb7 rev	CGAAGACTTTCCAGGCTCAC
HAR2154	Pik3c2g fw	CTGGCCAGGGTGAGTGTAAT
HAR2155	Pik3c2g rev	CGAAGAGCCGAGATGGATAG
HAR2156	Dusp19 fw	GGGAAGATGCCACAGTTCAT
HAR2157	Dusp19 rev	GGATTCGGACATATGGATGG
HAR2158	Brsk1 fw	TCGGGAATTTCATCTCCTTG
HAR2159	Brsk1 rev	GGCTCCGTTCTTCTCATCTG
HAR2160	Mkl2 fw	ACCCCAGCAGTTTGTTGTTC
HAR2161	Mkl2 rev	TGGAATGACTCAGCAAGTCG
HAR2162	Foxd4 fw	CTTGGGAGAGAGACCAGAGC
HAR2163	Foxd4 rev	TTAAAATTCGGGCAAGGTC
HAR2164	Mdn1 fw	GAGGAACCGTGGACTTGAAA
HAR2165	Mdn1 rev	GGACAACCGAGAGTCGAGAG
HAR2220	p300 fw	GAACGCCTCAGGAACAGGTA
HAR2221	p300 rev	CCCCTGATCATCTACCATGC
HAR2222	Rerg fw	TTGAAGATGCAACCACCAAA
HAR2223	Rerg rev	GTGAATTGCGGGTCTTGTTT

The PCR-products were ligated into pGEM-T-easy (Promega) and sequenced to confirm their identity and to determine the orientation of the insert with respect to the backbone. Details on the template, restriction enzyme, polymerase and probe length are shown in the table below.

Gene	Template	Polym.	Restriction enzyme	Probe Length
Abca13	A930002G16	T3	NdeI	469
Abcb7	PCR	Sp6	NcoI	428
Abra	C130068O12	T3	EcoRI	371
Akap5	A830007p05	T3	CfoI	450
Arl4c	A930033L07	T3	XbaI	1kb
Brsk1	PCR	Sp6	NcoI	458
CamK4	A730057B21	T3	HindIII	760
Cdkn2b	I920040F24	T3	CfoI	409
Depdc2	A430104n04	T3	NcoI	421
Dusp19	PCR	Sp6	NcoI	466
Epha3	B130048J04	T3	BglII	1kb
Erbb4	F530209F06	T3	XhoI	367
Etv4	G730044E08	T3	CfoI	1kb
Eya3	G730019b10	T3	BglII	820
Flt4	PCR	Sp6	NcoI	422
Foxd4	PCR	Sp6	NcoI	431
Foxo3a	E130305O06	T3	NdeI	324
Fra2(Fosl2)	F730016C03	T3	NheI	1,1kb
Gm967	PCR	SP6	NcoI	450
Gnai1	6330599a21	T3	NdeI	791
Gyk	2010011F10	T3	NcoI	506
Hoxb7	9230026O03	T3	BglII	288
Irx5	1200013N09	T3	BglI	929
Lhx6	C730046L05	T3	XbaI	860
Lipn1	F630108j21	T3	SpeI	415
Ly6g6e	2310011I02	T3	CfoI	900
Mdn1	PCR	Sp6	NcoI	455
Mkl2	PCR	T7	SalI	488
Mobkl1a	PCR	T7	SalI	1,3kb
Nr3c1	F830016H14	T3	HindIII	1,1kb
Pcaf	PCR	SP6	NcoI	500
Pik3r1	c53005k14	T3	Xba1	958
Pik3c2g	PCR	T7	SalI	405
Prkcb1	D230012a21	T3	EcoRI	920

Gene	Template	Polym.	Restriction enzyme	Probe Length
Prps2	B130019b02	T3	HindIII	593
Ptprn	A530097K05	T3	CfoI	510
Rabep1	6330437j23	T3	NheI	421
Rerg	PCR	Sp6	NcoI	723
Arhgap	I920042B01	T3	BglII	1,4kb
Rims	PCR	SP6	NcoI	650
Runx1	G830041K20	T3	NheI	1,6kb
Sgms1	C530027b15	T3	NcoI	1,1kb
Srpk3	1200011B22	T3	NcoI	1,3
Tcf4	Har 1025	T3	BglII	603
Tcf23	5031411E05	T3	BamHI	534
Utf1	2410026A04	T3	NheI	1,1kb
Wif1	HAR987	SP6	XhoI	500

From these templates 10 µg of DNA each were digested for 2h at 37 ℃ in a volume of 100µl with the appropriate enzyme (see table above) and restriction buffer to linearize the plasmid. After a phenol-chloroform extraction (1/10 Volume 3M NaAc pH 5,4 and 1 volume PCI-Mix pH 8) the aqueous phase was transferred into a new tube and DNA precipitated with 100% EtOH. After centrifugation the pellet was washed with 70% EtOH, dried, and resuspended in 10 µl MPW. Prior to precipitation 1µl of the template was checked on a gel to control the completeness of the digestion.

Probe preparation

From 1µg of the linearized DNA the RNA probe was prepared by addition of a mix of 2µl DIG-labeled nucleotide mixture (Roche), 2µl 10x Transcription Buffer (Roche), 0,5µl RNase inhibitor and 13µl MPW. Finally 1,5µl of the appropriate RNA-polymerase (as indicated in the table above) were added and the mix was incubated for 2h at 37 ℃. 1 µl of the product was checked on a TBE-gel. The DNA-template was digested by adding 1µl DNase (Roche) and incubated for 15' at 37 ℃. The RNA was then purified by LiCl/EtOH precipitation: 1µl Glycogen (Roche), 8µl LiCl-DEPC (5M), 100µl 1M TE-DEPC pH 8, 300µl EtOHabs (-20 ℃) were added to the RNA.

Precipitation was incubated for 1h or over night at -20 ℃. After washing with 70% EtOH-DEPC to remove excess salt on the next day the pellet was dried and resuspended in 100 µl MPW-DEPC.

Section *In-situ* Hybridisations

Embedding

Embryos were dissected in PBS and fixed over night in 4% PFA/PBS at 4 ℃. Embryos were then washed 2 times in PBS for 10 min and afterwards dehydrated in a series of ethanol (25% EtOH/PBS, 50% EtOH/PBS, 75% EtOH/MPW, and 100% EtOH) for 10 min each. The embryos were either stored at -20 ℃ or directly processed. For the transfer they were incubated in 100% EtOH for 1 hour and then cleared in Xylene two times for 10-30 minutes. Afterwards embryos were incubated in a 1:1 mix of Xylene/paraffin for 30 minutes at 60 °C, then for 3 times for 1 hour in paraffin at 60 ℃. The last step can also be elonga ted to overnight. The samples were then ready for embedding and cutting. Sections were cut on a microtome in 5µm sections.

Hybridisation & Staining

The slides were dewaxed by incubating them two times for 5’ in Xylene and afterwards were rehydrated by incubation in an EtOH/PBS-DEPC dilution series from 100% to 25% and finally PBS-DEPC (each for 5’). The sections were fixed with 4% PFA/PBS (10’), washed with PBT-DEPC (1x rinsed then incubated two times for 5’) and treated with ProteinaseK for 10’ (20µl from 10mg/ml in 200ml PBS-DEPC). After two washing-steps with PBT-DEPC for 5’ each, the slides were fixed again with 4%PFA/PBS for 5’, washed with PBT-DEPC (1x rinsed, then two times 5’) and afterwards acetylated by incubating them in 250ml of 0,1M TEA with 625µl Acetic Anhydride. The slides were then washed 3 times with PBT-DEPC (1x rinsed then two times 5’) and dried in the oven at 50 ℃.

1µl of the DIG labelled probe was mixed with 100µl hybridization solution/slide and incubated at 85℃ for 3’ to solve secondary structu res. The mix was then pipetted on

the slides and a coverslip was put on. Afterwards the hybridisation cassettes containing the slides were incubated over night at 65 ℃.

After the hybridization the coverslips were removed from the slides by rinsing them in 5x SSC then the slides were put in 1x SSC/50% Formamide for 30' at 65 ℃. As the next step the slides were incubated with TNE for 10' and treated with TNE+RNase A (20 μg/ml) for 30' at 37 ℃. Subsequently they were tran sferred in TNE and incubated for 10' at 37 ℃ followed by a 2x SSC and two 0,2 S SC incubations for 20' at 65 ℃ each.

Slides were then incubated two times in MABT for 5' at RT. For blocking a 20% HISS/MABT-mix was applied to the slides for 1h at RT. Afterwards the slides were incubated with α-DIG-AB (1:2000 Roche) in 2% HISS/MABT at 4 ℃ over night in a humidified box.

The next day slides were rinsed in MABT, washed three times for 5' each in MABT and then incubated with NTMT pH 9,5 for 10' at RT. BM-Purple AP substrate (Roche) was added for detection. The staining progress was observed following the color reaction by regular checks on the microscope. When sufficient staining was achieved slides were rinsed in NTMT pH 9,5 and washed two times in PBS for 5' at RT. For post-fixation slides were incubated with 4%PFA/PBS for 10' and then rinsed in PBS and dH_2O. DAKO-Glycergel was used for mounting.

Whole-Mount *In-situ* Hybridisations (WM-ISH)

For the WM-ISH embryos between E11.5 and E12 were used. With respect to the ex3 fl/fl mouse colony, I divided the embryos into two halves to be able to use 2 probes using only embryo, since there was a heavy demand for these embryos/mice in our lab.

After the harvest embryos were fixed in 4% PFA and on the next day dehydrated in a series methanol/PBT (25%, 50%, 75% 100% Methanol 5' each). Embryos were stored until usage at -20 ℃.

Whole mount staining procedure was carried out using glass scintillation vials. At the beginning of the procedure embryos were rehydrated in the reversed series of methanol/PBT used for dehydration. Embryos were then washed in PBT two times for 5'. For bleaching they were incubated with 6% H_2O_2/PBT for 1 hour at room temperature.

After the bleaching embryos were again washed 3 times in PBT for 5’ and then treated with ProteinaseK to improve their permeability in the staining procedure. The length of the treatment is dependent on the stage of the mouse embryos. For the stage of E11.5 treatment of 15’ is recommended, but depends on the activity of the batch of ProteinaseK. For detection of ectodermally expressed genes its advisable to reduce length of treatment. Treatment was carried out using a concentration of 10µg/ml ProteinaseK in PBT. To stop the ProteinaseK digest embryos were incubated for 10’ in 2mg/ml glycine in PBT. Followed by two washing steps with PBT for 5’. before re-fixation of embryos with 4%PFA/0,2% glutaralaldehyde in PBT for 20’, which was followed again by two washing steps in PBT for 5’ each.

Subsequently embryos were incubated for 10’ in a 1:1 mix of hybridisation solution and PBT. Then the embryos were incubated for another 10’ in hybridisation solution at room temperature before the pre-hybridisation at 70 ℃ for 1 hour. For hybridisation 1ml of hybridisation solution was mixed with 5µl of the DIG-labeled RNA probe and embryos were incubated over night at 70 °C.

To wash away unspecifically bound RNA probe three washing steps for 30’ at 70 ℃ in solution I and three washing steps in solution III were carried out for 30’ at 65 ℃ each. The embryos were then washed 3 times for 5’ in TBST.

While the washing steps were carried out, a preabsorption mix for the antibodies was prepared. Herefore mouse embryo powder - app. 5mg/500µl TBST and 500µl TBST/*In-situ* reaction – was incubated on a heatblock at 70 ℃ a nd 700rpm. The mix was then cooled on ice and 10µl HISS, 1µl anti-DIG antibody, 200µl BM blocking reagent and 290µl TBST were added per 500µl embryo powder solution and incubated for 1h at 4 ℃. The mix was then centrifu ged for 4’ at 8000rpm to separate embryo powder from the supernatant. The supernatant from all Eppendorf tubes was then pooled and 200µl HiSS, 400µl BM blocking reagient and 1400µl TBST were added per 500µl of the initial volume. This leads to a total volume of 2ml/*In-situ* vial with an antibody dilution of 1:2000. 2ml of this mixture were added to the embryos after the last TBST washing step and vials were incubated over night at 4 ℃.

On the next day embryos were washed with TBST three times for 5’, 5-8 times for about 1h at room temperature and then overnight at 4 ℃ to reduce unspecifically bound antibody.

For the staining reaction embryos were washed 3 times in NTMT for 10‘ and then incubated in BM Purple AP substrate in the dark. When the reaction was judged

complete by visual inspection embryos were washed twice in NTMT and twice in PBT for 5' each, then post-fixed in 4% PFA/0,1% glutaraldehyde and stored in PBS.

4.4 Total RNA-Isolations from embryonic mouse limb buds

Embryos were harvested at E11.5 and limb buds dissected. PBS was sucked off, the tissue shock-frozen in liquid nitrogen and stored at -80 ℃ until further usage.
0,2 ml Trizol were added and the tissue homogenized with a micro pistil in a 1,5 ml Eppendorf tube. After homogenization 0,8 ml of Trizol were added and the samples incubated for 5' at room temperature. After addition of 0,2 ml of chloroform and inverting for 15 sec the sample was again incubated for 2' and then centrifuged at 4000 rpm, at 4 ℃ for 15 '. The upper layer was transferred into a new tube and 0,5 ml of isopropanol were added to precipitate the RNA. After 10' incubation at RT the RNA was pelleted by centrifugation at 4000 rpm, 4 °C for 10'. The pellet was then washed with 75% ethanol; vacuum dried and resuspended in DEPC treated water. To ensure complete resuspension tubes were incubated at 55 ℃ for 10' and afterwards the RNA quantified by photometer measurement.

4.5 First Strand cDNA synthesis

For synthesis of cDNA 1µg of total RNA was used. 1 µl of oligo dT (HAR 068; 500ng/µl), 1µl dNTPs (10mM; Eppendorf) were used per reaction and dH2O was added to a volume of 10µl. To denature the RNA samples were heated at 65 ℃ for 5' and cooled on ice for 5', then 4µl of 5X buffer FS (Invitrogen-Y00146), 2µl of 0,1M DTT (Invitrogen-Y00147) and 1µl of RNase inhibitor (Promega-N211A 16546404) were added, the samples mixed by pipetting and incubated at 42 ℃ for 2' before 1 µl of RT-superscript II (Invitrogen-C#18064-022) was added. The samples were then incubated for 50' at 42 ℃. To inactivate the reaction, samples were heated for 15' at 70 ℃. 1 µl of RNaseH was then added and samples incubated for 20' at 37 ℃ to digest the RNA template strand. cDNA was stored at -20 ℃ until use. For all samples the –RT control was prepared in the same way, without the reverse transcriptase.

4.6 Semi-quantitative RT-PCR

To ensure equal amounts of cDNA as template loading contros were performed amplifying the housekeeping gene Glyceral-Aldehyde-3-Phosphate (Gapdh) a central component of glycolysis. For better visualisation of alterations in the expression ratio between the WT and GOF a 1:5 dilution was used in addition to undiluted cDNA. The PCR conditions and primers are shown below:

PCR-Mix

Takara Taq (RR001A) (5 units/µl)	0,2µl
10x PCR Buffer (incl. 1,5 mM $MgCl_2$)	2µl
dNTP Mixture (2,5 mM each)	2µl
Template	1µl for Gapdh, 3µl all other genes
Primer 1 (10 pM)	1µl
Primer 2 (10 pM)	1µl
Milli Q H_2O	Up to 20µl

PCR-Program

95 ℃		5’
94 ℃		45’’
60 ℃	**29 Cycles (Gapdh), 34 cycles (all other Genes)**	45’’
72 ℃		40’’
72 ℃		5’
4 ℃		

Primers

All Primers for semi-quantitative PCR were designed using Primer3 (Rozen and Skaletsky 2000) and span at least 1 Intron/Exon boundary. Product lengths varied between 400-500bp (Gapdh: 700bp). In addition PCRs were carried out for all primers at the same conditions however with the –RT control to test for genomic DNA contaminations.

Stock #	**Gene**	**Sequence**
HAR84	Gapdh fw	CAAATGGGGTGAGGCCGGTGCTGAGTAT
HAR85	Gapdh rev	CGGCATCGAAGGTGGAAGAGTGGGAGTT
HAR1888	Pik3r1 fw	TGACGAGAAGACGTGGAATG
HAR1889	Pik3r1 rev	CATCGCCTCTGTTGTGCAT
HAR1919	Foxo3 fw	CTGTCCTATGCCGACCTGAT
HAR1920	Foxo3 rev	CCTCGGCTCTTGGTGTACTT
HAR2033	Dusp19 fw	GTTCCTTGACGAAGCTCTGC
HAR2034	Dusp19 rev	AGTCGTCTCCATCCTGTGCT
HAR2122	Tcf4 fw	TCATATCCCACAGTCCAGCA
HAR2123	Tcf4 rev	TTCCAAACGGTCTTCGATTC
HAR2124	Wif1 fw	CTCCCTGGATAAAGGCATCA
HAR2125	Wif1 rev	TGGTTGAGCAGTTTGCTTTG
HAR2130	Eya3 fw	TTTCGCTTCAAGCACAAATG
HAR2131	Eya3 rev	CCGTTCCAATTCACTGTCCT
HAR2132	IRX5 fw	TACAGCACCAGCGTCATTTC
HAR2133	IRX5 rev	ACCTGGGTGAGGGTCATCTT
HAR2136	Prkcb1 fw	GGATCGCTGCTGTATGGACT
HAR2137	Prkcb1 rev	CCCATGAAGTCATTCCTGCT
HAR2138	Gnai1 fw	GGCAGTGGTCTACAGCAACA
HAR2139	Gnai1 rev	GATCCACTTCTTCCGCTCTG
HAR2140	Runx1 fw	CTTCCTCTGCTCCGTGCTAC
HAR2141	Runx1 rev	GCTGAGGGTTAAAGGCAGTG

After the PCR 3µl of loading buffer were added, the tubes were vortexed and centrifuged. Then 15µl of each sample were loaded on the gel.

4.7 Luciferase-Reporter Assays

For the luciferase reporter assays 293T cells were used. 24 hours before the transfection $8x10^4$ cells/well were seeded into 24 well plates.
For each condition tested 4 wells were transfected with the same trasfection-mix, therefore the mix was prepared for 4 wells at a time. First water was added in a calculated amount resulting in an end volume of 50µl/well. Then per well 0,5µg of the luciferase reporter construct and 0,05µg of the Renilla expression vector for normalization were added. The factors to be tested were added in concentrations as indicated in the results section, typically ranging from 0,25µg to 1µg per well. Finally 2M 7µl of $CaCl_2$ per well were added. All constructs used are shown in table below. The mixture for the 4 wells (200µl) was then vortexed and added drop wise to a 5ml polystyrene tube containing 200µl of 2x HBS pH 7,05, while bubbling with a 2ml plastic pipette. From this mix 100 µl were added to each well and cells were incubated for 24h at 37 ℃.

Luciferase measurements were made on the following day. The media was removed and 100 µl luciferase buffer (Promega Dual-Glo Luciferase Assay) mixed 1:1 with DMEM 1% FCS was added per well. The plate was incubated for 15‘ on a shaker and afterwards 50µl of the solution were pipetted on a 96-well plate. After measurement in the luminometer (Synergy Szabo-Scandic), 50 µl of the Dual-Glo & Stop (1:100) were added and incubated again for 10’ before the measurement. In addition a blank with Luciferase buffer (Promega Dual-Glo Luciferase Assay) mixed 1:1 with DMEM was treated the same way as the samples. All steps steps after addition of the Luciferase Buffer were carried out in the dark.

The blank was subtracted from the measured values and the signal of the Renilla was used to normalize the results. The average of the 4 wells was calculated if present the strongest outlier was not included in the calculation. For each condition at least 3 independent measurements were conducted and the average and standard error was calculated. For the p-value calculation a two tailed t-test for unpaired samples with same variance was used.

Stock #	Construct
722	Renilla expression vector
755	β-catenin expression vector
763	Lef1 expression vector
876	2,9kb Sox9-Promoter pGL4.10
923	TCF1 expression vector
924	Tcf1/β-catenin-fusion exp. vector
1056	TCF4A longisof in pcDNA3.1
1057	IRX5 in pcDNA 3.1
1058	FRA2 in pcDNA3.1
1059	2,5kb CyclinD1-Prmoter pGL 4.10
1060	2,4kb Tcf7l2-Promoter pGL 4.10
1062	2,3kb TCF4A-Promoter pGL4.10
1064	1,9kb IRX5-Promoter pGL4.10
1065	1,9kb Fra2-Promoter pGL4.10

4.8 Cloning work

Ligation

Ligation was carried out with Takara Ligation Kit according to the protocol supplied by the manufacturer.

Transformation in E.coli

80 µl competent DH5α cells were added to the plasmid, incubated for 15‘ on ice , exposed to heatshock at 42 ℃ for 1’ and incubated 5’ on ice before plating. As only AMP-resistance was used for transfections it was not necessary to recover cells in LB before plating.

Miniprep/Maxiprep

Qiagen Miniprep/Maxiprep and Promega Maxiprep kits were used for these purposes and all steps were conducted according to the standard protocol provided by the manufacturer.

Agarose gel and Gel elution

The PCR-products and restriction digests were separated on 1% Agarose gels and Qiaquick Gel Purification kit was used for purification according to the protocol provided by the manufacturer.

Isolation of genomic DNA

A mouse tail was incubated with 0,5ml of 50mM Tris (ph8), 100 mM EDTA, 0,5% SDS and 25 µl of a 10 mg/ml solution of proteinaseK at 55 ℃ over night. On the next day an extraction with 0,5 ml phenol:chlorophorm:isoamylalkohol (PCI/Sigma) (pH 8) was repeated for two times and to the remaining aqueous phase 50µl of 3M sodium acetate (pH 6) and 0,5 ml 100% Ethanol were added to precipitate the DNA. The DNA was then washed with 70% Ethanol, dried and resolved in 50µl TE. Successful DNA isolation was checked on a 0,8% agarose gel.

BAC-Isolation

For the Isolation of BACs a single colony was picked, inoculated to 200 ml of LB+Chloramphenicol (25 µg/ml) and grown overnight. For the preparation a Qiagen Midi Kit was used according to the standard protocol with the only modification that the elution buffer was heated to 65 ℃ for more efficient elution.

Verification of PCR-products

Taq amplified PCR-products were ligated into pGEM-T (Promega) using the A/T overlap generated by the polymerase and transformed into E.coli. The bacteria were then grown over night or for at least 8 hours before the plasmids were isolated by

miniprep. The inserts of the isolated plasmid DNA were then verified using an appropriate restriction strategy and if necessary sequenced.

PCR-Conditions

All PCRs for cloning purposes were carried out with the following PCR-Mix and program according to (Ralser, Querfurth et al. 2006) who propose the following enhancer mix and PCR-conditions.

	Single reaction Mix (25µl)
dntps 2,5mM Takara	2,5
10x Buffer incl $MgCl_2$ Takara	2,5
Primer1 10pM	0,5
Primer2 10pM	0,5
TAQ (Takara)	0,2
Template	1
bsa 1mg/ml	0,275
Dmso (Sigma-Aldrich)	0,335
Betaine (3M)	4,5
DTT 1M (Invitrogen)	0,335
Sum	12,145
dH20	12,855
Total mix	**25**

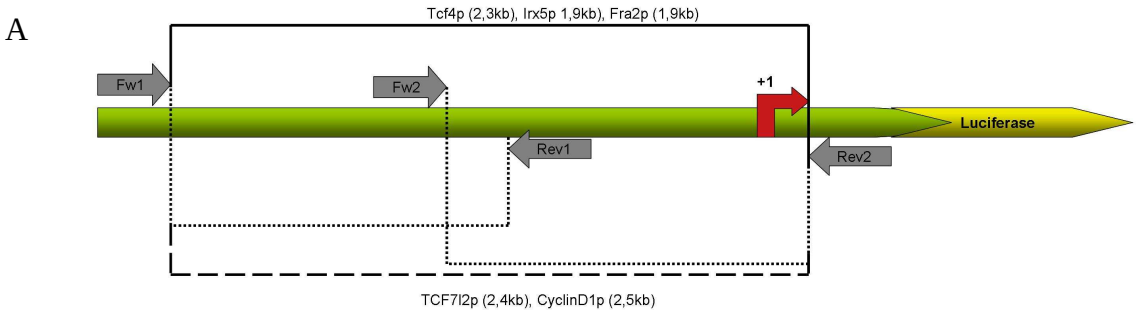
PCR-Program:

Initial denaturation	95 ℃	5’	
Denaturation	98 ℃	10’’	Touchdown phase 20 cycles
Annealing	62 ℃ -0,5℃/cycle	45’’	
Elongation	72 ℃	10’’ per 0,1 kb	
Denaturation	98 ℃	10’’	Amplification phase 15-20 cycles
Annealing	52 ℃	45’’	
Elongation	72 ℃	10’’ per 0,1 kb	
Final Elongation	72 ℃	5’	
Storage	4 ℃		

Cloning of promoter-fragments for Luciferase-Promoter Assays

All promoters in the thesis were cloned in a way that they include at least the first 30 bp of the transcript. This is to ensure that the transcriptional start site is within the construct and no minimal promoter provided by the luciferase vector was needed.
An overview of the cloning strategies including construct lengths and the Luciferase vector pgl4.10 is depicted in Fig. 29 A.
For CyclinD1 and Tcf7l2 the strategy was to ligate two overlapping PCR-products that contained a common endogenous restriction site. For the other genes primers were used that spanned the entire length as shown in Fig. 29 A. For ligation into pgl4.10 (Fig. 29 B) the necessary restriction sites were added to the primers or endogenous sites within the PCR-Products were used.

Stock#	**Gene**	**Purpose**	**Sequence**	**Comment**
HAR2166	Tcf4a	Rev2+XhoI	TTCTCGAGGGCACTGCGGCTTATAAAGA	
HAR2167	Tcf4a	Fw1	TCAGGCTGGCCTCTATGATT	Endogenous SacI site
HARXX!	Tcf4a	Sequencing	TGGGGGATAAAACACTGGAG	
HAR2212	Irx5	Fw1	GGACCAGAGCCGAGACACT	Endogenous SacI site
HAR2213	Irx5	Rev2+BglII	TTAGATCTCTCCCTCGCTCTTTGCAC	
HAR2231	Irx5	Sequencing	GAACTCTGCCTGGGACTCTG	
HAR2278	Fra2	Fw1	CCTGCAGAGCTGCACATTCT	Endogenous SacI site
HAR2279	Fra2	Rev2+Nhe1	AAGCTAGCCGCTCGCTCTCTTTCTCTCT	
HAR2283	Fra2	Sequencing	GCCTGCCCATTTTTCACTC	
HAR1600	Cdkn1	Fw1+Kpn1	TTGGTACCTTGGCTCCAGTCAGGTGG	
HAR1599	Cdkn1	Rev1	AGTTATTAGTCGCCCTTCC	
HAR1596	Cdkn1	Fw2	TGGAGAAACACCACCACC	
HAR1597	Cdkn1	Rev2+BglII	AAAGATCTGAGTCTGTAGCTCTCTGC	
HAR1602	Tcf7l2	Fw1+NheI	TTGCTAGCTCTCCCTCTTAGATACTGC	
HAR1603	Tcf7l2	Rev1	AACTGACGTTGGAAGTAGG	
HAR1604	Tcf7l2	Fw2	CTCCAGCTCTACTCATCC	
HAR1605	Tcf7l2	Rev2+HindIII	TTAAGCTTTCACCCACCAGCAGCAGC	



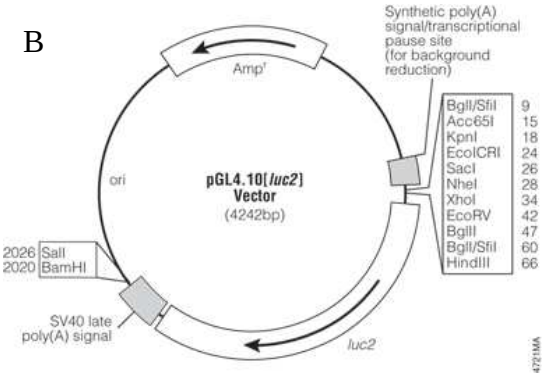


Fig. 29: Overview of the cloning strategies. On top 1 part PCR strategy on bottom 2 part PCR (A). Luciferase Vector Promega pgl 4.10 (B)

In case were I did not succeed to amplify the product by PCR using genomic mouse DNA as a template I used BAC-clones of the corresponding genomic loci as templates. BAC-IDs are shown in the table below

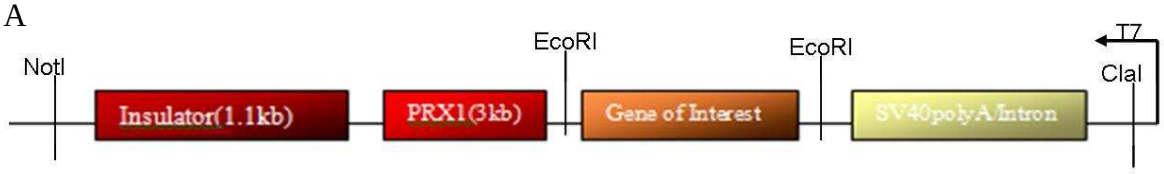
Construct	**Template**
Tcf4	Genomic WT-DNA
Irx5	RP24-69M8
CyclinD1	RP23-107I11
Tcf7l2	RP23-342F10
Fra2	RPCI-24-255I2

Cloning of cDNA-constructs

For the generation of functional cDNA clones containing the entire open reading frame (Luo, McMullen et al.) primers were targeted to the 5' and 3' regions neighbouring the coding region. This was particularly important as the 5' UTR contains the Kozak sequence (gccrccAUGG) which signals the start of translation to the ribosome (Kozak 1987) . Amplification of the full length cDNAs including the 3' UTR was not necessary as all the vectors used contained additional sequences providing the polyadenylation signals. Restrictions sites were added to the primers to facilitate integration of the resulting constructs into the following 2 vectors:

- An *in vivo* expression construct, for limb mesenchyme specific expression under the Prx1-promoter (Logan, Martin et al. 2002) (Fig. 30 A)
- pcDNA3.1(+) a vector for overexpression in tissue culture (Fig 30 B)

A



B

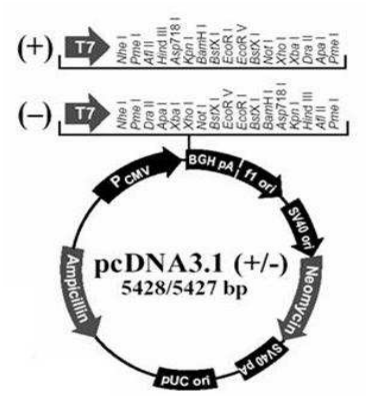


Fig. 30: Prx1-Promoter construct as received by M. Logan (A).

Invitrogen pcDNA3.1 expression vector (B)

Correct insertion into the Prx1 vector backbone was verified by sequencing from the T7 promoter in the backbone. The following primers were used for amplification of the constructs, with the PCR-program described above.

Primer	Gene+RE	Sequence	Construct Lenght
HAR2204	Fra2 fw+EcoRI,BamHI	TT**GAATTCGGATCC**GGGAAGAAAACCACCCTGTT	1,2kb
HAR2205	Fra2 rev+EcoRI	AA**GAATTC**TCGCTTGCAAGATGAATGAG	
HAR2206	Irx5 fw+EcoRI, Bgl2	AA**GAATTCAGATCT**AGAAGCCAGGTGCCCTCTC	1,6kb
HAR2207	Irx5 rev+EcoRI	AA**GAATTC**GCCAAGGCCATGGTTTTAAT	
HAR2209	Tcf4a rev+EcoRI	AA**GAATTC**AAACGGGGTTAAGGAGCAGT	2,3kb
HAR2216	Tcf4a fw+EcoRI,BamHI	TT**GAATTCGGATCC**CTCTTTTGGGGGAACACTCA	

After amplification the constructs were ligated into Promega pGEM-T-easy with using the A/T overlaps resulting from the Taq PCR amplification. A number of clones were sequenced to find error free coding regions. For Irx5 and Tcf4 the following sequencing primers were necessary to span the centre parts of the constructs.

HAR2224	Irx5	AGAAGATCATGCTGGCCATT
HAR2225	Tcf4a	TGCCATGGAGGTACAGACAA

For Tcf4a no clone without errors could be identified, therefore the error free 5', and 3' part originating from two different clones were combined using the internal XbaI site.

4.9 Preparation of Tcf4a plasmid for pro-nucleus injection

10µg of the plasmid were digested with NotI/ClaI (Fig 30 A) and separated on a gel. The 7kb band of the injection-construct was cut out and purified with the Qiagen Purification Kit according to the standard protocol provided by the manufacturer, with the slight modification that the elution from the column was carried out using injection buffer supplied by the mouse IMP/IMBA mouse service. Injections were carried out by the IMP/IMBA service.

4.10 Genotyping of transient transgenic embryos

For the genotyping Primers Har2122 and 2123 were used. Primers and Pcr-conditions as described in the Section 4.6 Semi-quantitative RT-PCR. 1µl of yolk sac preped genomic DNA was used as template. WT-DNA was used as negative control, the injected plasmid as positive control.

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Curriculum Vitae

Alexander Apschner

Date/Place of birth: 21.04.1982, Villach, Austria
Maritial status: not married

Education

1997-2002	Handelsakademie Villach
2002-2003	Civilian Service
2003-2004	Biotechnology and Food Sciences, University of Natural Resources and Applied Life Sciences, Vienna
2004-2010	Molecular Biology, University of Vienna

Research Experience

10/2008-10/2009	Diploma Thesis Institute of Molecular Pathology (IMP), Vienna Dr. Christine Hartmann Target genes of canonical Wnt-signalling in mouse skeletal development
07/2008-09/2008	Training European Molecular Biology Laboratory (EMBL), Heidelberg Dr. Darren Gilmour Dynamics of G-Protein-coupled-receptors during Zebrafish lateral line primordium migration
11/2007-03/2008	Training Institute of Molecular Pathology (IMP), Vienna Dr. Christine Hartmann Target genes of canonical Wnt-signalling in mouse skeletal development
07/2007-08/2007	Training University of Vienna Prof. Christoph Schüller Genomics of oxidative stress response in C. glabrata

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