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Identification and Characterization of Novel Binding Partners of the Lef/Tcf Transcription Factors.

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I declare that this work was done at the Department of Molecular Development and Cell Physiology from February 02, 2002 to August 30, 2005.

No other methods, equipment or reagents were used other that those described in this thesis.

Stephen Mbigha Ghogomu, 30th August 2005.

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1. Introduction.

The development of an organism from a fertilized egg into a multi-cellular organism starts with proper definition of the polarity. This polarity includes dorso-ventral, anterior-posterior and left-right symmetry. The embryological process is tightly regulated temporally and spatially and results from the interplay between several signalling pathways, which generate distinct signals defining the diverse array of cell types that make up an organism. Among the numerous pathways controlling this polarity the Transforming growth factor (TGF), delta-notch, retinoic acid and the Wnt/β-catenin (canonical Wnt signalling) pathways play crucial roles.

The scope of this study focuses on the modulation of the Wnt/β-catenin signalling pathway, which regulates multiple aspects of mammalian development, and aberrant signalling through this pathway has been linked to human developmental disorders and diseases including cancer. Although much has been done to dissect this pathway, much is left unknown with respect to the different components of this pathway and how it is regulated in the developing embryo. The nuclear transducers of this pathway are the Lymphoid enhancer factors (Lefs) and the T-cell factors (Tcfs). They have been characterised to have different functions despite the fact that they all have almost identical binding partners. Moreover, structural data on Lef/Tcfs are restricted to the most conserved motifs and do not reveal differences between family members. The fact that there exist numerous Lef/Tcf splice variants and cross-talks between the Wnt/β-catenin pathway and other signalling pathways further complicates the understanding of the regulation of individual Lef/Tcf-mediated signal transduction. The identification of sub type-specific Lef/Tcf binding partners and the different modes of regulation of these transcription factors remains an important cue to the understanding of this subtype specific activity.

1.1 The Wnt/β-catenin or canonical Wnt signalling pathway

In the simplest model of Wnt/β-catenin signal transduction, the binding of Wnt proteins to a frizzled transmembrane receptor inactivates the β-catenin degradation complex consisting of β-catenin, Axin, Adematous polyposis coli (APC) and glycogen synthase kinase (GSK)-3β. As a consequence, β-catenin accumulates in the cytoplasm, shuttles into the nucleus and together with the Lef/Tcfs activates Wnt/β-catenin target genes.

This simple mode of activation is complicated by many components that modulate the activity of the pathway at each level of the signal transduction cascade (Fig1).

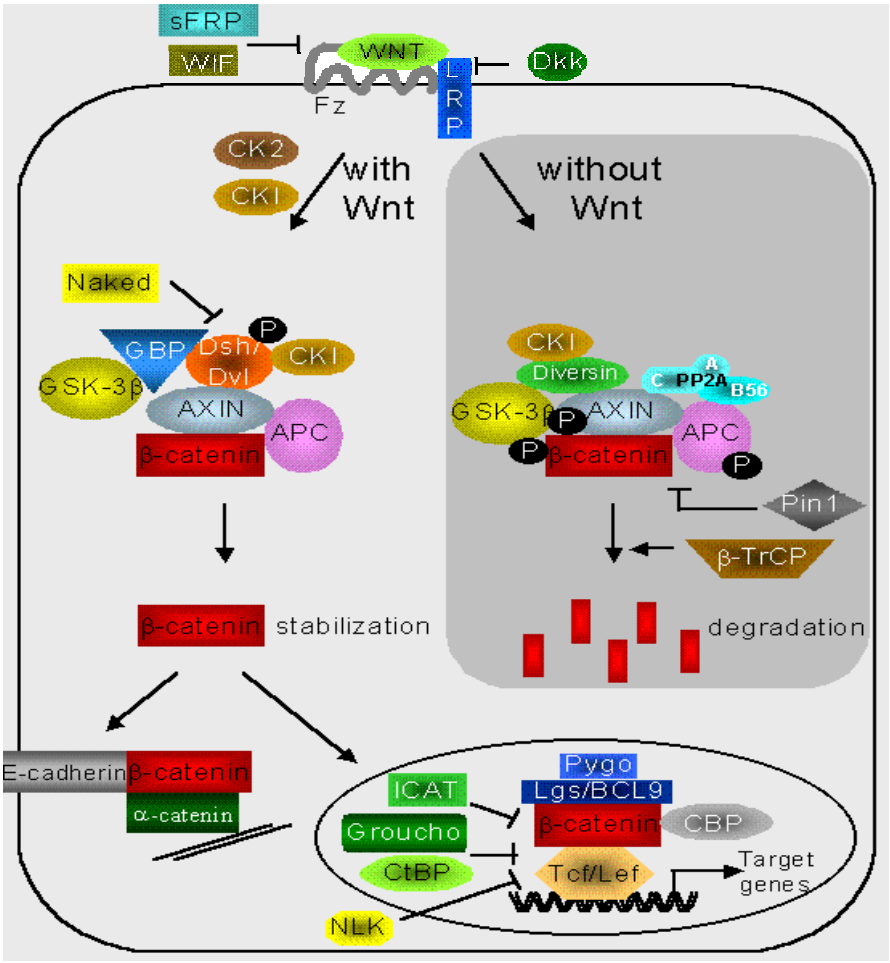

Fig. 1: The Wnt signalling pathway. Binding of Wnts to their transmembrane receptors Frizzled (Fz) and low density lipoprotein receptor-related protein (LRP) activates dishevelled (Dsh) following its phosphorylation by caseine kinases. Phosphorylated Dsh inactivates the β-catenin degradation complex comprised of β-catenin, Axin, Adematous polyposis coli (APC) and glycogen synthase kinase (GSK)-3β. Accumulated cytosolic β-catenin shuttles into the nucleus and together with the Lef/Tcf family of transcription factors activate Wnt target genes. This pathway is regulated in the cytoplasm as well as in the nucleus by diverse proteins acting either as activators or repressors. In the absence of the Wnt signals β-catenin is primed by phosphorylation and targeted by β-transducin repeat-containing protein (β-TrCP) for degradation in the protesome.
(http://www.infobiogen.fr/services/chromcancer/Deep/WNTSignPathID20042.htm).

Extracellularly, the Wnts are inhibited by binding partners which mimic Wnt ligand receptors e.g. the secreted frizzled-related proteins (sFRP) (Moon et al., 1997) and Wnt-inhibitory factor (WIF)-1 (Hsieh et al., 1999). These proteins compete with Wnts for binding to frizzled thereby restricting Wnt signalling. At the level of the Wnt-responsive ligand receptor interaction, Wnt proteins bind to the cysteine-rich domain

(CRD) of a seven transmembrane frizzled (fz) receptor (Bhanot et al., 1996). This binding is facilitated by a single-pass transmembrane molecule of the low density lipoprotein receptor-related protein (LRP) family (Lui et al., 2003). This stage is regulated by the inhibitor Dickkopf (Dkk) which forms a complex with and internalises LRP making it unavailable for Wnt reception (Mao & Niehrs 2003).

In the cytoplasm of the Wnt-responsive cell the pathway is regulated by kinases. Caseine kinase (CK)-1 and CK-2 activate *Drosophila* dishevelled (Dsh) or its vertebrate homologue, Dvl, by phosphorylation (Willert et al., 1997). In vertebrates, phosphorylated Dvl inhibits the activity of the β-catenin degradation complex. The result is the accumulation of cytosolic hypophosphorylated β-catenin which translocates into the nucleus and interacts with members of the Lef/Tcfs family of DNA-binding proteins leading to transactivation of Wnt target genes (Behrens et al., 1996). In the nucleus, β-catenin converts the transcriptional repressive Tcf/Groucho complex into a transcriptional active Tcf/β-catenin complex. In the absence of Wnts, Axin recruits CK1 to the multiprotein complex causing priming of β-catenin and initiation of the β-catenin phosphorylation performed by GSK-3β. Hyperphosphorylated β-catenin is then recognized by β-transducin repeat-containing protein (β-TrCP) and degraded by the proteosome, reducing the level of cytosolic β-catenin thereby inhibiting the Wnt/β-catenin pathway.

The tight regulation of the Wnt/β-catenin pathway is absolutely necessary since it has been demonstrated to regulate numerous important developmental processes. Wnt/β-catenin signalling is involved in the induction of neural crest marker genes, *Xslug* and *Xtwist*, in *Xenopus* (Chang et al., 1998), the renewal of neural crest stem cells (LaBonne et al., 1998) and the specification of cell fate in the adoption of a neuronal or pigmental phenotype by the neural crest cells (Dorsky et al., 1998). Wnt-β-catenin signalling is also involved in body axis formation and mesoderm patterning. β-Catenin and other components of the Wnt/β-catenin pathway is essential for body axis formation in mice (Huelsken et al., 2000), *Xenopus* (Moon and Kimelman, 1998) and Zebrafish Solnica-Krezel, 1999). In *Xenopus*, accumulation of β-catenin on the dorso-anterior side of the embryo is the earliest sign of axis formation and precedes gastrulation (Larabell et al., 1997). Overexpression of β-catenin induces formation of an additional embryonic axis (Heasman et al., 1994). Interestingly, members of the Wnt-signalling cascade have are also involved in establishing the body axis in Hydra,

a member of the evolutionary ancient metazoan Phylum Cnidaria (Hobmayer et al., 2000). These data suggest that Wnt signalling is a highly evolutionarily conserved process important for axial differentiation in the animal kingdom. This evolutionary conservation of Wnt/β-catenin signalling is also observed in the establishment of the dorsoventral polarity in the development of the chick limb buds (Kengaku et al., 1998), mouse limbs (Parr et al., 2005) and segment polarity in *Drosophila* (Klingensmith et al., 1994).

The need for tight regulation of the pathway is strengthened by the fact that aberrant signalling through this pathway has been linked to human developmental disorders and diseases including cancer. Mutation in the tumour suppressor adenomatous polyposis coli protein (APC) which is one of the components of the β-catenin degradation complex leads to accumulation of β-catenin, the root cause of colorectal cancer (Peifer, 1997). Moreover, mutations in β-catenin and Axin, all components of the pathway, have been found to be responsible for hepatocellular carcinoma (Taniguchi, 2002).

1.2 Lef/Tcf transcription factors

By virtue of their interaction with β-catenin, Lef/Tcf transcription factors are the nuclear transducers of an activated Wnt/β-catenin pathway. In vertebrates, there exist four family members: Tcf-1, Tcf-3, Tcf-4 and Lef-1. Generally, they are characterized by the presence of two highly conserved domains: the N-terminal β-catenin-binding domain of about 60 amino acids and a high mobility group (HMG) box domain of about 80 amino acids. These two conserved domains sandwich a core domain comprised of an alternatively spliced activating Exon and two poorly-characterised domains. (Fig. 2).

The HMG box domain does not only mediate DNA binding to the consensus sequence (A/T)(A/T)CAA(A/T)GG of Wnt target genes (Clevers et al., 1997) but also induces a sharp 120° bend in DNA to which it binds (Giese et al.,1995). Because of this bending ability and the observation that Tcfs can not directly activate transcription in reporter gene assays, it is proposed that Lef/Tcf family members serve an architectural function. Lef-1 appears unique in that it contains a context-dependent activation domain (CAD) (Carlsson et al., 1993) which can activate transcription in the presence of the co-factor ALY (Bruhn et al, 1997). Flanking the conserved alternatively spliced Exon are two motifs that mediate repression of

transcription (Gradl et al., 2002a). This could be either by changing the overall structure of Lef/Tcfs so that transactivation *via* β-catenin can no longer take place or recruitment of a specific co-repressor by these motifs (Gradl et al, 2002a). Moreover the serine-rich motive SXXSS is subject to phosphorylation events that regulate DNA/protein interactions (Pukrop, et al., 2001).

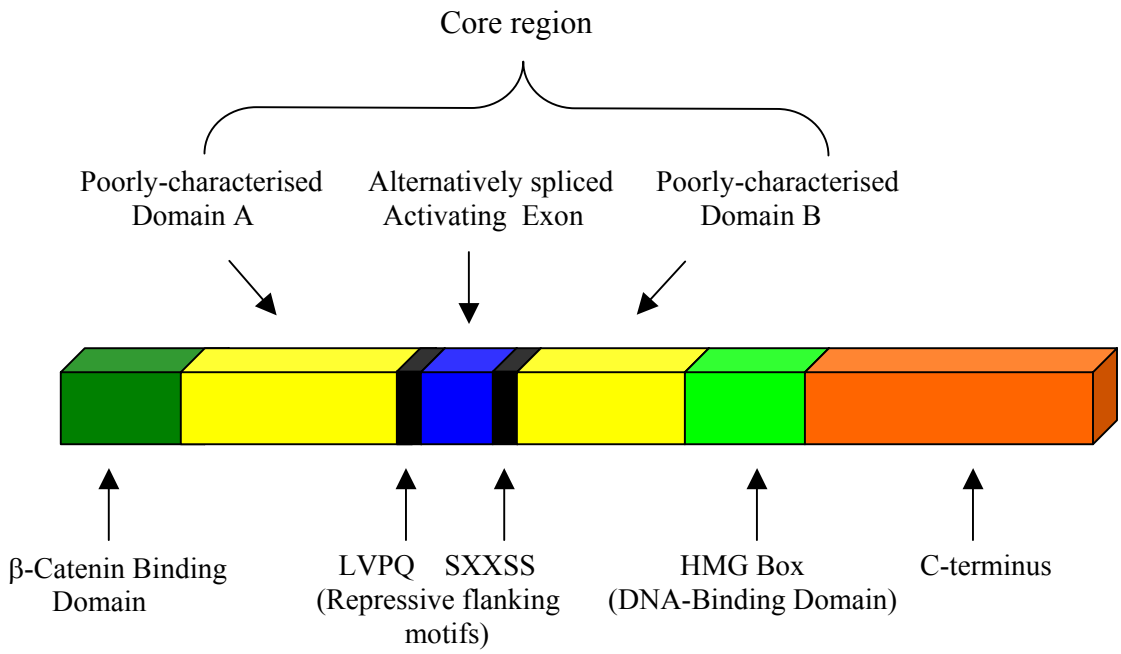

Fig 2: General structure of Lef/Tcf transcription factors showing the different functional domains. Lef/Tcfs possess two highly conserved domains: the β-catenin binding domain and the high mobility group (HMG) box. These two domains sandwich a core region comprised of an alternatively spliced activating Exon boarded by two flanking motifs and two poorly characterized domains. The length of the region C-terminal to the HMG box varies depending on the LefTcf subtype (from Gradl et al., 2002).

1.2.1 Alternative Splicing of Lef/Tcfs

Lef/Tcfs have been found to be differentially spliced, resulting into a multiplicity of isoforms which shape the level and time course of Wnt target gene expression. Usage of either alternative Exon splicing or splice donor and acceptor sites have been found to generate numerous isoforms.

The generation of C-terminal isoforms have been reported for hLef-1, hTcf-1 and hTcf-4 genes. Each of these tails has been given different alphabetic designation to distinguish between them. Apart from two short highly conserved basic and cysteine-rich domains in the E-tails, they diverge in amino acid sequence and length: 107 aa residues for hTcf-1E and 136 for hTcf-4E. Contrary to the E-tails, B and N tails are extremely short: hLef-1B: 25 aa; hTcf-1B: 24 aa; hTcf-4: 24 aa and hLef-1N: 11 aa.

Whereas the B- and the E-tails are predominant forms for hTcf-1 and hTcf-4, the B-tail is the predominant isoform of hLef-1 (van de Wetering et al., 1996) (Fig. 3). This hLef-1B results from splicing which excludes exon XI of the C-terminus while splicing which includes this exon results to hLef-1N. (Hovanes et al., 2000).

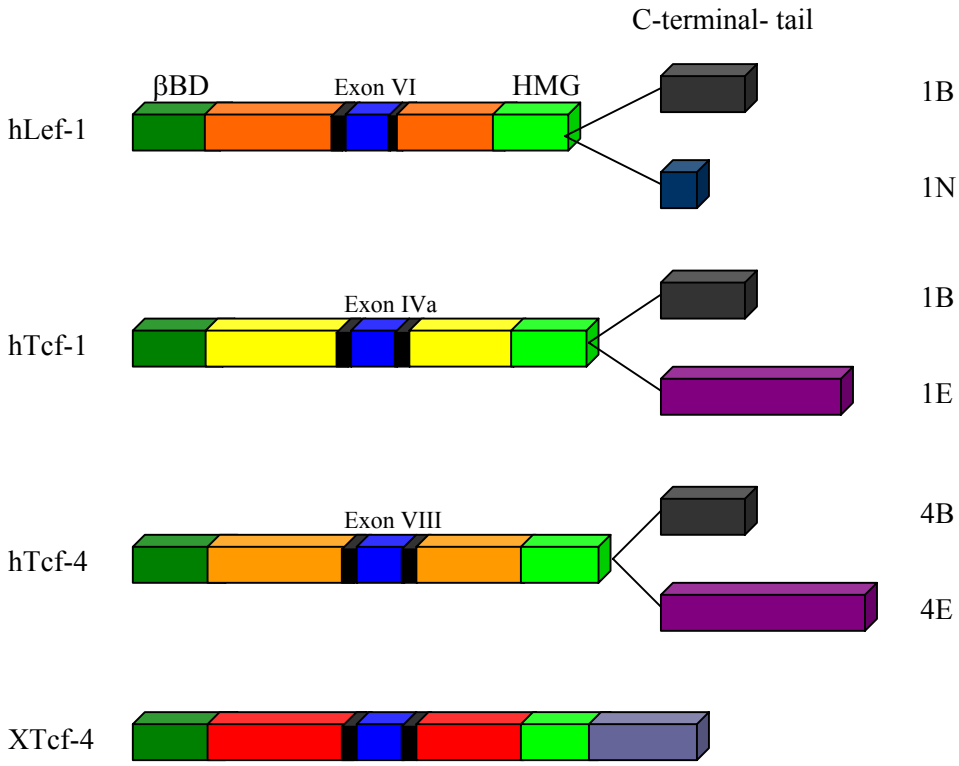

Fig 3: General view of some Lef/Tcf splice variants. The C-terminus is alternatively spliced resulting to 1B and 1E and 1N variants. (Clevers et al., 1997). The alternatively spliced Exon is represented in blue and corresponds to Exon VI (in hLef-1), IVa (in hTcf-1) and VIII (in hTcf-4). Alternative splicing of the flanking motifs (black) in *Xenopus* Tcf-4 yields XTcf-4A (two flanking motifs present), XTcf-4B (the right flanking motif missing) and XTcf-4B (the two flanking motifs missing) (Pukrop et al., 2001).

The difference in lengths of the C-terminal isoforms is the basis of functional diversity observed in these tails. HTcf-4E contains a PLSLV sequence after the aa_{124} of its E-tail that offers it the possibility for recruitment of the Wnt/β-catenin pathway repressor, C-terminal binding protein (CtBP) (Valenta et al., 2003). Apart from Tcf-3 that also possesses this CtBP-binding motif (Brannon et al., 1999), all other forms of Lef/Tcf lack this motif.

Alternative splicing of an activating Exon results to additional hLef-1, hTcf-1, hTcf-4 isoforms (Fig. 3). This alternatively spliced Exon corresponds to Exon VI in hLef-1, IVa in hTCF-1and VIII in hTcf-4 (Fig. 4).

The flanking regions of this alternatively spliced activating Exon comprise two different splice acceptor sites resulting to the presence or absence of an LVPQ and SFLSS motifs. These motifs are also differentially spliced in XTcf-4 giving XTcf-4A, 4B and 4C. (Pukrop, et al., 2001) (Fig. 3).

The conserved nature of some of the exons in Lef/Tcfs is remarkable (Fig. 4). Exon I codes for the β-catenin binding domain. In Lef-1 this exon is extremely large as it includes the entire 1.2 kb GC-rich 5´-untranslated region involved in post transcriptional regulation for proper translation of the Lef-1 coding sequences (Hovanes et al., 2000).

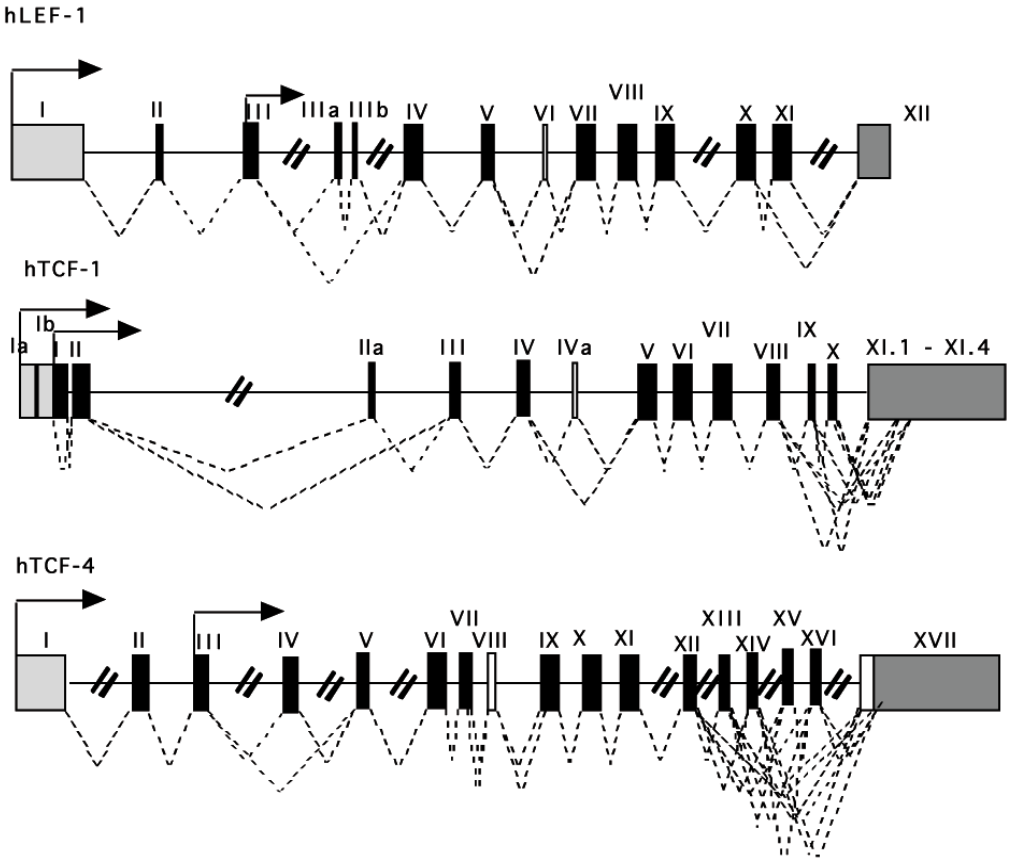

Fig 4. Genomic organisation of hLef-1, hTcf-1 and hTcf-4 according to Duval et al., 2000 and Hovanes et al., 2000. Possible translation start sites are indicated as arrows. The highly conserved β-catenin binding site is indicated in light grey boxes (extreme left). The exon VI of hLef-1 corresponds to Exon IVa in hTcf-1 and Exon VIII in hTcf-4. All other exons are indicated in bold. Dotted lines indicate splicing sites. (Gradl et al., 2002b).

However, some splice variants of Lef-1, hTcf-1 and Tcf-4 have been found to lack this β-catenin binding domain and are regulated by alternative promoter usage (Hovanes et al., 2000).

Exon II-VI codes for parts of the poorly characterised context activation domain (CAD) domain in hLef-1. The CAD renders Lef-1 unique in activation of target genes without recruitment of the transcriptional activator, β-catenin. In all Lef/Tcfs the region corresponding to this CAD is less conserved.
The Exons coding for the HMG box domain are very conserved. No naturally occurring Lef/Tcfs apart from Drosophila Tcf (dTcf) have the capacity to generate alternative DNA binding domains by splicing. This highly conserved gene structure points to a conservation in function (Dooijes et a., 1998). In all Lef/Tcfs the HMG box domain is encoded by 3 exons. In humans these exons are : VIII-X in Lef-1, VI-VIII in Tcf-1, X-XII in Tcf-4 (Hovanes et al., 2000).

1.2.2 Lef/Tcfs Expression patterns and Function

Considering the varied nature of developmental decisions regulated by Wnts one would expect a broad expression pattern of the nuclear transducers of the Wn/β-catenin pathway. Surprisingly, Lef/Tcf expression patterns during embryonic development are quite often specific and restricted to distinct organs and regions. The expression of XLef-1 starts only after mid blastula transition (MBT) and at later stages, it is expressed in the central nervous system, eyes, otic vesicles, head mesenchyme, neural crest derivatives, brachial arches, developing heart, tail bud and limbs. (Molenaar et al., 1998). In mammals *Lef-1* mRNA is readily observed in pro- and pre- B-lymphocytes (Travis et al., 1991) and in all stages of T-lymphocyte differentiation. Although, mice generated to carry a homozygous germ-line mutation in the *Lef-1* gene that eliminates its protein expression show no obvious defects in lymphoid cell populations at birth, they lack teeth, mammary glands, whiskers, hair and the mesencephalic nucleus of the trigeminal nerve, the only neural crest-derived neuronal populations. Together, the pattern of these defects suggests an essential role for Lef-1 in the formation of several organs and structures that require inductive tissue interactions (van Genderen et al., 1994).
The expression of Tcf-1 is observed at high levels in the animal hemisphere in cleavage and blastula stage embryos. During gastrulation XTcf-1 is differentially expressed with high levels in the animal cap and all parts of the marginal zone except for a narrow domain around the blastopore. At neurula stages expression is predominant in the neural plate. At tail bud stages expression is localized in specific areas of the brain, in the eyes, the otic vesicle, branchial arches and head

mesenchyme, somites, tail bud, pronephros and pronephric duct (Roel et al., 2003). Tcf-1 expression has also been demonstrated in mice thymocytes. Although very low expression levels are found in the prothymocytes high level expression occurs from the prethymocyte stage onwards (Oosterwegel et al., 1993). Germline mutations in Tcf-1 have been found to block thymocyte development in mice at the transition from the cluster of differentiation (CD)8+ immature single-positive to the CD4+/CD8+ double-positive stage. Most of the immature single-positive cells in the mutants are not in the cell cycle and the number of immunocompetent T-cells in peripheral lymphoid organs is reduced. These observations confirm that Tcf-1 controls an essential step in thymocyte differentiation (Verbeek et al, 1995).

Overlapping expression pattern of Tcf-1 and Lef-1 has been detected outside the lymphocytes in early mouse embryos. In neuroectodermal cell types (e.g. brain, neural crest) as well as in differentiating mesenchymal cells (e.g. in limb bud, pharyngeal arches, lung bud and urogenital system) (Oosterwegel et al., 1993). Tcf-1 expression overlaps with that of Lef-1. The expression of the two genes is shut off around birth in these tissues, at the moment when the final architecture of most organ systems has been established. Tcf-1 and Lef-1 are thus thought to play a role in the establishment of organs rather than in the maintenance of a particular cell type (Oosterwegel et al, 1993).

XTcf-3 is maternally expressed. Its expression pattern after MBT is very similar to that of XLef-1. It is expressed in the central nervous system, eyes, otic vesicles, head mesenchyme neural crest derivatives, branchial arches, developing heart, tail bud and in the limbs (Molenaar et al., 1998). Tcf-3 has been found to inhibit the induction the anterior/posterior axis in the mesoderm both in *Xenopus* (Molenaar et al., 1998) and in mice (Merrill et al., 2004).

During embryogenesis, *mTcf-4* displays a highly specific pattern of expression. It appears much later in development than do the other three family members, and its expression is essentially restricted to the di- and mesencephalon and the intestinal epithelium (Barker and Clevers, 2000). Disruption of the gene coding for Tcf-4 in mice results in death following birth. The neonatal epithelium is composed entirely of differentiated, non-dividing villus cells. Tcf-4 therefore is thought to control a genetic program that maintains the crypt stem cells of the small intestine (Korinek et al., 1998). Although Tcf-4 is expressed in the mouse brain, brain defects have not been observed in Tcf-4 knockouts. In *Xenopus*, Tcf-4 has been found to demarcate the

forebrain-midbrain boundary (König et al., 2000). The expression of this transcription factor depends on Xwnt-2b, which is itself under the control of XTcf-4. This autoregulatory loop is indispensable for proper development of the midbrain and the isthmus (Kunz et al., 2004).

As a summary, specific Wnt-driven patterning events are determined by the unique tissue distribution of different Tcf/Lef family members.

Besides the unique role of the individual Lef/Tcfs in the development of different organs, double knockout experiments reveal functional redundancy of these transcription factors. Null mutations in both Lef-1 and Tcf-1 cause a severe defect in the differentiation of paraxial mesoderm and lead to the formation of additional neural tubes. These phenotypes are identical to those reported for Wnt-3a-deficient mice. In addition, Lef-1/Tcf-1 double knockout embryos have defects in the formation of the placenta and in the development of limb buds, which fail to express Fgf-8 and to form an apical ectodermal ridge. These observations provide evidence for a redundant role of Lef-1 and Tcf-1 in Wnt signalling during mouse development. (Galceran et al., 1999).

Tcf-4 and Tcf-1 also demonstrated functional redundancy. Tcf-4 and Tcf-1 double knockouts show severe caudal truncations with no hind limb and tail, duplications of the neural tube, defects in hindgut expansion, and an anterior transformation of the gastro-intestinal tract (transformation of the duodenum into the stomach) (Gregorieff et al., 2004). These data suggest that further Lef/Tcf double as well as triple knockout experiments may reveal further redundancy.

1.2.3 Binding partners of the Lef/Tcf transcription factors

1.2.3.1 β-catenin

Since Lef/Tcfs have no intrinsic transactivation domain, apart from a context-dependent transactivation domains in Lef-1 (Carlsson et al., 1993), Tcf proteins do not function as classical transcription factors. Sole binding of Lef/Tcfs to DNA is not sufficient to cause transcriptional activation (Eastman and Grosschedl, 1999). Instead they bind to a number of auxiliary proteins, thereby recruiting essential functional domains to the regulatory regions of target genes (Bruhn et al., 1997). Promoter activation is only achieved after complex formation with β-catenin to generate a functional bipartite transcription factor. Recent studies have revealed the mechanism by which this bipartite complex activates the promoter. In this complex, Lef/Tcfs

provide the DNA-binding moiety through their HMG box while β-catenin provides the transcription activation domain through its C-terminus (Barker and Clevers, 2000). The N-terminal domain of β-catenin comprised of approximately 130 amino acids contains putative GSK-3β binding sites which regulate its priming for ubiquitination (Dajani et al., 2001). The central region of 12 imperfect repeats of 42 amino acids known as armadillo repeats (since they show homology with repeats found in the Arm protein of *Drosophila*) mediate binding to Lef/Tcfs. These armadillo repeats are arranged to form a super helix of 12 repeats with each repeat (except repeat 7) comprised of 3 helices (Fig. 5).

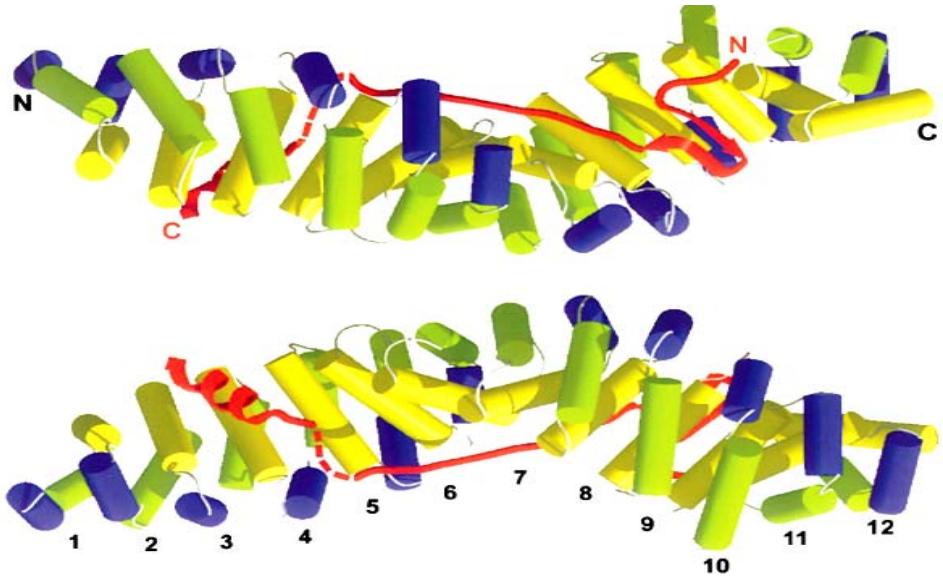

Fig. 5: The overall structure of the XTcf-3-β-catenin binding domain/β-catenin armadillo repeat complex. Two views of the complex are related by a 180° rotation about the β-catenin super helical axis. The armadillo repeat of β-catenin forms a super helix made up of 12 repeats. Each repeat, except repeat 7, consists of 3 helices (shown in blue, green and yellow) respectively. The β-catenin binding domain of XTcf-3 is shown in red and consists of (from N- to C-terminus) a β hairpin module, an extended region that contains β-strand and an α-helix. Unresolved structures are shown in dashed lines. The β-catenin binding domain of XTcf-3 wraps around the armadillo repeat region of β-catenin in an anti parallel fashion along the major axis of the super helix (Graham et al., 2000).

Through their β-catenin binding domain (CBD), each Lef/Tcf forms an elongated structure that runs anti parallel along the major axis of the positively-charged (Lysine-rich) groove of the super helix. The CBD mediates physical interactions with the super helix in three ways: through the acidic N-terminal β-hair pin domain, the central β-strand and the C-terminal α-helix.

The function of this complex is tightly regulated by a multitude of binding partners which either co-activate or co-repress gene transcription. The functions of these binding partners are discussed in detail in the following.

1.2.3.2 Co-activators of the Lef/Tcfs

The co-activators of the Lef/Tcfs include a variety of proteins involved in either chromatin modelling, activation through the HMG box or activation of the Lef/Tcfs in an unusual way.

As co-activators involved in the modelling of the chromatin are the cyclic AMP receptor-binding (CREB) protein (CBP). In Wnt-stimulated *Xenopus* cells, the transactivating domain of β-catenin interacts with CBP and this synergistically stimulate transcription of Wnt target genes (Takemaru and Moon, 2000) (Fig. 6).

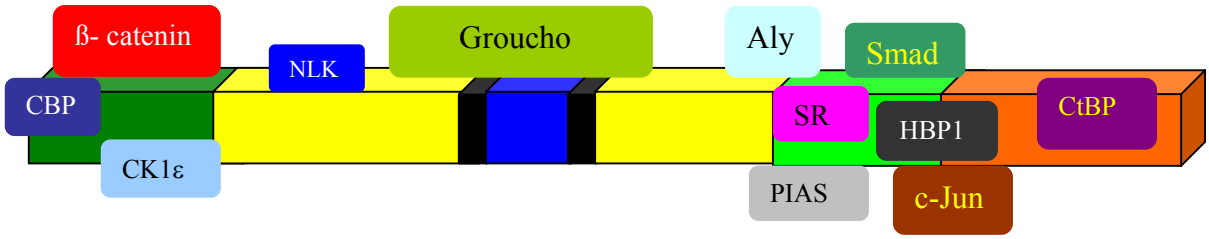

Fig 6: Lef/Tcf function is regulated by a multitude of binding partners: β-catenin furnishes the activation domain, the CREB-binding proteins (CBP) favour an open chromatin structure, the smads bind to HMG-box while the Caseine kinases (CK1ε) phosphorylate the β-catenin binding site. Groucho, C-terminal binding protein (CtBP) favour a closed chromatin structure, the HMG box-containing protein-1 (HBP1), Protein inhibitors of activated STAT1 (PIAS), Steroid receptors (SR) mask the HMG box,. ALY specifically binds to both the HMG box and core region of Lef-1. (http://www.stanford.edu/~rnusse/pathways/binding.html).

CPBs are histone acetylases and are thought to acetylate nucleosomal histones resulting to a more open conformation of the DNA. This allows the transcription machinery to gain access to the promoter region to initiate transcription (Goodman and Smolik, 2000). The enhancing effect of CBP on the transcriptional potential of β-catenin-bound Lef/Tcfs is however limited. Only a modest increase in transcriptional level is observed indicating that they might act in concert with more factors to achieve efficient gene activation.

The smads have been found to enhance this complex in transactivation by binding to the HMG box (Fig. 6). Smads are the nuclear transducers of the transforming growth factor (TGF)-β signalling pathway. They are comprised of highly conserved Mad homology (MH) N-terminal and C-terminal domains (referred to as MH1 and MH2

domains respectively) separated by a less conserved linker domain (Heldin et al., 1997). It has been reported that through these MH1 and MH2 domains smads interact with the HMG domain of Lef/Tcfs to synergistically activate the *Xenopus* homeobox gene, *twin* (Labbé et al., 2000, Nishita et al., 2000). Moreover smads recruit transcriptional co-activators such as P300 and CBP to target promoters and may provide a transactivation domain to transcription factors (Derynck et al., 1998).

The ubiquitously expressed nuclear protein ALY has also been shown to interact with the core region and the HMG box of Lef-1 (Fig.6). Although nothing as yet indicates that ALY is involved in the Wnt/β-catenin pathway, the complex Lef-1/ALY together with acute myeloid leukaemia (AML) factor-1 activate enhancers of specific target genes, such as the T-cell receptor- enhancer (TCR). This association increases the affinity for both Lef-1 and AML proteins to bind DNA. ALY therefore mediates transcriptional activation by facilitating the functional collaboration of multiple proteins in the TCR alpha enhancer complex (Bruhn et al., 1997).

An unusual way of regulating Tcf-3 is observed in its activation by casein kinase (CK)1ε. With the evidence that a substantial amount of Tcf-3 is extra-nuclear (Lee et al., 2001) its activity is enhanced by binding to CK1ε. This kinase phosphorylates XTcf-3 at conserved phosphorylation sites in the β-catenin-binding region (Fig. 6). The outcome of this phosphorylation is the increase in affinity of Tcf-3 for cytoplasmic β-catenin. Phosphorylated Tcf-3 therefore competes with Axin and APC for binding to β-catenin resulting to decreased degradation of β-catenin. These phosphorylation sites are also present in other members of the Lef/Tcf family suggesting that similar phosphorylation procedures might take place to enhance β-catenin binding (Lee et al., 2001).

Another more specific phosphorylation dependent regulation of Tcfs is observed in intestinal cancer. The binding of Tcf-4 to the proto-oncoprotein, *c-Jun*, is only possible following phosphorylation of the of c-Jun protein by c-Jun N-terminal kinases (JNK). Phosphorylated c-Jun then interacts with Tcf-4/β-catenin complex. This interaction is mediated *via* the HMG box of Tcf-4 and the conserved serines 63 and 73 of c-Jun. The complex Tcf4/β-catenin/c-Jun regulates transcription of *c-jun* gene favouring intestinal cancer progression (Nateri et al., 2005).

1.2.3.3 Co-repressors of Lef/Tcfs

Numerous experiments have shown that Lef/Tcfs do not only function as transactivators when activated by Wnt/β-catenin signalling. They can either actively associate with co-repressors to inhibit target gene expression in the absence of Wnt/β-catenin signalling or bind to other partners to modulate other signalling pathways.

Among the co-repressors are proteins altering the chromatin structure, some mask the conserved domains and others inhibit the Lef/Tcf function in an unusual manner.

In the list of co-repressors of Lef/Tcfs that alter the chromatin structure are the *Drosophila* Groucho and their human homologues, transducin-like enhancer of split (TLE)1-4 proteins and the C-terminal binding proteins (CtBP). The Grouchos are general transcriptional repressors of the Lef/Tcfs. These proteins are characterized by a conserved C-terminal WD-repeat domain which mediates interactions with DNA-bound Lef/Tcfs (Brantjes et al., 2002). The interaction domain in the Lef/Tcfs is the core region comprised between the β-catenin binding site and the HMG box. It is believed that Groucho family proteins repress transcription through their glycine/proline-rich variable region which recruits histone deacetylase Rpd-3 to the template. These enzymes are known for their function as silencers of transcription by altering local chromatin structure (Chen and Courey, 2000). Although Groucho and β-catenin have different binding sites they compete for binding to Lef/Tcfs (Daniels and Weiss, 2005). In the absence of a Wnt/β-catenin signal, Tcf/Groucho complexes act as repressors of gene transcription. Upon activation by Wnt signals, β-catenin replaces Groucho and thus results into a complex that activates transcription of Wnt target genes. The almost ubiquitous expression pattern in *Xenopus* (Ishitani et al., 1999) coupled with the fact that Grouchos bind indiscriminately to Lef/Tcfs suggest that these proteins might not contribute to the functional diversity of Lef/Tcfs.

Unlike the Grouchos, CtBP acts as a co repressor only to a subset of Tcfs. It binds only to XTcf-3 (Brannon et al., 1999) and to one of the splice variants of hTcf-4 (Tcf-4E) (Valenta et al., 2003). This interaction is mediated by the PLSLV/T motif located in the C-terminal of both transcription factors. These sites have also been found in hTcf-3 and hTcf-1E but their functional interaction has not yet been confirmed. CtBP acts as a histone deacetylase resulting in a closed chromatin structure thereby inhibiting the transcriptional machinery (Valenta et al., 2003). The fact that this co-repressor binds only to a subset of Tcfs suggests its role in defining differences in

Lef/Tcf target gene activation. However elimination of the C-terminus of XTcf-3 and thus CtBP binding, has not been found to have any effect on the inhibitory character of XTcf-3 (Gradl et al., 2002a). This suggests that additional repressive motifs might exist in XTcf-3.

Another mode of repression is to binding and mask the HMG box. This is observed in the regulation of the promoters *c-myc* and *cycline D1*. These genes are responsible for malignant proliferation following inappropriate activation of Wnt/β-catenin pathway. The HMG-box-containing protein-1 (HBP1) is described as an ubiquitously expressed protein that binds to the Lef/Tcfs through its repressive domain (aa_{220}-aa_{401}). Unexpectedly, this interaction is not mediated by competition with Lef/Tcfs for binding to target DNA but by direct binding to the HMG box of Lef/Tcfs (Sampson et al., 2001). Also, HBP1 shows no preference to any Lef/Tcf subtype indicating that it might be a general Lef/Tcf repressor and can not contribute to the functional specificity observed in Lef/Tcfs.

A second regulatory mechanism through masking of the HMG box is by sumolylation and subsequent sequestration into subnuclear structures. Inhibitors of signal transducers and activators of transcription (STAT1) have been found to bind to Lef-1 and repress its transcriptional activation potential by sequestration. A candidate inhibitor of STAT1 is PIAS1 (Protein inhibitor of activated STAT1) (Chung et al., 1997). PIAS1 binds to the HMG domain of Lef-1 and acts by conjugating lysine residues of Lef-1 with the small ubiquitin-like modifier 1 (SUMO1) protein. This sumoylation targets Lef-1 to nuclear bodies where it is sequested thereby antagonizing Wnt-induced transcriptional activation (Sachdev et al., 2001).

Although the CBP has been found to be a co activator of the Wnt target genes in Wnt-stimulated cells, it acts as a co-repressors in unstimulated cells. In *Drosophila*, it has been found to acetylate a conserved lysine (Lys 25) in the β-catenin-binding domain in *Drosophila* Tcf (dTcf) thereby reducing dTcf affinity for armadillo (*Drosophila* homologue of β-catenin). It is speculated that in this case CBP serves as a negative control mechanism to keep Tcfs passive and therefore eliminates inappropriate activation of Tcfs (Waltzer and Bienz, 1998).

Phosphorylation-dependent binding of proteins to Lef/Tcf is a further unusual type of repression. The Nemo-like kinase (NLK) has been shown to interact directly with Lef/Tcfs. This interaction induces phosphorylation of Lef/Tcfs on two conserved

serine/threonine residues in the core domain thereby, reducing the affinity of the β-catenin/Lef/Tcf complex to bind DNA (Ishitani et al., 2003).

1.3. Integration of the The Wnt/β-catenin signalling pathway into a cross-regulatory network.

Some of the binding proteins of nuclear β-catenin and Lef/Tcfs do not only modulate the response of cells to Wnt/β-catenin signalling but also comprise adaptor proteins through which the Wnt/β-catenin pathway cross-talks with other signalling pathways in numerous developmental processes .

As examples for such cross-talks, the smads bridge Wnt/β-catenin signalling to TGFβ- signalling at many levels. Through smad-2 the activin/Vg-1 of the TFG-β cooperates with the Wnt/β-catenin pathway to induce Wnt/β-catenin target genes (*siamois* and *Xnr3*) (Crease et al., 1998). Reciprocally, and still through smad-2, the Wnt/β-catenin pathway enhances the induction of TGF-β target genes (*goosecoid and chordin*) (Crease et al., 1998). Moreover, the existence of Wnt- and TGF-responsive elements in the *Xgoosecoid* promoter suggests that Wnt signals may provide a cooperative stimulus for the induction of genes responsive to the activin/Vg-1 signalling (Watabe et al., 1995).

Activin/Vg-1 signalling pathway has been demonstrated in the vegetal cell of the pre-gastrula embryo and in the presumptive mesoderm (Weeks et al., 1987) while the Wnt/β-catenin signalling pathway is present at the dorsal side of the embryo (Wylie et al., 1996). The two pathways overlap in the dorsal vegetal domain of the embryo which corresponds to the domain in which genes of the Spermann organiser (*siamois, Xnr3, goosecoid and chordin*) are induced (Fig. 7). Since the Spermann organiser has been found to induce the primary body axis the cooperation between these pathways could impart a fine level of patterning information across the early organiser domain (Crease et al., 1998).

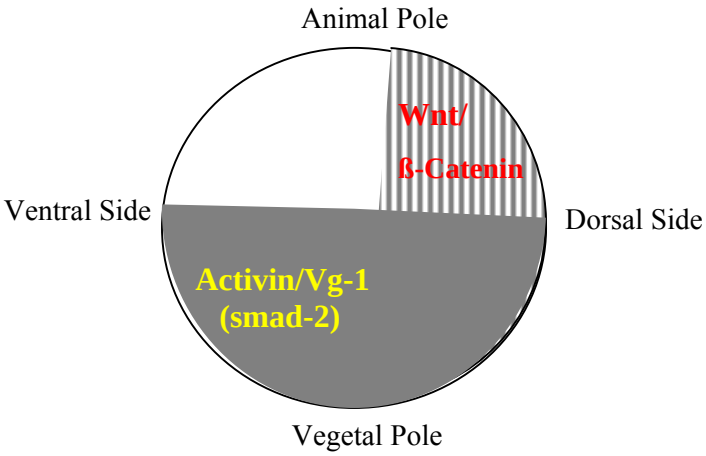

Fig. 7: Cross-talk between Wnt/ß-catenin signalling and transforming growth factor (TGF)-β pathway. Wnt/β-catenin signalling pathway at the dorsal side of the embryo cross-talk with the activin/Vg-1 signalling through smad-2 at the vegetal pole to induce the Spermann organiser at the dorsal vegetal pole. (Crease et al., 1998).

An alternative TGF-β pathway that modulates the Wnt/β-catenin pathway is the mitogen-activated protein (MAP) kinase pathway. This pathway activates the Nemo-like kinase (NLK) *via* TGF-β activated kinase (TAK)-1. NLK has been found to directly phosphorylate and inactivate the Lef/Tcf transcription factors (Ishitani et al., 2003). The consequence of this inhibition is the down-regulation of Lef/Tcf target gene expression.

Cooperation with other pathways also occurs following direct binding to transcription factors. The direct binding of smads to Lef/Tcfs relates the bone morphogenic protein (BMP) pathway (another TGF-β pathway) to the Wnt/β-catenin pathway and plays an important role in the control of critical developmental processes. In *Xenopus*, the collaboration between these pathways directly and synergistically affects expression of the *twin* gene during establishment of the Spermann organizer. This Interaction thus permits tight control in the specification dorsal axis. (Labbé, 2000, Nishita et al., 2000).

The collaboration between the Wnt/β-catenin signalling and the TGF-β signalling is not always synergistic. It is also antagonistic in the control of developmental processes. Through smad/Lef/Tcf interaction for example, the Wnt/β-catenin pathway inhibits the expression of bone morphogenic protein (BMP)-4 at the dorsal side of the early *Xenopus* embryo during neural development (Backer et al., 1999). This inhibition is a regulatory step in the induction of the primary body axis in *Xenopus*. Reciprocally, it is shown in mice that through phosphorylated smad-1, -5, -8, the

PTEN/Akt pathway inhibits the Wnt/β-catenin pathway by limiting nuclear translocation of β-catenin. The consequence is the inhibition of Wnt/β-catenin-induced intestinal stem cell self-renewal (He et al., 2004). All these observations points to a complex regulatory network of cross-regulation between TGF-β and Wnt/β-catenin pathways in the control of developmental processes (Fig. 8).

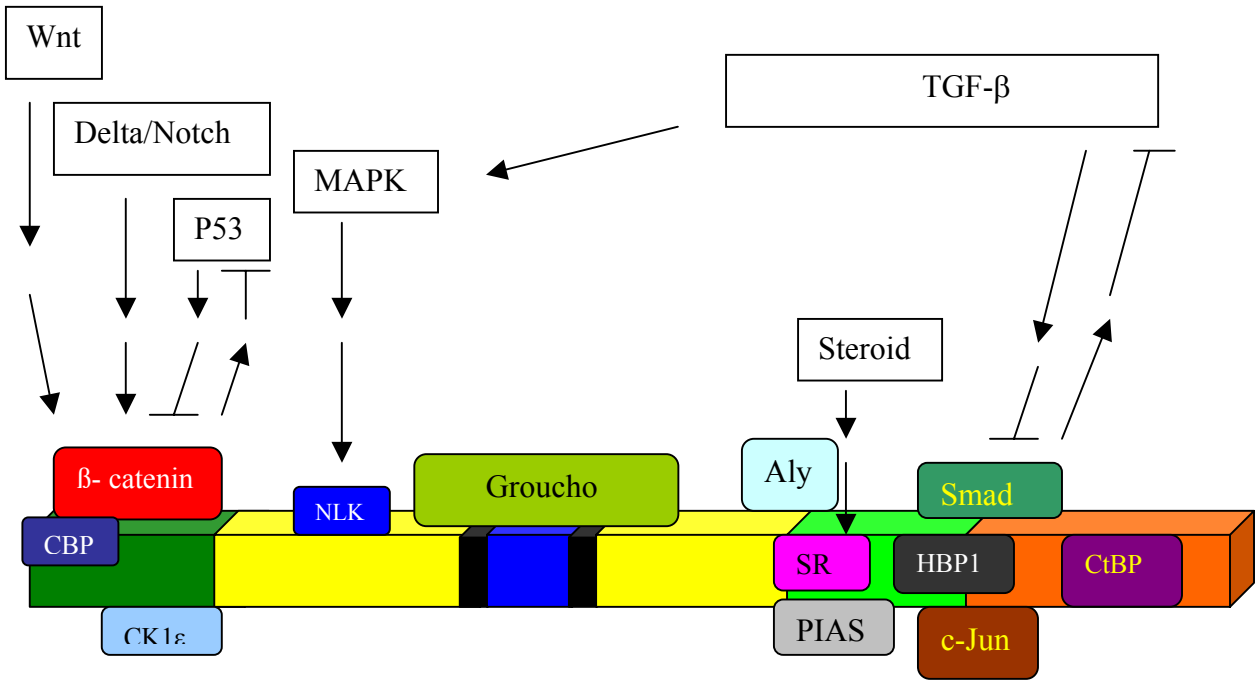

Fig. 8: Many signalling pathways converge at or diverge from the Wnt/β-catenin signalling cascade thereby establishing a cross-regulatory network. Delta/Notch pathway enhances the Lef/Tcf-driven transcription through interaction with β-catenin. p53, through a negative feedback process down-regulates β-catenin levels while β-catenin also down-regulates p53 levels. Steroid signalling establishes cross-talks with the Wnt/β-catenin signalling through steroid receptors (SR). The TGF-β signalling regulates Lef/Tcf driven transcription either through smads or through the Nemo-like Kinase (NLK) of mitogen-activated protein kinase (MAPK)

Further regulatory network modulating the Wnt/β-catenin signalling cascade is established through the interaction with the Delta/Notch signalling pathway. Wnt-responsive transcription factors of the Lef-1/Tcf family are involved in a direct regulation of the *Delta-like1 (Dll1)* gene of the Notch signalling pathway, thus providing a molecular link between the Wnt /β-catenin and the Notch signalling pathway in somitogenesis (Galceran et al., 2004). Also, Notch has been found to directly bind through its N-terminal tail to the active form of β-catenin thereby regulating β-catenin levels (Fig. 8). This modulation provides two functions: it establishes a threshold for Wnt/β-catenin signalling that is likely to play an important

role in the patterning of tissues and the assignation of cell fates during development (Martinez et al., 2002) and, in addition it provides a stringent regulation of the activated form of β-catenin. The second function might be crucial in pathological situations and might contribute to the understanding of Notch as a tumour suppressor (Radtke and Raj, 2003).

One important cross-talk demonstrates a negative feedback process between β-catenin and p53. Excess of β-catenin induces accumulation of active p53 (Damala et al., 1999). Reciprocally, activated p53 down-regulates β-catenin thereby counteracting oncogenic effects (Sadot et al., 2001) (Fig. 8). Disruption of this feedback process affects tumorigenesis driven by deregulated β-catenin activity. The high frequency of p53 inactivation observed in colorectal cancer (Peifer, 1997) and hepatocellular carcinoma (Taniguchi et al., 2002) underlies the significance of the mutual control mechanism. The fact that hTcf-4 is a direct downstream target of p53 (Rother et al., 2004) further supports the cross-regulatory mechanism (Fig. 8).

The binding of β-catenin and Lef/Tcfs to steroid receptors establishes a link between Wnt/β-catenin signalling to steroid signalling. A mechanism by which Wnt/β-catenin signals may regulate expression of a subset of androgen receptor (AR)-regulated genes during male development and in prostate cancer has been reported. This is mediated through a cooperative binding interaction between AR and Tcf-4 on regulatory elements of these genes. The AR/Tcf-4 complex serves as a potent transcriptional repressor in resting cells but is a very sensitive target for β-catenin signalling (Amir et al., 2003). Interaction between estrogen receptors (ER), Tcf-1, and Tcf-4 has also been reported. Whereas Tcf-4 antagonises ER activity in rat mammary cells Tcf-1 activity is synergistic. As Tcf-4 being the most prominent Tcf family member in the breast, it is suggested that *in vivo,* the antagonistic interaction is dominant in this tissue (El Tanini et al., 2001).

Reciprocally, steroid receptors have also been found to regulate the Wnt/β-catenin pathway. Competition between glucocorticoid repressors (GR) and Tcfs for β-catenin binding (Song et al., 2003) leads to repression of the Wnt/β-catenin pathway. This cross-talk is further emphasised by glucocorticoids which suppress Wnt/β-catenin signalling by inhibition of phosphorylalation of glycogen synthase kinase (GSK)-3β (Smith and Frankel, 2005) or by the enhancement of dickkopf-1 production in osteoblasts (Ohnaka et al., 2005).

In summary, the establishment and stability of cell fates during development depend on the integration of multiple signalling pathways. Whereas some of these pathways are synergistic, others antagonise each other. This makes it very important to search for binding partners that bridge the different pathways to Wnt/β-catenin signalling.

2. Aim of this Study.

The individual Lef/Tcf transcription factors have specific biological functions. This functional diversity is not explained by the known binding partners they recruit. Many of the known binding partners are not specific to any Lef/Tcf subtype. The hypothesis of this work is that since tissues are capable of responding to Lef/Tcf-mediated signals in a subtype-specific manner, then less conserved motifs within this family of transcription factors might act by recruiting different co-factors and this might be at the origin of their functional diversity. A candidate region for recruitment of these co-factors is the core region comprised between the β-catenin binding site and the HMG box. This core region is less conserved in the Lef/Tcf family of transcription factors and might render each family members unique in its function through recruitment of different co-factors.

The aim of this work is therefore to identify and characterize proteins that bind specifically to this core region of Lef/Tcfs and provide individual family members with specific characteristics. Because Wnt/β-catenin signalling is embedded in a complex regulatory network, identified proteins will be investigated for their role as adaptor proteins in the communication with other signalling pathways.

3. Materials.

Antibiotics:

- Ampicillin Na-salt (Serva, Heidelberg)
- Kanamycine (Amplichem, Darmstadt)
- Penicillin/Streptomycin (Invitrogen)
- Tetracycline (Sigma-Aldrich Chemie GmbH, Steinheim)

Antibodies:

- Anti Myc primary antibodies (Cell culture, Zoology Institute II)
- Goat anti mouse peroxidase secondary antibodies (Dianova, Dörentrup)
- Anti Lef-1 (Dinova)
- Anti Tcf-3 /-4 (Dinova)
- Anti Hic-5 (BD Biosciences)

Bacterial strains:

- **Bl21 DE3** strain: BL21 (DE3)*hsdS gal (c*I*ts*857 *ind*1 *Sam*7 *nin*5 *lac*UV5-T7gene*1*) (Promega GmbH, Manheim)
- **JM109** strain: endA1 recA1 gyrA96 thi hsdR17(rk-,mk+) relA1 supE44 I-D (lac-proAB) [F' traD36 proAB laqIqZDM15]. (Promega GmbH, Manheim)
- **XL1-Blue MRF**˘strain: D(mcrA)183 D(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac [F˘proAB lacIqZDM15 Tn10 (Tetr)]. (Stratagene)

Kits:

- ECL + Western Blotting Detection system (Amersham GmbH, Freiburg)
- pGEM^{R}-T Easy Vector System (Promega GmbH, Manheim)
- QIAGEN^{R} Plasmid Midi Kit (Qiagen GmbH, Hilden)
- QIAprep^{R} Spin Miniprep Kit (Qiagen GmbH, Hilden)
- QIAquick^{R} Gel Extraction Kit (Qiagen GmbH, Hilden)
- Random primed DNA labelling kit (Boerhringer, GmbH, Manheim)
- TNT^{R} SP6 Coupled Reticulosite Lysate System (Promega GmbH, Manheim)
- TNT^{R} T7 Coupled Reticulosite Lysate System (Promega GmbH, Manheim)

Chemicals:

Unles otherwise stated, all chemicals were of molecular biology grade and from Amplichem GmbH, (Darmstadt), Roth GmbH (Karlsruhe), Fluka Chemie GmBh (Taufkirchen), Merck GgaA (Darmstadt), Sigma Aldrich Chemie GmbH (Taufkirchen).

- Agarose (Invitrogen GmbH, Karlsruhe)
- Ammoniumpersulfate (Bio-Rad Laboratories GmbH, München)
- Bactotryptone (Invitrogen GmbH, Karlsruhe)
-Complete, Mini, EDTA-free protease Inhibitor cocktail tablets (Roche Diagnostics, Mannheim)
- [α^{32}P]dCTP (Amersham GmbH, Freiburg)
- 100bp DNA ladder Molecular weight marker (New England Biolabs, Frankfurt a. m.)
- 2-log DNA Molecular weight marker (New England Biolabs, Frankfurt a. m.)
- ^{35}S-methionine (Amersham GmbH, Freiburg)
- Deoxyribonucleoside triphosphates (dNTPs)(Promega GmbH, Manheim)
- Dithiotritol (DTT) (Invitrogen GmbH, Karlsruhe)
- Dulbecco´s modified eagle´s medium (DMEM) (BioWhittaker^{TM} Belgium).
- Fetal Calf serum (Invitrogen GmbH, Karlsruhe)
- Glass beads (425-600μm) (Sigma-Aldrich Chemie GmbH, Steinheim)
- Glutathione (GHS) sepharose 4B (Amersham GmbH, Freiburg)
- Nickel nitrilo triacetic acid (NTA)-agarose (Qiagen GmbH, Hilden)
- Phenol (Promega GmbH, Manheim)
- Rnasin^{R} ribonuclease inhibitor (Promega, gmbH, Manheim)
- Select agar (Invitrogen GmbH, Karlsruhe)
- Sephadex G50 spin columns (Amersham GmbH, Freiburg)
- Sodium deodecyl sulfate (SDS) (Serva Electrophoresis, Heidelberg)
- Tripsin / EDTA (Invitrogen GmbH, Karlsruhe)
- Tween-20 (Serva, Heidelberg)
- Yeast extract (USB Cleveland, USA)

Enzymes:

- Advantage polymerase (Clontech, Heidelberg)
- Dnase I Rnase-free (Roche Diagnostics GmbH, Manheim)
- Klenow (Promega GmbH Manheim)
- Rnase A (Amplichem, Darmstadt)

- Shrimp alkaline phospatase (SAP) (Promega GmbH, Manheim)
- T4 DNA ligase (Promega GmbH, Manheim)
- Reverse transcriptase (Gibco BRL GMBH, Karlsruhe)

Constructs:

- For subcloning in bacterial expression vectors:

-pET41b from D. Gradl (karlsruhe)

-pETM-30 from G. Stier (Heidelberg)

.pGex-Hic-5 from from M. Stallcup (South Callifornia)

- For eukaryotic cell transfection:

- Androgen receptor in pSG5 from A. Cato (Karlsruhe)
- Cytomegalovirus (CMV)-β- galactosidase from D. Gradl (Karlsruhe)
- mhic-5 in PSG5 from C. Chang (New York)
- mMBP1in pcDNA3.1 from W. M. Gallagher (Dublin)
- MMTV Luciferase reporter from Olver Kassel (Karlsruhe)
- TopFlash promoter from J. Hollander (Basel)
- Xfibronectin reporter in from D. Gradl (Karlsruhe)

- For Invitro transation:

	Polymerase used for translation
mLef-1 in pSP64T3 from J. Behrens (Erlangen, Germany)	T7
hTcf-3, hTcf-4, mTcf-3, hTcf-1in pcDNA3 from W. Birchmeier (Berlin)	T7
XLef-1, XTcf-3, XTcf-4 in pCS2-Myc (D. Gradl)	SP6
TOLLIP in pcDNA3.1 from S. Ghosh (Yale, USA)	T7
DLXIN-HA in pcDNA from S. Kani (Japan)	T7
ARC-105 pDH105 from Y. Kato (Tallahassee, USA)	SP6
ROR2-HA in pcDNA from S. Kani (Japan)	T7
RNF-4 in pcDNA3 from K.A Engelka (Philadelphia, USA)	T7

Equipment :

- Blot apparatus, Mini transblot (Bio-Rad, München)
- Centrifuges:
  - Biofuge fresco (Kendo, Langenselbold)
  - Multifuge^{R} 3S-R (Kendo, Langenselbold)
- Gel documentation:
  - DIANA II (Raytest, Straubenhardt)
  - Gel Max (Intras, Göttingen)
- Fluorecence binoculars:
  - MZFLII (Leica Microsystem, Bensheim)
  - Digital Camera Retiga Color 12 bit (QImaging, Burnaby, Kanada)
  - Software, Openlab 3.1.2 (Openlab, Heidelberg)
- Fluorecence microscope:
  - DMIRE2 and CTR MIC (Leica Microsystem, Bensheim)
  - Digital Camera C4742-95-12 ERG (Hamamatsu, Heidelberg)
  - Software, Openlab 3.1.2 (Openlab, Heidelberg)
- Hybridisation incubator 7601 (GFL, Burgwedel)
- Incubator shaker, orbital shaker (Thermo Forma, Marietta, USA)
- Luminometer, Lumat LB 9507 (Berthold Technologies, Bad Wilbad)
- PAGE apparatus, Mini Protean II^{TM} (Bio-Rad, München)
- PCR cycler, Personal cycler and UNO II (Biometra , Göttengen)
- pH meter pH 521 (WTW, Weilheim)
- Phosphoimager BAS 1500 (Fuji, Japan)
- Sonifier: UW2070 (Bandelin electronic, Berlin)
- Spectrophotometer, Bio-photometer (Eppendorf, Hamburg)
- Spectrophotometer, Ultraspec 2100 pro (Amersham, Freiburg)
- Water bath: Victor Recker (Krankenhaus-laborbedarf, Berlin)
- Weighing balances, LC2201P and ISO9001 (Satorius)
- Micropipette puller (Sautter Instruments, Novato, USA)

Primers:

All primers were synthesised by either biomers.net GmbH (Ulm) or BD Bioscience (Heidelberg).

-For cloning into BTM 116 vector :

Xlef1*Bam*HIFwd:	5´-ATAGGATCCTTAATGAGACTGAGATTATCCCC-3´
Xlef1*Pst*IRev :	5´-ATACTGCAGAGGTTTCTTTATGTGAGG-3´
XTCF3*Eco*RIFwd:	5´-ATAGAATTCTCAGAGGTGGAGAGGCGTCCC-3`
HMGBack3,4*Sal*I:	5´-ATAGTCGACYAGDGGYTTCTTWATGTGVGG-3´
XTCF4A,B,C.*Eco*RIFwd:	5´-ATAGAATTCTCCGAGACCGAGCGGCG-3
HMGBack3,4*Sal*I:	5´-ATAGAATTCTCCGAGACCGAGCGGCGACCCCCC-3`

-For cloning into pETM-30 expression vector:

Lef-1*Nco*1forw:	5´-TATGCCATGGAGACTGAGATTATCCCC-3´
TCF-3*Nco*1forw:	5´-TATGCCATGGAGGTGGAGAGGCGTCCC-3´
TCF-4NCbluntforw:	5´- GAG ACC GAG CGG CGA CCC-3´
HMGBACK2:	5´- ATA CTC GAG YAG DGG YTT CTTWAT GTG VGG-3´
HicLD*Nco*1Fwd:	5´- TCACCCATGGGTTAGG-3´
HicLD*Xho*1Rev:	5´- ATATCTCGAGGTGTGG-3´
HicLimNco1Fwd:	5´- GGCGCCATGGTAAACC-3´
HicLimXho1Rev:	5´- ATATCTCGAGTTCAGG-3´

- For cloning into PCS2-myc vector:

mHic*Xho*IIStart:	5´- CGTCTCGAGCACATGTCACGGTTAGGGGC-3`
mHic*Xho*I:IStop:	5´- CGACTCGAGTCACGCGAAGAGCTTCAGG-3`
mBP1*Xho*IFwd:	5´- ATATACTCGAGATGCTCCCTTTTGCC-3`
mMBP1*Xho*I Rev:	5´- TATATCTCGAGGAAGGTATAGGCTCC-3`
XHic*Nco*Istart:	5´- ATATCCATGGTAATGTCGAATACGACG-3`
Xhic*Xho*IStop:	5´- TATACTCGAGCTACCCATACAGTCGGG-3`

-For sequencing clones in pVP16 vector:

pVP16 Fwd:	5´-GGT ACC GAG CTC AAT TGC GG-3`
pVP16 Rev:	5´-AGC TAG CTT CTA TCT ATC TAG CG -3´

Libraries:

-Mouse Day 10 cDNA library.

The library screened was kindly provided by Jürgen Behrens. The library constructed is in the *Not*I site of pVP16 vector and in frame with the VP16 protein (AD). The plasmid carries an ampicillin-resistance gene for antibiotic selection and a leucine gene for growth selection in cultured media. It also has an NLS that allows for nuclear targeting of the hybrid protein.

-*Xenopus* Tad pole stage λ-zap cDNA library.

This was kindly provided by Michael Kühl (Ulm).

Solutions:

- Bacteria Culture Media:

LB-Agar:

1.5% Agar in LB medium. Autoclave at 120 °C for 20 min.

LB medium:

10g Bactotryptone, 5g yeast extract, 10g NaCl, add water to 1l. Autoclave at 121°C, 20 min. Store at +4°C.

SOB medium:

20g Bactotryptone, 5g yeast extract, 0.5g NaCl, 2.5g $MgCl_2.6H_20$, add distilled water to 1l. Autoclave at 121°C, 20 min. Store at +4°C.

2YT-agar:

2YT medium, 1.5% agar.

2YT medium:

16g tryptone, 10g yeast extract, 5g NaCl. Autoclave at 121°C, 20 min. Store at +4°C.

- Bacteria Transformation Media:

TFB1

30 mM potassium acetate, 10mM $CaCl_2$, 50mM $MnCl_2$, 100mM RbCl, 15% Glycerol, adjust pH to 5.8 with acetic acid. Filter-sterilize (0.2 μm).

TFB2

100 mM MOPS, 75mM $CaCl_2$, 10mM RbCl, 15% Glycerol, adjust pH to 6.5 with 1M KOH, Filter-sterilize (0.2 μm).

- DNA Analysis Buffers:

TAE:

40 mM Tris/acetate, pH 8.3, 1mM EDTA.

TE buffer:

10 mM Tris-Cl, pH 7.5 1 mM EDTA.

- Phage library Screen Media:

Denaturing solution:

0.5 M NaOH, 1.5 M NaCl.

Hybridisation solution:

6x SSC, 5x Denhardt´s solution, 1% SDS, 100μg/ml Salmon sperm DNA.

Neutralisation solution:

0.5 M Tris-HCl pH 7.0.

1.5M NaCl.

Pre- hybridisation solution:

6x SSC, 0.5% SDS, 100μg/ml Salmon sperm DNA.

20x SSC:

3 M NaCl, 300 mM Na-citrate pH 7.0. Autoclave at 121°C, 20 min.

SM buffer:

0.58g NaCl, 0.2g $MgSO_4.7H_20$, 5.0 ml of 1M Tris-HCl (pH7.5), 0.5ml of 2% (w/v) gelatin, add distilled water to a final volume of 100ml. Autoclave at 121°C, 20 min. Store at +4°C.

- Protein Analysis Media:

Bacteria Lysis buffer:

400 mM NaCl, 50 mM NaH_2PO_4,,15 mM Imidazole, adjust pH with NaOH to 8.

Destain:

Stain solution without Coomassie Blue

Dialysis buffer:

400 mM NaCl, 50 mM NaH_2PO_4, 10% Glycerol, 5mM $MgCl_2$.

Elution buffer 1:

400 mM NaCl, 50 mM NaH_2PO_4, 50 mM NaH_2PO_4, 100 mM Imidazole, Adjust pH with NaOH to 8.0

Elution buffer 2:

400 mM NaCl, 50 mM NaH_2PO_4 , 200 mM Imidazole, adjust pH with NaOH to 8.0.

Elution buffer 3:

400 mM NaCl, 50 mM NaH_2PO_4 300 mM Imidazole, adjust pH with NaOH to 8.0.

1x PBS:

137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2PO_4, 1.5 mM KH_2PO_4, pH7.5.

PBS-Tween.

1x PBS, 0.05% (v/v) Tween 20.

NOP buffer:

10 mM Tris/HCl pH 7.8, 150 mM NaCl, 1 mM $MgCl_2.6H_20$, 0.75 mM $CaCl_2.2H_20$, 2% Nonidet P_4O. Store at +4°C.

Reduction solution:

10% ethanol, 0.25% of 37% Formaldehyde, 6% citric acid.

SDS Sample buffer (6X):

300 mM Tris.Cl (pH 6.8), 600mM dithiothreitol, 12% SDS, 0.6% Bromophenol blue, 60% glycerol.

Silver solution:

1). Dissolve 0.6g $AgNO_3$ in 10 ml water

2). 1.9 ml 1 M NaOH, 15 ml 32% Ammoiaque, add 96 ml water.

Drop 2) in 1) until the solution becomes colourless and fill up to 100ml with distilled water. Protect against light.

Coomassie Stain.

0.25g Coomassie Brilliant Blue R250, 90ml Methanol:water (1:1), 10 ml acetic acid.

Transfer buffer:

25 mM Tris, 192 mM glycine, 20% (v/v) methanol.

Tris/ glycine Electrophoreses buffer.

25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS.

Wash buffer:

400 mM NaCl, 50 mM NaH_2PO_4, 20 mM Imidazole, Adjust pH with NaOH to 8.

Reporter Gene Assay Media:

β-galactosidase 100x Mg solution:

0.1 M MgCl2, 4.5 M ß-Mercaptoethanol.

Luciferase assay buffer:

20 mM tricine, 1.07 mM $(MgCO_3)_4$ $Mg(OH)_2.5H_20$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT.

5x Luciferase assay cell lysis buffer:

125 mM tris, pH 7.8 (corrected with H_3PO_4), 10 mM CDTA, 10 mM Dithiotritrol (DTT), 50% glycerin, 5% triton.

Luciferase assay substrate for 10 ml assay buffer:

2.12 mg Coenzyme A, 1.32 mg Lusiferin, 2.92 mg ATP.

1x O-nitrophenyl-ß-Galactopyranoside (ONPG) solution:

6µl of 100x Mg solution, 132µl 1x ONPG, 60µl cell extract, 402µl of 0.1 M Sodium Phosphate, pH 7.5.

0.1 M Sodium Phosphate, pH 7.5:

41 ml 0.2 M $Na_2HPO_4.2H_2O$, 9 ml 0.2 M $NaH_2PO_4.H_2O$, 50 ml H2O.

- Yeast Culture and Analysis Media:

10 x LiAc:

1 M Lithium acetate.

10 x TE:

100 mM Tris pH 7.5, 10 mM EDTA.

Yc Medium:

0.12% yeast nitrogen base, 0.5% ammonium sulfate, 1% succinic acid, 0.6% NaOH, 2% glucose, 0.1% (adenine, arginine, cysteine, leucine, lysine, threonine, tryptophan, uracil), 0.005% aspartic acid, histidine, isoleucine, metheonine, phenylalanine, proline, serine, tyrosine, valine). Autoclave at 121°C, 20 min, cool to < 50°C and add ampicillin to 50µg/ml, Store at +4°C.

Yc Medium-Agar:

Yc medium, 2% agar. Autoclave at 121°C, 20 min, cool to < 50°C and add ampicillin to 50µg/ml. Pour plates and allow to harden. Store at +4°C.

YEPD medium:

1% yeast extract, 2% tryptone, 2% Dextrose (D-glucose). Autoclave at 121°C, 20 min, cool to < 50°C and add antibiotic to 50µg/ml, Store at +4°C

Yeast lysis buffer:

2.5 M LiCl, 50 mM Tris.HCl, 4% Triton X-100, 62.5 mM EDTA.

Z-buffer:

60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0.

1x LiAc/40% PEG-3350/1xTE:

100 mM Lithium Acetate, 40% PEG-3350, 10 mM Tris-HCl, pH 7.5. Autoclave at121°C for 20 min.

-Cell Culture media:

APBS:

2.7 mM KCl, 0.15 mM KH_2PO_4, 103 mM NaCl, 0.7 mM Na_2PO_4, pH 7.5.

Blocking solution:

1% BSA in APBS/ 2 mM $CaCl_2$.

Danaturation solution:

1% Triton in APBS/ 2 mM $CaCl_2$.

Fixing solution:

10% Paraformaldehyde in APBS/ 2 mM $CaCl_2$.

10x HeBS:

1.36 M NaCl, 0.05 M KCl, 7 mM Na_2HPO_4, 1% (w/v) glucose, 0.2 M Hepes, pH 7.0 adjusted with NaOH

1x HeBS:

1: 10 dilution of 10x HeBS, pH 7.12 adjusted with NaOH.

Wash solution:

APBS/ 2 mM $CaCl_2$.

- Embryo Cultivation and Manipulation Solutions:

10x MBSH (Modified Barth's Saline): 880 mM NaCl, 100 mM HEPES, 10 mM KCl, 24 mM $NaHCO_3$, 8 mM $MgSO_4$, 4 mM $CaCl_2$, 3.3 mM $Ca(NO3)_2$ (pH 7.3)

Cysteine solution: 2% (w/v) L-Cysteine Hydrochloride in 0.1x MBSH (pH 8.2)

Human Chorionic Gonadotropin (HCG): 3300 IU/mg; stock solution: 1 mg/ml in sterile 0.5% NaCl.

4. Methods.

4.1 Molecular biology methods

4.1.1 DNA methods

4.1.1.1 DNA Purification.

DNA purification can be carried out by binding to silica gel matrix under high salt condition and low pH followed by washing steps and DNA elution under low salt concentration and basic pH.

For purification of DNA fragments from agarose gels, this was done using the QIAGEN QIAquick^{R} gel extraction kit. Each DNA fragment is excised from the gel using a sharp clean blade and transferred into a clean 1.5ml tube. The gel is melted at 50°C in 3 gel volumes of buffer QG and then 1 volume of isopropanol added. To bind DNA to the silica matrix, the sample is applied to the QIAquick column, centrifuged at 13,000 rpm for 1 min to eliminate impurities and washed with buffer PE. The bound DNA is eluted with nuclease-free water in a volume of 50μl.

For purification of plasmid DNA, QIAGEN small-scale purification (Minipreps) or large scale purification (Midipreps) were employed depending on necessity.

For purification of PCR products, the QIAquick PCR purification kit (Qiagen) was used. To one volume of PCR product, 5 volumes of DNA binding buffer (PB) are added and the mixture is applied to the QIAquick column. Following centrifugation at 13,000rpm to eliminate impurities, the column-bound DNA is washed with 0.75 ml of buffer PE. DNA is eluted with nuclease-free water in a volume of 30μl.

4.1.1.2 Sequencing

DNA sequencing was done using the enzymatic method of Sanger et al., in 1977 which is based on *in vitro* DNA synthesis carried out in the presence of chain-terminating nucleoside triphosphates. This method uses standard primers (T3, T7, SP6, M13) or sequence-specific primers and four dideoxyribonucleoside triphosphates as terminators in separate DNA synthesis reactions on the same primed single-stranded DNA template. These four dideoxyribonucleoside triphosphates are covalently linked to different fluorescent dyes and blocks the addition of subsequent deoxyribonucleoside triphosphate and thus prevents their incorporation into a DNA chain due to the absence of a 3' hydroxyl group. As a result, the chain-extension reaction yields a series of DNA fragments in each of the tubes.

These fragments are then separated in four parallel lanes by polyacrylamide gel electrophoresis. This leads to a series of bands, each with the fluorescence spectrum indicating a successive base in the DNA being sequenced.

4.1.1.3 Quantification of DNA and RNA

Due to their characteristic absorption maximum at 260 nm, nucleic acids can be photometrically quantified. Contaminations with proteins which have an absorption maximum at ultraviolet wavelengths of 280 nm can influence the result. Therefore, the ratio of absorptions at both 260 nm to absorption at 280 nm (A260/A280) greater than 1.9 for DNA, or greater than 1.8 for RNA is indicative of samples that are free of these contaminants. For uncontaminated nucleic acid solutions the law of Lambert-Beer is applied: 50 µg/ml double-stranded DNA has an OD_{260} of 1.0 while 40 µg/ml single-stranded DNA or RNA has an OD_{260} of 1.0.

4.1.1.4 Restriction digestion of DNA

This was done according to Sambrook et al, 1989. In a final volume of 20µl, plasmid DNA (10µg) is digested overnight at 37°C using appropriate restriction enzyme (1U), buffer (1x) and water. The digests are separated on a 1% agarose gel in 1x TAE buffer at 100V.

4.1.1.5 Dephosphorylation of 5´- Phosphate residues from DNA

Shrimp alkaline phospatase (SAP) removes 5´-Phosphate residues from DNA and prevents vector self-religation.

In a 1.5 ml reaction tube 2.5 µg of linearised vector DNA is incubated at 37°C for 30 min. with 1x SAP buffer, 0.5U SAP and water in a final volume of 20µl. The reaction is further incubated with additional 0.5U SAP 37°C for 30 min. The reaction is then stopped by inactivating the enzyme at 65°C for 15 min. 20ng of the dephosphorylated plasmid is used for ligation reactions.

4.1.1.6 Polymerase Chain Reaction (PCR)

Amplification was performed with Advantage polymerase mix which is a mixture of Taq polymerase and Deep Vent polymerase with a proofreading activity to lower error frequency.

To a final volume of 50µl, reaction buffer (1x), nucleotide mix (2.5mM each), primer (25µM each) $MgSO_4$ (25mM), 10ng DNA template, 1U Advantage Enzyme mix and water is added. The PCR is run for 36 cycles with an initial predenaturation step of 94°C, 2 min. All cycles are comprised of a denaturation step of 94°C, 2 min., an annealing step of 55°C, 2 min. and a polymerisation step of 72°C but with a time of 2 min that increases every three cycles by 20 sec. Following a final elongation step of 7 min that terminates the reaction the sample is stored at 4°C until analysed on an agarose gel.

4.1.1.7 Recovery of DNA from agarose gels

Nucleic acids have a consistent negative charge and can be separated in an electric field based on the difference in their nucleotide lengths. In Ultra-violet light DNA bands can be detected by fluorescent ethidium bromide which intercalates between the stacked bases of DNA (Sharp et al., 1973).
The digest or PCR product are analysed on a 1% (w/v) agarose gel in 1x TAE buffer containing 0.05mg/ml ethidium bromide at 100V. The gel is visualised under long wavelength ultra-violet (312 nm) lamp in order to minimise radiation damage to DNA. The fragment sizes are characterised by comparison with the Ready-Load™ 100bp DNA ladder standard molecular weight marker. Bands are documented with the Gel Max (Intras) or Diana II (raytest) documentation system.

4.1.1.8 Sticky-End Ligation of DNA Fragments into Plasmids

Ligation of cohesive DNA termini can be achieved by T4 DNA ligase which associates the free 3´-hydoxyl terminus of one nucleotide of a DNA fragment to the free 5´-phosphate terminus of another. Sticky-end ligation has the advantage of controlled insert integration with predefined orientation. Ligation is done as described by Sambrook et al., 1989.
Briefly, 100pg of linearised dephosphorylated plasmid vector to insert ratio of 1:5 is incubated with T4 DNA ligase (1u/10µl) and 1X ligase buffer in a total volume of 10µl at 14°C for 16h. 5µl of the ligation mixture is used in transforming 100µl competent *E. coli* strain JM109 cells prepared by Calcium Chloride method.

4.1.1.9 DNA probe Labelling

A labelled radioactive probe was used for screening of the *Xenopus* tail bud library. This labelling is done using the Random primed DNA labelling kit and is based on the hybridisation of a mixture of all possible hexanucleotides to the DNA to be labelled. The probe is synthesised from the 3´OH termini of the random hexanucleotide primer using Klenow enzyme. Modified deoxnucleoside triphosphates eg ^{32}P-dCTP present in the reaction mixture are incorporated into the newly synthesised complementary DNA strand.

A 510 bp fragment of Hic-5 previously obtained by RT-PCR from *Xenopus* tissue culture cell (XTC) using degenerate primers is denatured at 100°C for 10 min. Into a 1.5 ml reaction tube, 25ng of the denatured DNA, 1μl each of 0.01 mM dATP, dGTP, dTTP, 2μl of 10x reaction mixture, 5 x10^{-6} mM [α^{32}P] dCTP, distilled water is added up to 19μl and 1μl of 2U Klenow enzyme. The mixture is incubated at 37°C for 30 min. and the reaction stopped by heating to 65°C for 10 min. Removal of non-incorporated dexoxyribonucleoside triphosphates is performed by chromatography on Quick Spin Column, Sephadex^{R} G-50 (Amersham).

4.1.1.10 Determination of the appropriate phage dilution to use for plating

It is necessary to determine the appropriate phage dilution to use for plating in order to avoid confluence of plaques. Cells previously stored in 10mM $MgSO_4$ are further diluted to an OD_{600nm} of 0.5 with 10mM $MgSO_4$ and 7 samples of 200μl taken into sterile 15ml reaction tubes. To these tubes, 100μl of phage library diluted in phage storage medium (SM) with dilutions ranging from 1:10^3-1:10^9 are added respectively. Cells are incubated at 37°C for 20 min to allow the phage to attach to the cells and plated on 150 x 20mm 2YT agar plates with 3ml 2YT top agar. Plates are allowed for the agar to set for 10 min. and then incubated for 6-8 h at 37°C for plaques to appear. The phage dilution of 1:10^5 was found to give about 5,000 plaque forming units (pfu) per plate and without confluence. This dilution was subsequently used for plating.

4.1.1.11 Plaque Lifts

The phage library is diluted 1:10^5 in SM buffer and 100μl used to infect 16 samples of 200ml each of XL-1B *E. coli* at an OD of 0.5 in 10mM $MgSO_4$. samples are

incubated at 37°C for 20 min. and plated with 3ml 2YT top agar on at least two days old 2YT agar plates and incubated at 37°C for 8h. The plates are placed at 4°C overnight to harden so as to prevent subsequent sticking of agar onto the nitrocellulose membrane. Plates are given numbers and oriented by punching through the agar with a cut pipette tip. To allow transfer of colonies to the nitrocellulose membrane, a circular nitrocellulose membrane is placed on each the plates for 2 min. The membrane is also given corresponding numbers and oriented by pricking through the membrane with a needle to correspond to the punched agar wells. The membrane is lifted and the bound DNA is denatured in denaturing solution for 2 min., neutralised in neutralisation solution for 2 min. and rinsed for 30 sec in 0.2M Tris-HCl (pH7.5)/2x SSC. The membrane is blotted on Whatman 3MM paper and DNA cross-linked to it by backing in the oven at 85°C for 2h. Filters are blocked in 50ml prehybridisation buffer for 4h at 65°C with rotation and incubated with 10^{-6} mM ^{32}P-labelled probe in 50ml hybridisation buffer. Filters are washed successively with 50ml changes of 6x SSC/0.1%SDS, 2x SSC/0.1%SDS and 0.2x SSC/0.1%SDS at 65°C for 2h each wash with rotation. Filters are then dried and exposed to the phosphoimaging plate overnight and revealed by a phosphoimager. The image is superimposed with the corresponding agar plate and colonies giving signals are cored and stored in 1ml phage storage medium (SM) containing 40ml chloroform at 4°C for phages to be eluted from the agar. The eluted phages are further purified in three rounds of plaque lifts.

4.1.1.12 *In-vitro* excision

The λ-Zap Library is designed such that a pBluescript vector carrying the library insert is cloned between a replication initiation site and a replication termination site in the λ-Zap II vector. Inside *E. coli* the filamentous (f1) bacteriophage (helper phage) proteins recognise these replication initiation sequences and introduces a nick at that point so that replication starts. This replication continues until it reaches the termination sequence where it stops. The single stranded DNA comprised of the pBluescript and the insert is circularised and automatically creates a functional origin of replication as in bacterophages and can be replicated. This procedure circumvents the sub cloning step.

Separate 50ml overnight cultures of XL1-Blue cells are cultured overnight at 30°C in LB medium containing 50μg tetracycline. Cells are harvested at 1,000xg and resuspended in 25ml 10 mM $MgSO_4$ and further diluted with 10 mM $MgSO_4$ for OD_{600nm} to read about 1.0. In a 15ml tube each, 200μl XL1-Blue cells (OD_{600nm} of approximately 1.0), 250μl of phage stock and 1μl helper phage (R408) are added and incubated at 37°C for 15 min. to allow phage to attach to the cells. 5ml 2YT broth supplemented with tetracycline are added and tubes incubated at 37°C for 3h with shaking. The tubes are heated at 70°C for 20 min. to lyse the λ-phage particles and spun at 4000g for 15 min to pellet debris. The supernatant containing the pBluescript phagemid packed as filamentous particles is decanted into sterile 15ml tubes. To 200μl of XL1-Blue cells in a 1.5ml reaction tubes, 10μl of the pBluescript phagemid are added and incubated at 37°C for 15 min. Samples are plated on 50μg/ml ampicillin agar plates and incubated overnight at 37°C. The colonies that grow are those that have been transformed by the pBluescript. Colonies are re-grown in liquid culture medium and pBluescript extracted by miniprep method, sequenced and blasted against the NCBI data bank.

4.1.2 RNA Methods

4.1.2.1 RNA extraction from *Xenopus* embryos

Total RNA from whole embryos are isolated using Trizol Reagent. Injected embryos are allowed to develop to the desired stage and are then used for RNA isolation or immediately frozen in liquid nitrogen and stored at -70°C for later use.

1 ml of Trizol Reagent is sufficient for the total RNA isolation from 10-20 embryos, equivalent to 50-100 mg of tissue. Trizol Reagent is a mixture of phenol and guanidine isothiocyanate, and is able to dissociate nucleotides from proteins and maintain the RNA integrity. After washing the ultrasonic homogeniser pistil in Trizol Reagent and rinsing in water whole embryos samples are rapidly homogenized in 500μl of Trizol Reagent until all pieces are crushed. For removal of proteins, 100μl chloroform are added to the homogenate and vigorously agitated by hand for 15 sec. After centrifugation for 15 min. at 10,000g (maximum speed) at 4°C, the upper aqueous phase contains the RNA and is transferred into a sterile tube. A second chloroform extraction is performed and RNA is precipitated at -80 °C for at least 1h with two volumes of absolute ethanol and 1/10th volume 3M sodium acetate solution.

Following 30 min. centrifugation at 10,000g the RNA pellet is washed with 70% ethanol, dried at RT re-dissolved in an appropriate volume of DEPC H_2O.

4.1.2.2 *In vitro* transcription

4.1.2.2.1 Reverse transcription PCR

The reverse transcriptase, a retroviral DNA polymerase, uses RNA as a template to transcribe isolated mRNA into cDNA which can be later used for PCR. This RT-PCR permits semi-quantitative analysis of expression of RNAs of interest during embryonic development when compared to a standard housekeeping gene.

Into a nuclease-free microcentrifuge tube 1 µg of total RNA and 1µl Random Hexamer (0.5 µg/µl) are added. After mixing by pipetting, the mixture is heated at 70°C for 10 min. and quickly chilled on ice to allow Random Hexamer to bind to mRNA. The sample is briefly centrifuged and 4 µl of 5x *first strand* buffer, 2 µl 10 mM DTT, 1 µl 10 mM dNTP mix and 1U RNase inhibitor are added to the sample. After gently mixing, 0.5µl (100 U) of reverse transcriptase (Superscript II, Gibco BRL) is added. The reaction is incubated at 42°C for 50 min. then inactivated by heating at 70°C for 15 min. and stored at -70°C or used directly for PCR. To control specificity of reverse transcription, -RT is also performed using all the other components together with DEPC H_2O instead of the reverse transcriptase.

4.1.2.2.2 Preparation of mRNA for injection

Messenger RNA (mRNA) can be transcribed from a DNA template using RNA polymerase and free ribonucleoside triphosphates. mRNA was prepared using the mMESSAGE mMACHINE^{TN} kit (Ambion Ltd)

In a total reaction volume of 20µl, linearised and PCR-purified plasmid (1µg) is incubated for 2h at 37 °C with 2x Nucleoside triphosphates, 1x reaction buffer, 1 unit RNA Polymerase enzyme specific for SP6 or T7 and water. The DNA template is then digested by adding10U DNase 1 and incubating further for 15 min. at 37 °C. The RNA is cleaned by passing it over a RNeasy column (Qiagen) and eluting with 30µl of RNase-free water.

4.1.3 Protein Biochemistry Methods

4.1.3.1 Induction of encoded peptides

Large amounts of eukaryotic proteins can be obtained in *E. coli* BL21 DE3 by placing the gene under the control of a Lac-Z operon in a T7 RNA polymerase vector. The lactose analogue Isopropyl-(β)-D-thiogalactopyranoside (IPTG) can then be used to derepress the promoter for the gene to be expressed.

An overnight culture of a single transformed colony is diluted 1: 100 in 500ml LB medium containing the appropriate antibiotic and cultured at 37°C with vigorous shaking until the OD_{600} read 0.7. Protein expression is induced by adding IPTG to a final concentration of 1 mM and shaking continued at but 30°C (to reduce protein degradation) for 4 h. An aliquot of 300μl is taken out after the 4h induction, centrifuged at 13,000 rpm for 1 min., re-suspended in 60μl SDS PAGE loading buffer and heated at 95°C for 20 min. This sample is run on a 10% SDS PAGE gel and induced protein band of interest identified. The rest o the bacteria is centrifuged in aliquots of 100ml at 5,000rpm for 5 min. and pellet frozen at -80°C until used for protein extraction.

4.1.3.2 Protein extraction

Transformed and frozen bacteria are thawed on ice for 30 min. and re-suspended in1ml of PBS supplemented with 400 mMNacl containing protease inhibitors. To disrupt the cell wall lysozyme is added to a concentration of 1mg/ml and incubated for 30 min on ice. To shear RNA and DNA, RNAse A, and DNAse I are added to a final concentration of, 10μg/ml and 5μg/ml respectively and the sample incubated on ice for 15 min. The sample is transferred to 2 ml reaction tubes and centrifuged at 13,000g for 30 min. to eliminate cell debris. The supernatant (crude lysate) is saved as 200ml crude lysate samples in liquid nitrogen until used for protein purification or GST pull-down assays.

4.1.3.3 Purification of Histidine-tagged proteins

Recombinant proteins provided with a 6x Histidine-tag can coordinatively bind to transition metals (Ni, Co, Zn) attached to an agarose column. Proteins without the 6x Histidine-tag bind weakly and can be washed off with low imidazole concentrations.

The bound recombinant protein can be eluted with high concentrations of imidazole whose structure is similar to that of histidine and thus competes with the histidine side chain for binding to the metal.
200ml crude bacterial lysate of induced proteins are rotated with 50µl of washed Nickel nitrilo triacetic acid (NTA)-agarose in binding buffer pH 8.0 at 4°C for 2h. Following 3 times washing steps each with 2ml wash buffer the protein is eluted 3x with 1ml elution buffer. The eluted protein is dialysed with 3x 200ml dialysis buffer to eliminate the imidazole. Samples are stored as 200µl samples in liquid nitrogen until used for GST pull-down experiments.

4.1.3.4 Immobilisation of GST-tagged proteins on sepharose columns

Glutathione-S-transferase (GST) enzymes catalyse the detoxification of reactive electrophiles through the reactive cysteinyl residue in their cofactor glutathione (GSH). The high affinity of GST for GSH has been employed in the purification of GST-tagged proteins on GSH sepharose gels.
200ml crude bacterial lysate of induced proteins are incubated with 50µl of washed GSH sepharose gel in phosphate buffered saline (PBS) supplemented with 400 mM NaCl containing protease inhibitors at 4°C for 2h. Following three washing steps each with 2ml PBS supplemented with 400 mMNacl the gel is used for GST pull-down assays.

4.1.3.5 GST Pulldown assays

Bacterially expressed GST-tagged proteins are immobilised on 50µl of Glutathione sepharose 4B beads for 2h at 4°C in NOP buffer and incubated with NOP-lysates of transfected or untransfected human kidney epithelial cells (h293). Binding reactions are rolled for 2h at 4°C and washed 3 times with NOP buffer. Following 3x 15 min. washing steps, the beads are boiled in SDS sample buffer for 5 min. and subjected to 10 or 12 % SDS PAGE and run at 100V. At the end of the run the gel is stained with either Coomassie brilliant blue or Silver. For Coomassie-stained gels the band of interest is cut out, digested with tripsin and analysed by MALDI-TOF. MALDI-TOF analysis was done in collaboration with Marc Genzel, EMBL, and Heidelberg. For Western blotting proteins are transferred onto nitrocellulose membrane, probed with anti myc antibody 9E10 and revealed by chemiluminescence reaction.

^{35}S-labelled *in vitro*-translated proteins are also incubated with GST-tagged proteins immobilised on sepharose beads in NOP buffer. Following five washes with NOP buffer and elution with 20μl SDS sample buffer, the proteins are separated on a 10% SDS PAGE gel. After Coomassie staining the gel is subjected to phosphorImager analysis to visualise bound protein.

4.1.3.6 *In vitro*-translation

The use of in vitro translation systems have advantages over in vivo gene expression when the overexpressed product is toxic to the host cell, when the product is insoluble or forms inclusion bodies, or when the protein undergoes rapid proteolytic degradation by intracellular proteases. The *in-vitro* translated protein can be radio-labelled with ^{35}S-Methionine so that emitted radioactive rays can be detected by a phosphoimaging film.
In-vitro translation of protein was done as described by the TNT^{R} coupled reticulocyte lysate system (Promega).

4.1.3.7 Sodium Deodecyl Sulphate (SDS) Polyacrylamide Gel Electrophoresis (PAGE)

Under denaturing conditions proteins are separated in to their individual polypeptide subunits. Most commonly, the strongly anionic detergent SDS in combination with reducing agents and heat are used to dissociate the protein before they are loaded on the gel. The SDS molecules interact with the proteins to form negatively charged complexes containing a constant ratio of approx. 1.4mg of SDS per mg of protein. At this ratio, the charge density per unit length of proteins is equal. As a result, all proteins are separated in accordance with charge density and consequently with respect to the sizes of the polypeptides. By using markers of known molecular weight, it is therefore possible to estimate the molecular weight of the polypeptide chains. In this work a 10 or 12% separating gel and a 5% stacking were prepared according to the table 1.

Table1: Recipe for preparation of the Stacking gel (5%) and the separating gel (10% or 20%).

Solution	5% Stacking Gel	10% Separating Gel	12% Gel Separating Gel
Water	1.4ml	1.9 ml	1.6 ml
29% Acrylamide,1% bisacrylamide	0.33 ml	1.7 ml	2.0 ml
1.5 M Tris-Cl pH 8.8	-	1.3 ml	1.3 ml
0.5 M Tris-Cl pH 6.8	0.25 ml	-	-
10% SDS	0.02 ml	0.05 ml	0.05 ml
Ammonium persulfate (1mg/ml)	0.02 ml	0.05 ml	0.05 ml
TEMED (Tetramethyetheledіamine)	0.002 ml	0.002 ml	0.002 ml
Total volume	2.0 ml	5.0ml	5.0ml

The separating gel is poured between two glass plates and allowed to polymerise at room temperature (RT). In a similar manner the stacking gel is poured and a ten-teeth comb inserted. The gel is allowed to polymerise and the comb removed to create wells in which the samples will be applied. The gel is mounted into the electrophoresis apparatus containing 1x Tris-glycine electrophoresis buffer and 10-20μ samples applied. The gel is run with at 100V and is either blotted, stained with Coomassie Brilliant Blue R250 or by the silver method to reveal bands.

4.1.3.8 Coomassie Staining

Coomassie Brilliant Blue R250 is a dye that binds to proteins by hydrophobic and ionic interactions and can be used for detection of protein amounts as less as 100ng. The gel is immersed in at least 5 gel volumes of dye and slowly rocked for about 20 min and rinsed with tap water. The gel is distained in three changes of distain solution dried and scanned for documentation.

4.1.3.9 Silver Staining

Silver ions form complexes with aspartic acid, cystein and glutamic acid and are reduced by alkaline formaldehyde to elemental silver resulting into a brown staining. Silver staining is at least 100 fold more sensitive than coomassie since protein amounts as less as 0.1ng protein can be detected.

The gel is fixed overnight in 100ml methanol: acetic acid: water (50:12:38). The gel is then washed in 100ml ethanol: acetic acid: water (10:5:85) for 20 min and then in excess water for 20 min. It is reduced with100ml Na_2B4O_7 (1.9%w/v)/4% gluteraldehyde/distilled water and washed 3x 20 min. with distilled water. The gel is incubated in silver solution for 20 min in the dark in order to avoid oxidation by light. After 4 washing steps of 1 min. each, bands are developed in reduction solution under visual control. The reaction is then stopped with 7% acetic acid, washed 2x 20 min. with water, stored in 7% acetic acid for eventual documentation.

4.1.3.10 Analysis of protein bands from SDS PAGE by mass spectrometry

This is a procedure where a protein or peptide is degraded under controlled conditions using a chemical reaction or specific enzymes. After purification by means of High Performance Liquid Chromatography (HPLC), protein fragments subjected to a Matrix-Assisted Laser-Desorption Ionization (MALDI). MALDI is based on the bombardment of sample molecules into ions with a laser light to bring about ionization. The sample is pre-mixed with a highly absorbing matrix compound which transforms the laser light into excitation energy leading to sputtering of analyte and matrix ions. In this way the analyte molecules are spared from excessive direct energy which may otherwise cause decomposition. The Time Of Flight (TOF) analyser then separates the ions according to their mass (m) to charge (z) ratio by measuring the time it takes for ions to travel through a region known as the flight or drift tube. The heavier ions move slower than the lighter ones. TOF values are then matched with those of the World Wide Web databases where masses of proteolytic peptides expected from enzymatic degradation of proteins of known sequences can be found.

4.1.3.11 Western Blotting

Electrophoretically separated proteins can be transferred from a gel to a solid support (nitrocellulose membrane) and probed with antibodies that specifically recognise the proteins.

At the end of the SDS page run, the gel is removed and placed in transfer buffer. Four pieces of Whatman 3MM paper and a piece of nitrocellulose (NC) membrane are cut to the exact size of the SDS polyacrylamide gel. The NC membrane, pieces of Whatman 3MM paper and two porous pads are allowed to soak in transfer buffer

by capillarity in order to avoid trapping of air bubbles. The electrodes are assembled to sandwich the gel, NC membrane, pieces of Whatman 3MM paper and the two porous pads in the following order:

Cathode

Porous pad

Two pieces of Whatman 3MM paper

Nitrocellulose membrane

Gel

Two pieces of Whatman 3MM paper

Porous pad

Anode.

The set is mounted into a Western blotting apparatus (Bio Rad) containing electrode buffer and equipped with a cooling system. Transfer is done at 111V for 1h.

4.1.3.12 Staining proteins immobilised on nitrocellulose (NC) membrane by Ponceau S.

To provide visual evidence that transfer of proteins has taken place the NC membrane is stained with Ponceau S which specifically binds to protein band giving them the characteristic purple colour.

Proteins are transferred onto transfer NC membrane and incubated with Ponceau S for 5-10 min. with gentle agitation. The membrane is then washed with several changes of distilled water at room temperature and the positions of proteins used as molecular weight standards are marked with a waterproof ink. The membrane is then proceeded to immunological staining.

4.1.1.13 Immunological staining

Proteins are transferred onto nitrocellulose membrane and potential non specific binding sites of the membrane were blocked in 5% (w/v) non fat dried milk for 1h and the filter incubated overnight with rotation in anti-myc primary antibody (9E10). Following 3x 15 min washes in PBS/0.05%Tween 20 the filter is incubated for 2h at RT with gentle agitation in the secondary antibody (Goat anti-mouse antibodies coupled to horse radish peroxidase). Following three washing steps of 15 min each in PBS/Tween, bands are revealed by chemiluminescence reaction (ECL, Amersham).

4.1.1.14 Chemiluminescence

Luminescence is the emission of light by a substance. It occurs when an electron returns to the electronic ground state from an excited state and loses it's excess energy as a photon. Chemiluminescence occurs when a chemical reaction produces an electronically excited species which emits a photon in order to reach the ground state. In the presence of peroxides, horse radish peroxidase (HRP) catalyse the oxidation of lumigen PS-3 acridian substrate to acridium ester intermediates which react with peroxide under slight alkaline conditions to give an excited product. This product emits light as it returns to the ground state and is detected by a CCD camera (DIANA, Raytest) as a distinct protein band.

4.2 Microbiology methods.

4.2.1 Bacteria manipulation methods

4.2.1.2 Preparation of competent bacteria cells by $CaCl_2$ method.

From empirical experimentation, the efficiency of plasmid up-take by *E. coli* is improved by treatment with ice-cold solutions containing $CaCl_2$ (Cohen et al., 1972). The cells, directly from a frozen stock (stored at -70°C in 15% glycerol) are streaked out on an antibiotic-free LB agar plate and incubated overnight at 37°C. A colony of 1-2 mm in diameter is transferred into a sterile 1l flask containing 5 ml LB medium and incubated with shaking overnight at 37°C. Cells are then diluted 1:100 with LB medium supplemented with 20 mM $MgSO_4$ and cultured with vigorous shaking at 37°C until an OD_{600} of 0.4-0.6 was reached. Cells are then harvested by centrifugation at 4,500g for 5 min. at 4°C and gently re-suspended in 0.4x original volume of ice-cold transformation buffer (TFB) 1 and incubated on ice for 1h. Cells are again harvested at 5,000g for at 4°C then re-suspended in ice-cold 1/25 original volume of TFB 2. Following incubation on ice for 30 min. the cells are saved as 100µl samples and frozen immediately at -80°C.

4.2.1.3 Transformation of competent *E. coli* cells

To 100µl aliquot each of competent cells thawed on ice 1- 5ng plasmid DNA are added and incubated further on ice for a 30 min. After a heat-shock of 90 sec at 42°C, the cells are placed on ice for 2 min. To allow expression of the antibiotic

resistance gene of the plasmid, 900μl of LB medium are added and incubated with vigorous shaking at 37°C for 45 min. Cells are then harvested at 2000rpm for 2 min., re-suspended in 100μl of LB medium, spread on LB-agar plates containing the appropriate antibiotic and incubated overnight at 37°C.

4.2.1.4 Blue/white colony screening

When *E. coli* cells are transformed with a vector carrying *E. coli* Lac-Z gene, the enzyme is expressed as β-galactosidase enzyme following induction by Isopropyl-(β)-D-thiogalactopyranoside (IPTG). This enzyme metabolizes its substrate 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) to a blue product. Insertion of foreign DNA into the lac-Z gene leads to insertional inactivation of the lac-Z gene product which can no longer metabolise its substrate and white colonies instead of blue are observed.

Ligation mixture (5μl) of pGEM-T and insert is used to transform competent *E. coli* JM109 cells. The cells are plated on 50μg/ml ampicillin LB agar plates containing 20 mg/ml X-gal in dimethyl formamide and 200 mg/ml IPTG and incubated O/N at 37°C. White colonies are picked and plasmid isolated using the Qiagen kit and sequenced.

4.2.1.5 Library amplification

Twenty five vials of 100μl *E.coli* JM109 strain are transformed each with 5ng of library DNA by $CaCl_2$ method. Colonies are scrapped and re-suspended in 5ml LB medium. Plasmids minipreps are made and pooled into a single 1.5ml tube to constitute the amplified library.

4.2.1.6 Preparation of *E.coli* XL-1Blue bacteria for phage infection

The *E.coli* bacteria strain XL-1Blue is streaked on a 2YT agar plate containing 50μl tetracycline and incubated overnight at 37°C. Four colonies are used to inoculate 30ml of antibiotic-free 2YT medium and incubated at 37°C with shaking until the OD_{600nm} was about 1.0. Cells are then centrifuged at 2,000rpm at 4°C for 10min. and re-suspended in an equal volume of 10 mM sterile $MgSO_4$. Cells are used immediately for phage library tittering, plaque lifts or stored at 4°C for a maximum of 24h.

4.2.2 Yeast Two hybrid methods

4.2.2.1 Yeast two-hybrid assay

Yeast two-hybrid assays represent a versatile tool to study protein interactions *in vivo.* Two-hybrid assays are based on the fact that many eukaryotic transcriptional activators consist of two physically and functionally separable domains: a DNA-binding domain (DBD) that specifically binds to a promoter and an activation domain (AD) that directs RNA polymerase II to transcribe the gene downstream of the DNA-binding site. While these domains may be part of the same protein (as in the case of the native yeast GAL4 protein), they can also function as two separate proteins. The transactivation function can be restored when the DBD and the AD from the same or different transcription factor(s) are brought together by two interacting heterologous proteins which are the bait and the prey (Fig. 9).

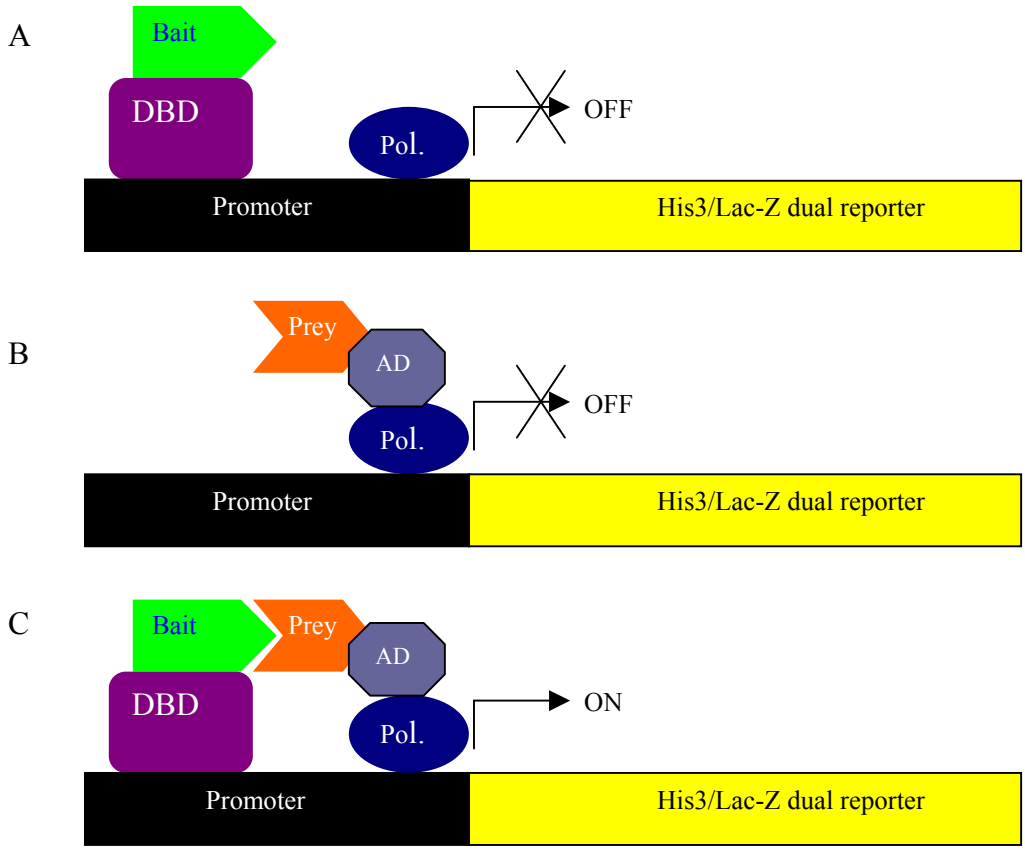

Fig. 9: Principle of the yeast two hybrid system. A) Binding of the DBD-bait fusion alone to the promoter (A) or activation domain (AD)-prey fusion protein alone to the DNA polymerase II (Pol.) does not lead to target gene reporter. C) bait/prey interaction brings the DBD and the AD to close proximity and thus reconstitutes a functional transcription factor which activates the promoter. (Chien et al., 1991 with modification from Ghogomu S.M).

The bait is always a well characterized protein while the prey constitutes the inserts of a cDNA library. Binding of the DBD-bait fusion alone or the activation domain (AD)-prey fusion is not sufficient to activate target gene reporter. Interaction between the bait and the prey brings the DBD and the AD to close proximity and a functional transcription factor is reconstituted which activates the reporter. Reporter activation is read either as growth on a histidine-deficient medium or appearance of blue colonies following β-galactosidae assay. In this work, an *Escherichia coli* repressor protein (LexA) served as DBD while VP16 protein (a herpes simplex virus protein that acts as a transcriptional activator in mammalian cells) served as AD.

4.2.2.2 Preparation of sheared denatured salmon sperm DNA

In a 250ml flask 0.5g of salmon sperm DNA is dissolved in 50ml TE buffer by pipetting up and down using a 10ml pipette and incubated overnight at 4°C. The DNA is sheared by sonifying twice for 30 sec. at 3.0 kV. Samples of 12.5ml are extracted with 12.5ml of TE-saturated phenol (to precipitate proteins) by mixing, centrifuging at 10,000g for 5 min. at 4°C and transferring the upper aqueous phase to fresh 50ml tube. The sample is next extracted with 12.5ml of phenol:chloroform:Isoamyl alcohol (25:24:1) mixture and finally with 12.5ml of chloroform alone to remove residual phenol. In a 250ml centrifuge tube, the DNA is precipitated with 2.5 volume of ice-cold (-20°C) 95% ethanol and 1/10 volume of 3 M sodium acetate pH 6.0. The DNA is harvested at 10,000g for 5 min at 4°C, washed once with 100ml of 70% ethanol and air-dried. The DNA is dissolved in 50ml sterile TE (10mg/ml) and denatured by boiling in a water bath for 20 min. Samples are cooled on ice and stored as 2ml samples at -20°C.

4.2.2.3 Testing the bait for non specific activation of reporter constructs

The bait is not supposed to non specifically transactivate the reporter constructs in the L40 strain or interact with either the nuclear localisation sequence (NLS) or the activation domain (AD) of the library plasmid. As a consequence the transformed strain should not grow in the absence of histidine or exhibit detectable β-galactosidase activity.

L40 yeast strain is streaked on an antibiotic-free YEPD plate and incubated at 30°C for 3 days. A single colony is used to inoculate 10ml of YEPD medium and incubated

overnight with shaking at 30°C. Cells are diluted to an OD_{600nm} of 0.5 and cultured for an additional 3h. Cells are harvested at 2,500rpm at room temperature (RT) and washed by re-suspension in 40ml TE followed by centrifugation. Cells are re-suspended in 2ml of 100 mM Lithium acetate (LiAc)/0.5x TE and incubated at RT for 10 min. Into a 1.5ml reaction tube containing 100μl yeast suspension in LiAc/0.5xTE, 1ug plasmid DNA (bait) and 100ug denatured, sheared salmon sperm DNA, 700μl of 100 mM LiAc/40% Polyethylene glycol-3350/1x TE are added. The mixture is vigorously agitated and incubated at 30°C for 30 min. Following addition of 88μl DMSO, the sample is mixed, heat shocked at 42°C for 7 min. and cells harvested at 2,500prm for 10sec. Cells are washed by re-suspension in 1ml TE buffer and re-centrifugation. The pellet is re-suspended in 100μl TE and plated on 150 x 20mm agar plate deficient in tryptophane, uracyl and lysine (-Trp, -Ura and –Lys) and containing 100μg/ml ampicillin). Plates are incubated at 30°C for 3 days after which colonies are then assayed for β-galactosidase activity.

4.2.2.4 β-Galactosidase assay

A dry circular nitrocellulose membrane is placed onto the yeast colonies for 2 min. The membrane is removed and placed colony-side-up on a pre-cooled aluminium boat floating in a sea of liquid nitrogen. After 30 sec the boat and the membrane are immersed in the liquid nitrogen for 5 sec. The membrane is then removed and placed colony-side-up at RT until thawed. In a 150 x 20mm Petri dish, 6.4ml of Z-buffer containing 25mg/ml X-gal are added. A circular 3MM Whatman filter is placed in the buffer followed by the thawed nitrocellulose filter colony-side-up avoiding trapping of bubbles. This is covered with the bottom of the dish and incubated at 30°C for 6h after which colonies analysed for observed for blue/white selection.

4.2.2.5 Large scale library transformation

A colony of L40 transformed with the bait plasmid is cultured overnight in 5ml of Yc medium lacking Triptophane, Uracil and Lysine (Yc/-Trp, -Ura and –Lys). The culture is diluted with the same medium to an OD_{600nm} of 0.3-0.5 and re-cultured for 4 hours. Cells are washed by re-suspension in 500ml TE followed by centrifugation at RT for 10min at 5,000rpm. Cells are re-suspended in 20ml of 100 mM (LiAc)/0.5 x TE and incubated at RT for 10min. Cells are mixed with 2ml of denatured sheared salmon

sperm DNA, 250μg library plasmid DNA (prey), 140ml of 100mM LiAc/40% polyethylene glycol-3350/1 x TE and incubated at 30°C for 30 min. The mixture is vigorously agitated and incubated at 30°C for 30 min. Following addition of 17.6ml DMSO, the sample is mixed, heat shocked at 42°C for 7 min. with occasional swirling and cells harvested at 5,000rpm for 10sec. Cells are washed by re-suspension in 500ml TE buffer and re-centrifugation. The pellet is re-suspended in 1l YEPD medium and cultured with aeration at 30°C for 1h to allow expression of antibiotic resistance genes and the reporters. Cells are washed with TE buffer, re-suspended in 1l Yc/(-Trp, -Ura and –Lys) medium and cultured with aeration overnight at 30°C. Cells are harvested, washed twice with 500ml TE and re-suspended in 10ml TE. 100μl samples are plated on twenty 150 x 20mm ampicillin Yc/(-Trp, -His, -Ura, -Leu and –Lys) agar plates and incubated at 30°C for 3 days. Colonies are then assayed for β-galactosidase activity.

4.2.2.6 Perching of yeast strain transformed L40 of bait plasmid

The scourge of the two hybrid system is the notorious ``false positives´´ arising from independent activation of the promoter by the bait or the prey. The best strategy for elimination of false positives is to test colonies for interaction with other LexA fusion partners. This is done by analysing the specificity of the interaction by mating colonies with a set of strains containing interacting and non interacting baits. But before mating, each colony is perched of the bait plasmid. Extended growth of L40 transformed with both bait and prey plasmid in the absence of tryptophane (Trp) allows segregation and survival of the daughter cells without the bait plasmid. Cells from each colony are thus streaked on –Leu, +Trp plates and individual colonies are picked to both -Leu, +Trp and -Leu, -Trp (negative control). Colonies that grow on the -Leu, +Trp plates and not on the -Leu, -Trp plates are those that have lost the bait plasmid (Leu+,Trp-) and can be used for mating analysis.

4.2.2.7 Mating of yeast strains

In a 1.5ml tube containing 0.5ml of YEPD medium supplemented with 100μg/ml ampicillin, each colony perched of its bait is mixed with AMR70 yeast strain previously transformed respectively with the bait plasmid or the LexA-Lamin plasmid (negative control). Lamin is a nuclear protein found to interact with very limited

number of proteins. Cultures are grown overnight with aeration at 30°C and 10μl of each are spread on YEPD ampicillin plates and incubated for 3 days at 30°C. Diploids of bait/prey plasmid giving positive β-galactosidase activity on –Trp, -Leu ampicillin plates and negative β-galactosidase activity with corresponding LexA-lamin diploids are selected as potential interactors. They are stored at 4°C for not more than two weeks to avoid formation of spores following meiosis in conditions of limited nitrogen.

4.2.2.8 Extraction of library plasmids from diploids and analysis

Before bait plasmid extraction diploids are first perched to eliminate the bait plasmid by repeated growth on +Trp culture medium.

Single colonies are grown in 5ml +Trp, -Leu medium overnight at 30°C with shaking. Cells are harvested at 2,500rpm for 5 min, re-suspended in 0.3ml of yeast lysis buffer and transferred into 1.5ml tubes. A volume of 150μl glass beads and 0.3ml of phenol/chloroform (1:1) are added taking care to remove all glass beads adhering near the top of the tube as they can be caught when the lead is closed and cause leakage of toxic phenol. The tube is vigorously agitated for 3 min and centrifuged at 1,400rpm for 1 min. The aqueous phase is transferred into a fresh 1.5ml tube and plasmid DNA precipitated with 0.1 volume 3 M sodium acetate and 1.5 volume of ethanol. The precipitate is dissolved in 25μl TE buffer and used for transformation of chemically competent *E.coli* strain JM109. Plasmid minipreps are made and sequenced using the M13 universal primers. Sequences are blasted against the NCBI data bank and potential binding partners selected based on their nuclear localisation and known role in embryonic development.

4.3 Cell culture methods

4.3.1 Cell culture and transfection

Addition of $CaCl_2$ and DNA into a buffered saline/phosphate solution containing cultured eukaryotic cells generates a $CaPO_4$/DNA co-precipitate can be taken-up by the cells via endocytosis or phagocytosis (Gorman et al., 1985).

Human epithelial kidney (h293) or human cervix carcinoma cells (Hela) are routinely grown in 5ml Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum in the presence of 7% CO2 at 37°C in 250ml tissue-culture flasks (Greiner).

One days prior to transfection, cells are trypsinised, seeded to semi-confluence in culture tubes and incubated at 37°C. 4h prior to transfection, the medium is exchanged with 5ml fresh complete medium. Following medium change, cells are incubated further incubated at 37°C for 4h. The different plasmids are mixed with HEPES buffered saline pH7.21 and 2.5 M $CaCl_2$ solution is added drop-wise. The mixture is incubated at RT for 30 min. so that co-precipitates of $CaPO_4$/DNA should form. This mixture is added to the respective cells and incubated at 37°C for 4h. Following medium change, the cells are incubated at 37°C for 48h and harvested in 1.5 ml PBS. 450μl are saved to be used for luciferase assay and 900μl saved to be used for β-galactosidase assay. For analysis of the glucocorticoid response transfected cells are treated with various concentrations of dexamethasone while for analysis of the androgen response, cells are incubated with 10 nM dihydoroxytestosterone (DHT).

4.3.2 Luciferase assay

The luciferase assay is based on the principle that a luciferase reporter gene under the control of a defined promoter is expressed when the promoter is specifically activated. The expressed luciferase enzyme then transforms its substrate, Luciferin, to oxyluciferin with the emission of light at a particular wavelength which can be measured with a luminometer.

Cells saved as 450μl samples are centrifuged at 5,000prm at 4°C for 5 min. and re-suspended in 125μl Luciferase assay lysis buffer. The extract is passed through a narrow gage syringe to facilitate cell lysis. To 20μl of the extract 100μl of luciferase assay substrate are added. Light emission is quantified as relative light units (RLU) in a luminometer (Berthhold Technologies).

4.3.3 β-Galactosidase assay

Cells saved in aliquots of 950μl in PBS are centrifuged at 5000prm at 4°C for 5 min and pellet re-suspended in 150μl 0.25 M Tris pH 7.8 and passed through a narrow gage syringe to facilitate cell lysis. To 60μl of each extract are added 6μl of 0.1 M $MgSO_4$/4.5 M β-mercaptoethanol solution, 132μl of o-Nitrophenyl-β-glucopyranoside (ONGP), 402μl 0.1M sodium phosphate buffer pH 7.5. A similar sample is prepared but without cell extract to serve as negative control. Samples are incubated at 37°C

for 30 min to obtain the light yellow colour of the reaction product. Reactions are stopped by adding 1 ml of 1 M Na_2CO_3 and OD_{420} measured.

4.3.4 Immunocytochemistry

In a 6-well culture plate, three cover slides are introduced and coated with a drop of Alzian blue per slide for 15 min at RT. Excess die is removed by exhaustive exchanging the die with DMEM medium containing 10% fetal calf serum. 2.5 ml of tripsinised human cervix carcinoma (Hela) cells are then seeded in each well and incubated at 24°C and 7% CO_2. Cover slides are then removed and washed with 3x 10 min changes of APBS/ 2mM $CaCl_2$. The cell are fixed with 10% paraformaldehyde in APBS/ 2mM $CaCl_2$ for 10 min and intracellular proteins denatured in 10% Triton in APBS/ 2mM $CaCl_2$ for 8 min. Unbound protein sites on the cover slides are blocked with 1%BSA in APBS/ 2mM $CaCl_2$ for 30 min and washed with 3x 10 min. changes of APBS/ 2mM $CaCl_2$. Slides are then placed in the anti-Tcf-3/Tcf-4 or anti-Hic-5 primary antibody overnight at 4°C. After 3x 10 min. washes, slides are placed in Goat anti-mouse secondary antibody coupled to Cy3 for 2h at RT. Slides are washed and observed in a drop of elvanol under the fluorescence microscope.

4.4 Developmental Biology methods

4.4.1 Induction of egg maturation

Human choriongonadotropin (HCG) hormone induces egg maturation in *Xenoups* within 14-18h.

The dorsal lymph sacs of *Xenopus laevis* adult females are injected with 50U of HCG hormone and after 14-18 h another hormone dose of 600U is given. After about 6-8h the eggs are gently squeezed out manually from the oviducts of the frog and collected in a Petri dish. Subsequent squeezing can be done after every 1 hour for a maximum number of 4 batches.

4.4.2 Extraction of the Testes

A male frog is anesthetized by placing it in fumes of a 0.05% (v/v) amino-benzoic acid-ethylestermethane solution for 30 minutes. Using a sharp blade the urino-genetal system is exposed and the testes are observed as orange coloured bodies

lying ventral to the kidneys. These testes are isolated and stores in 1x MBSH solution at 4°C for a maximum of 4 days.

4.4.3 *In-vitro* fertilization

A piece of testis is macerated in 1-1.5 ml 1x MBSH. 100-200µl of this testis stock suspension is diluted to 0.1x MBSH and distributed over the squeezed eggs. About 30 min later, the Petri dish is filled with 0.1x MBSH and kept at 14-16°C. Success of fertilization is determined by the upward turning of the pigmented animal pole. Approximately 90-120 min. after fertilization, the embryos start to cleave. The jelly coat is then removed by gently spinning the embryos in 2% cysteine solution pH 8.0 for 2 min. Afterwards the eggs are intensively rinsed with 0.1x MBSH to remove excess cysteine.

4.4.4 Microinjections

The glass needle is fixed onto a micromanipulator that is connected to an air pump. The injection volume is determined by measuring the diameter of a water droplet expelled into mineral oil from the glass needle tip using a standard square meter. For injection the embryos are transferred into an agarose-coated dish containing 0.1x MBSH and fixed in the wells by gently eliminating the buffer. Messenger RNA is injected in a volume of 4-10 nl into the two ventral blastomeres of 4-cell stage *Xenopus laevis* embryos. Dorsal and ventral blastomeres can be distinguished at the 4-cell stage by their size and pigmentation: dorsal blastomeres being less pigmented and smaller than ventral ones. After injection, the embryos are kept in 1x MBSH for about 1-1.5 hours at RT for wound to heal and then cultured in 0.1x MBSH at 14-18°C.

4.4.5 Analysis of embryos

Embryos are sorted out under the light microscope for any eventual phenotype and statistical data collected.

5. Results

5.1 Cloning of GST fusions of *Xenopus* Lef/Tcf Transcription factors

In order to identify novel binding partners, a method of choice is the use of recombinant proteins incubated with crude cell lysates followed by identification of bound protein on SDS PAGE gel and analysis by MALDI-TOF. A prerequisite for these studies is to isolate large quantities of highly purified fusion proteins. Thus, the core domain of *Xenopus* Lef-1 (aa_{63}-aa_{274},), XTcf-3 (aa_{63}-aa_{328}) and XTcf-4 (aa_{63}-aa_{353}) as well as the activating exons of XTcf-3 (aa_{193}-aa_{249}) and XTcf-4 (aa_{220}-aa_{316}) in XLef/Tcfs were inserted into *Xho*I/*Nco*I site of the pETM-30 expression vector (Fig. 10).

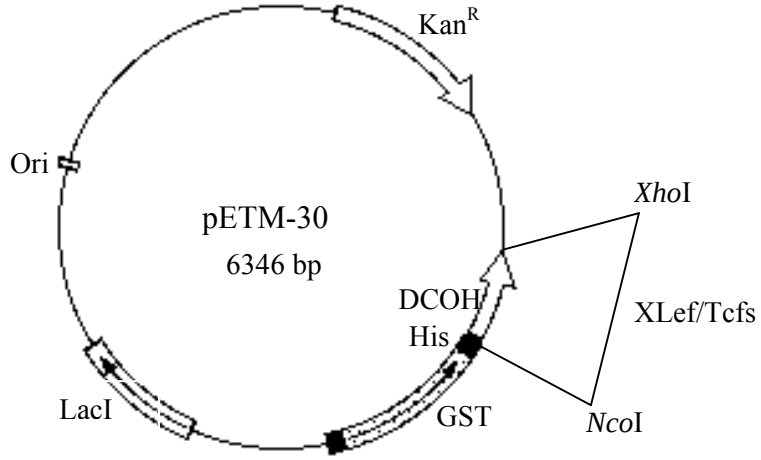

Fig. 10: pETM-30 expression plasmid. Core domains of XLef-1, XTcf-3, XTcf-4A and XTcf-4B were cloned into the *Nco*I/*Xho*I sites. The vector also possesses the kanamycine resistance gene (Kan^R) for selection in culture media, the LacI gene which codes for the repressor of the Lac operon, the histidine tag (His) and the GST tag (GST) for purification on affinity columns. The origin of replication (Ori) is represented.

Following cloning, correct insertion of the fragment into the vector was verified by restriction digestion and agarose gel analysis. Restriction analysis of the core domains of Lef/Tcfs is represented in Fig. 11. The sizes of the core domains correspond to the predicted sizes. XLef-1 fragment consists of 650 bp, XTcf-3 of 800 bp, and XTcf-4A and -4B each of 850 bp. In-frame cloning of the inserts was verified by sequencing.

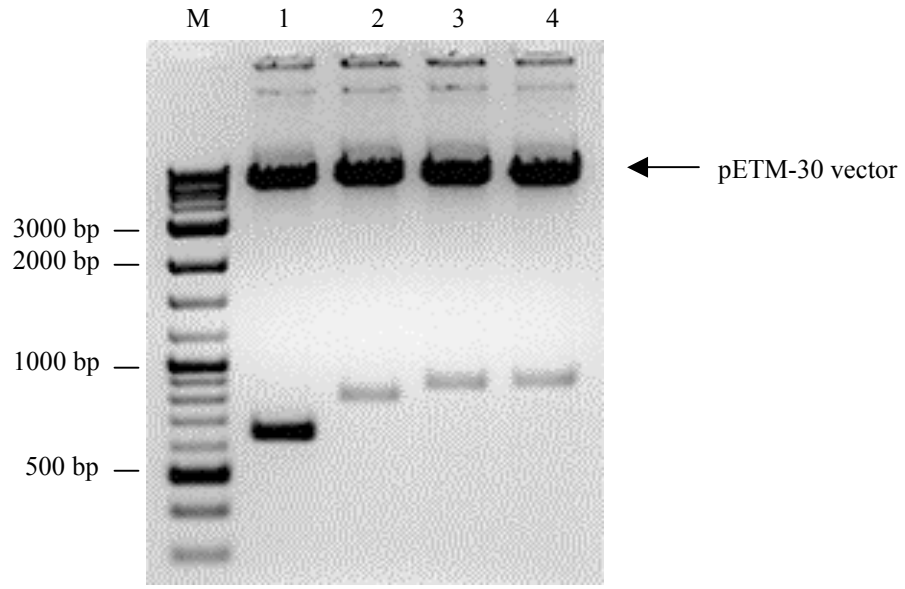

Fig. 11: Restriction analysis of the core domain of transcription factors in pETM-30 vector with XhoI and NcoI. The core domain of XLef-1 (lane 1), XTcf-3 (lane 2), XTcf-4A (lane 3) and XTcf-4B respectively were excised from the pETM-30 vector with *Xho*I and *Nco*I enzymes and analysed on a 1% agarose gel. The 2-log molecular weight marker (M) with molecular weight of most intense bands is indicated.

Similarly, the Domain A of Lef-1 (aa_{67}-aa_{213}), XTcf-3 (aa_{63}-aa_{221}) and XTcf-4C (aa_{63}-aa_{260}) were cloned into the *Xho*I site of pET41b expression vector respectively. Lef/Tcfs cloned in pET vectors allows high-level purification and tandem affinity purification via His and GST tags.

5.2 Induction of GST-fusions proteins

GST-fusion constructs of *Xenopus* Lef/Tcf were used to transform bacterial strain BL21 DE3 and induced with 1.0 mM IPTG. Cell lysates were analysed on a 10% SDS PAGE gel. A gel sample of the results for the induction of Lef/Tcf core domain fusions and the control vector is shown on Fig. 12A with the induced bands corresponding to the most intense band of each lane. The sizes of the fusion proteins correspond to those predicted, for XLef-1 55 kD, XTcf-3, 60 kDa, XTcf-4 65 kD and the DCOH-GST fusion about 40 kD. Apart from these prominent bands, other bands although faint were observed on the gel. These faint bands represent either the bacterial proteins or degradation products of the fusion proteins.

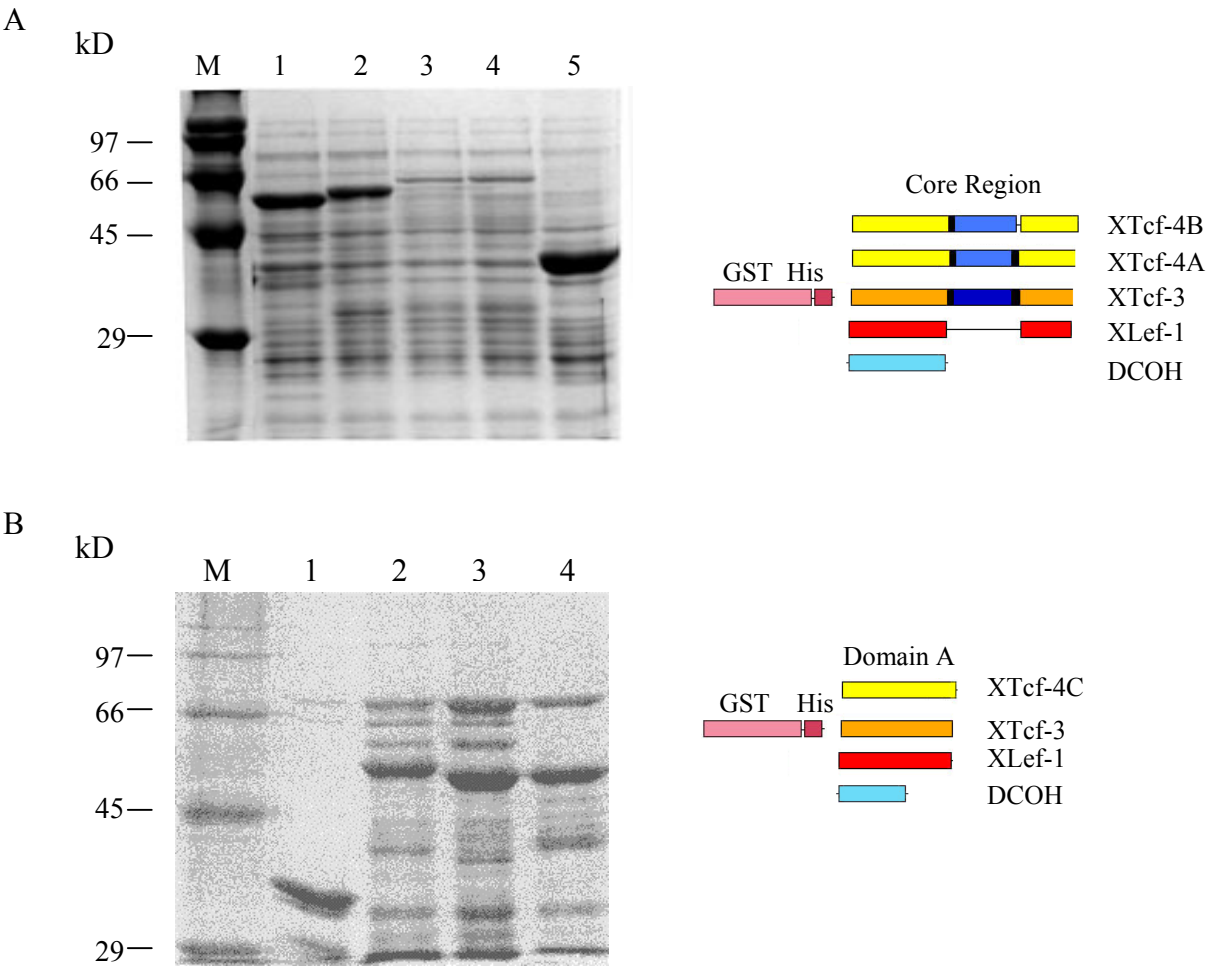

Fig. 12: Analysis of lysates for encoded proteins of Lef/Tcfs on a SDS PAGE gel. A) 12% gel: Lysates from bacteria transformed with pETM-30-DCOH (Lane 5) or recombinant vector containing the core domain of XLef-1, XTcf-3, XTcf4-A and XTcf-4-B (Lanes 1, 2, 3 and 4 respectively). B) 10% gel: Lysates from bacteria transformed with pET 41b (lane 1) or recombinant vector containing the Domain A of XTcf-4C, (lane 2), XLef-1 (lane 3) and XTcf-3 (lane 4). The most intense band of each lane corresponds to the induced peptide. Lane M is the SDS 6H molecular weight marker. Schematic representation of constructs used is indicated.

Domain A fusion proteins of XLef-1, XTcf-3 and XTcf-4C in pET 41b were induced in a similar way and expressed protein bands corresponded to predicted sizes. GST fusions XLef-1 had a size of 51 kD, XTcf-3 (53 kD) and XTcf-4C (57kD) (Fig. 12B).

5.3. GST pulldown assays using lysates from human kidney epithelial 293 cells.

In order to identify potential binding partners of the Lef/Tcfs, GST pulldown assays were carried out. Bacterially expressed Lef/Tcf proteins were immobilised on glutathione sepharose 4B and incubated with or without lysates of kidney epithelial

293 cells which served as a protein pool. These cells were chosen because sequence data (peptide mass data) for human proteins is available and the XLef/XTcf functional diversity was established using these cells (Gradl et al., 2002a). Subtype-specific binding partners should be present in these cells. Following several washing steps the bound proteins were eluted with sample buffer and analysed on a 10% or 12% SDS PAGE gel. Gels were stained with Coomassie blue dye (Fig. 13A) or Silver (Fig. 13B).

The binding of the Lef/Tcf fusion proteins on the sepharose gel was efficient and this is testified by the presence of a strong band on each lane having the right molecular weight on the SDS PAGE gel. Faint bands existing in both the control and the test samples correspond to either bacterial proteins that bound to the sepharose gel or they represent degradation products from the fusion protein. In addition to these bacterial impurities and the degradation products the other faint bands in the samples incubated with lysates of transfected cells should correspond to the putative Lef/Tcf binding partners.

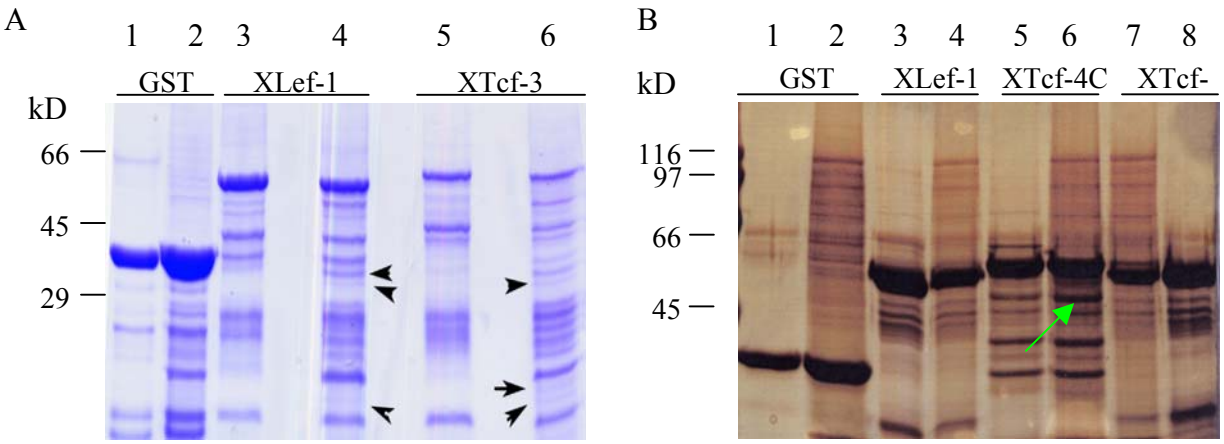

Fig. 13: Analysis of GST pulldown eluates on SDS PAGE gels. A) Coomassie-stained 12% SDS PAGE gel of eluates from glutathione sepharose-immobilised GST-DCOH (Lane 1), core domains of XLef-1 (lane 3), and XTcf-3 (Lane 5). Corresponding eluates from immobilised GST-DCOH, XLef-1 and XTcf-3 (Lanes 2, 4, 6 respectively) incubated with lysates h293 cells. Arrowheads point to bands corresponding to potential binding partners of XLef-1 and XTcf-3 that were cut and analysed by MALDI-TOF. B) Silver-stained 10% gel of eluates from glutathione sepharose-immobilised GST-DCOH (Lane 1), core domains of XLef-1 (lane 3), XTcf-3 (Lane 5) and XTcf-4A (Lane 7). Corresponding eluates from immobilised GST or core domains of XLef/Tcfs (lanes 2, 4, 6, 8) incubated with lysates of h293 cells. Green arrow points to a potential XTcf-3 binding partner. Molecular weights in kD are indicated.

These bands of interest (indicated by arrow heads in Fig. 13A and by a green arrow in 13B) were cut out and in collaboration with Marc Genzel (EMBL, Heidelberg)

proteins were eluted from the gel, digested with trypsin and analysed by MALDI TOF. Results revealed proteins such as F1 ATPase, ribosomal proteins and many others which were all of cytoplasmic and mitochondrial localization. None of these proteins were described to be involved in early development and there was no indications that any of them could shuttle into the nucleus where the Lef/Tcfs transduce Wnt/β-catenin signals. Purification of Lef/Tcf proteins on Nickel nitriloactetic acid (Ni-NTA) columns prior to binding and improvement of elution conditions did not improve the pull-down. Drawbacks encountered with the bacterial expression system imposed a switch in procedure to a yeast two-hybrid system that circumvents the problem of protein impurities and degradation.

5.4 Yeast two-hybrid screen.

5.4.1 XLef-1 and not XTcf-3 and XTcf-4 independently activates the β-galactosidase reporter

Since the read-out of the two-hybrid system makes use of a transcription event, one of the most crucial initial experiments is to check whether the bait alone is able to initiate transcription independent of the prey. To exclude the latter, the core domain of XLef-1 (aa_{63}-aa_{274},) was inserted into the *Bam* HI/*Pst*I, while XTcf-3 (aa_{63}-aa_{328}) and XTcf-4 (aa_{63}-aa_{353}) were inserted into the *Eco*RI/*Sal*I sites of the BTM116 vector in frame with the DNA binding domain of LexA (Fig. 14). Correct insertion was verified by restriction digestion and sequencing.

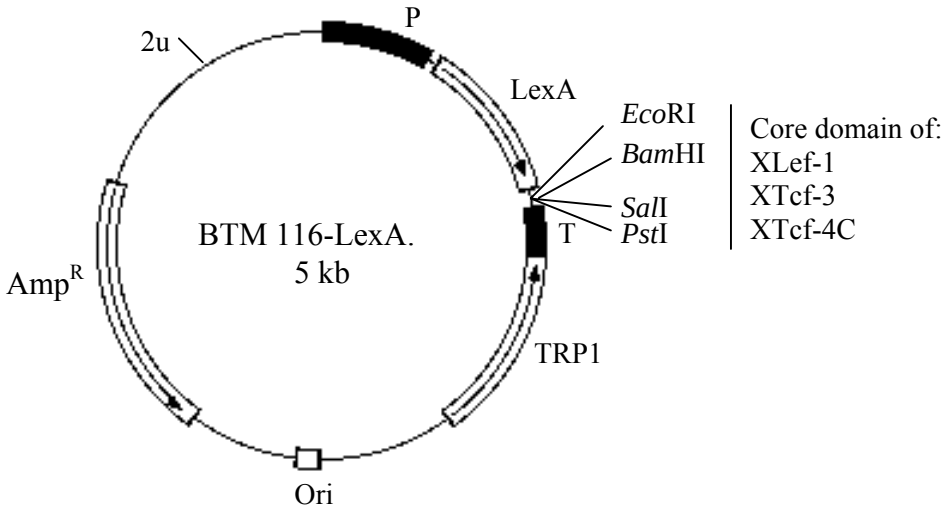

Fig. 14: BTM116 plasmid carrying the LexA DNA binding domain. Core domains of XLef-1was cloned into the *Bam*HI/*Pst*I sites and XTcf-3 and XTcf-4 in the *Eco*RI/*Sal*I sites. P is the promoter driving LexA fusion protein and T is the termination sequence. The vector also possesses the tryptophane (TRP)1 and ampicillin resistance (Amp^R) genes for selection in culture media and a portion of the yeast 2*u* circle plasmid for replication in yeast hosts.

To be certain that these constructs do not independently activate the reporter gene, they were assayed for Lac-Z activation by β-galactosidase test. This assay revealed that LexA-XLef-1 fusion proteins activated the β-galactosidase gene independent of a prey (blue colour of β-galactosidase). In contrast, LexA-XTcf-3 and LexA-XTcf-4 fusion proteins did not activate the transcription of the β-galactosidase gene (absence of blue colour) (Fig.15).

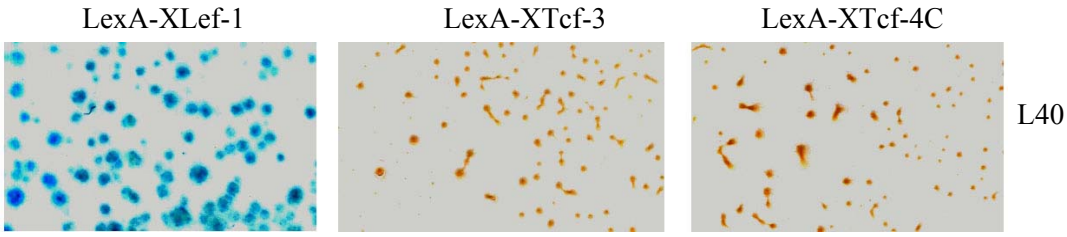

Fig. 15: LexA-XLef-1 construct independently activates the β-Galactosidase promoter (Blue colour of β-Galactosidase). Yeast strain L40 were transformed with LexA-XLef-1 LexA-XTcf-3 and LexA-XTcf-4 construct in BTM116 vector and transformats were assayed for β-galactosidase activity. Whereas LexA-XLef-1 activates the Lac-Z promoter (presence of blue colour of X-gal product), LexAXTcf-3 and LexA-XTcf-4 do not independently activate the promoter (absence of blue colour).

This ability of LexA-Lef-1 to activate transcription independently of the prey made it useless as bait. The inability of LexA-XTcf-3 and LexA-XTcf-4 to activate transcription qualifies them for use as baits in the yeast two-hybrid screen.

5.4.2 Numerous library proteins interact with the core domains of XTcf-3 and XTcf-4

To identify proteins interacting with XTcf-3 and XTcf-4 core domains, L40 yeast strain previously transformed respectively with LexA fusion constructs in BTM116 vector were further transformed with the mouse embryonic day 10 cDNA library. This library was kindly provided by Jürgen Behrens. The mouse library was used because no *Xenopus* library adapted for yeast two hybrid system was available. Moreover, this mouse library had previously been used to isolate authentic binding partners of β-catenin such as Lef-1 and conductin (Behrens et al., 1996 and 1998).
Many proteins of the library interacted with the core region of XTcf-3 and XTcf-4 since many colonies grew on selective medium and possessed β-galactosidade activity (Fig. 16).

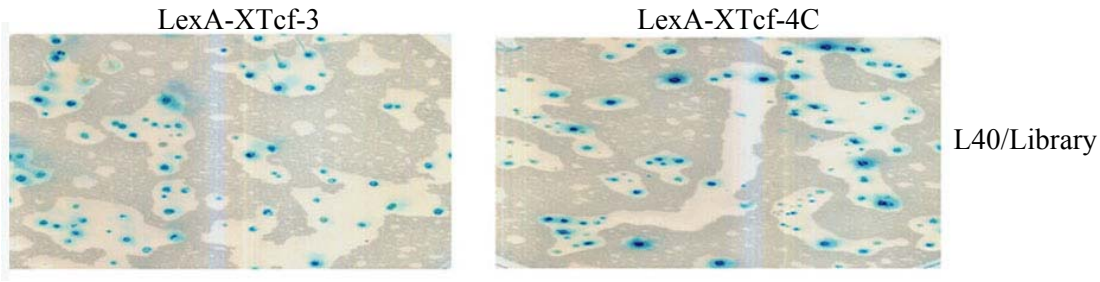

Fig. 16: Interaction of the bait and the library prey in L40 yeast strain. XTcf-3 and XTcf-4C interact with the library prey to activate β-Galactosidase promoter (presence of blue colour of colonies).

5.4.3 Elimination of False positives by mating

The best strategy for elimination of false positives is to test colonies for specificity of interaction with other LexA fusion partners. Colonies transformed with both the bait and the prey were made to loose the bait plasmid by perching and then analysed by β-galactosidase assay. Colonies that lost the bait plasmid show negative β-galactosidase assay while those that remain blue did not loose the plasmid (Fig. 17).

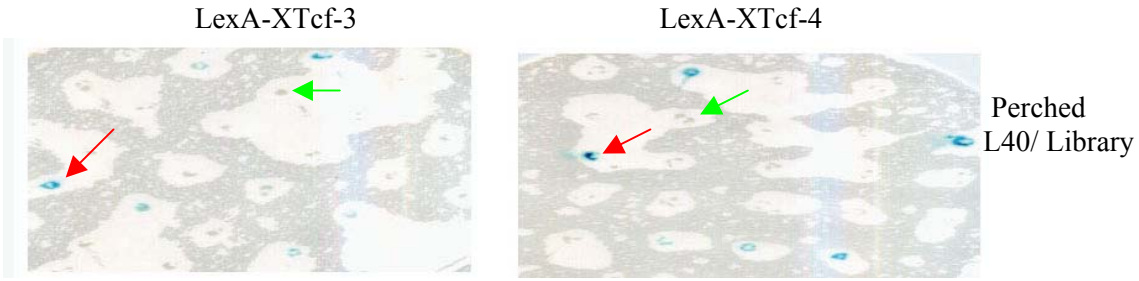

Fig. 17: Perching of L40 yeast colonies of the bait plasmid. Colonies were repeatedly grown on medium containing tryptophane and emerging colonies were tested for β-galactosidase activity. Colonies that lost the bait plasmid are represented by brownish white spots (green arrow) while colonies that retain the bait plasmid are represented by blue spots (red arrows).

Colonies loosing the bait plasmid were mated with AMR 70 yeast strain previously transformed with the interacting (LexA-Tcf-3/4) and non-interacting (LexA-Lamin) baits respectively. Diploids were spotted on a grid so that a transcription factor interacting with a particular prey matches with its corresponding Lamin counterpart. Following β-galactosidase assay, prey interacting with XTcf-3 or XTcf-4 (Blue colonies) but not with Lamin (brownish white colonies) were considered positives (Fig. 18).

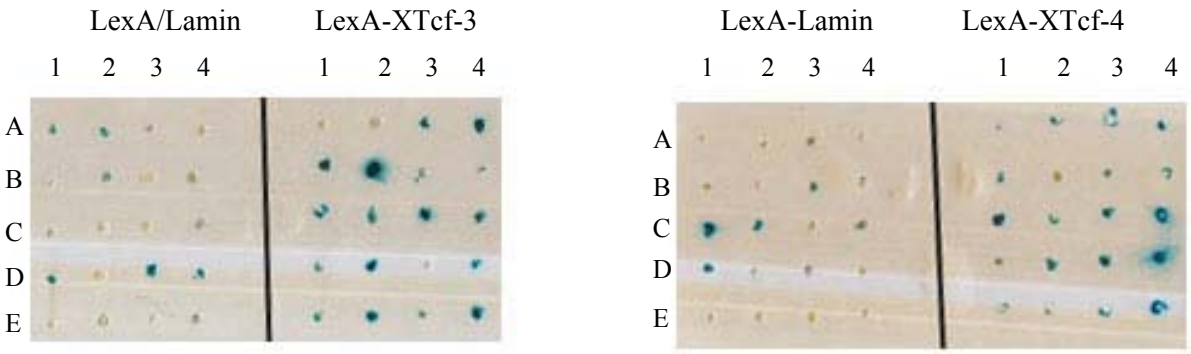

Fig. 18: Elimination of false positives by mating. L40 yeast train transformed with both bait and library plasmids are spotted on a grid so that a transcription factor interacting with a particular prey is matched with its corresponding lamin counterpart. Only prey interacting with XTcf-3 or XTcf-4 (blue colonies) but not with Lamin (brownish white colonies) are considered positives. Examples of some true interacting partner of XTcf-3 are colony 4A, 2C, and 2D and for XTcf-4 are 4A, 1B and 4E.

Prey plasmids from the positive colonies were extracted and sequenced. Sequencing results revealed twenty six potential interacting partners. Eleven of them including arsenic ATPase, Ribonucleoprotein A1 and ATP sythase were either of cytoplasmic or mitochondrial localization and were considered as false positives since they are not localized to the nucleus where the Lef/Tcfs transduce Wnt/β-catenin signals. Four candidates revealed homology with unknown expression sequence tags (ESTs) and so could not be characterized further. Potential binding partners of XTcf-3 included Lipopolysaccharide-induced tumour necrosis factor (TNF)-α factor (LITAF), melanoma-associated antigen D1 (MAGED1 or DLXIN), Retinoic acid orphan receptor 2 (ROR 2), and Toll-interacting protein (TOLLIP) while potential binding partners of XTcf-4 were hydrogen peroxide-inducible clone 5 (Hic-5) [Androgen Receptor Activator (ARA-55)], Activated Recruited cofactor 105 (ARC 105) [Glutamine-rich transcriptional adaptor protein (TIG-1)], Ring finger protein 4 (RNF-4), Mutant-p53-binding protein-1 (MBP1) and DLXIN. These potential binding partners exhibit diverse sub-cellular localizations. Whereas MBP-1 is reported to be localized in the extracellular space and in the nucleus, ROR2 is transmembrane while LITAF is nuclear. TOLLIP and ARC 105 are uniquely of cytoplasmic localization and DLXIN, Hic-5 and RNF-4 are cytoplasmic and the nuclear. (Tables 2 and 3). All these clones were further analysed in GST pull-down assays.

Table 2: Potential interacting partners of XTcf-3. The subcellular localization and the roles played by each of these partners in the cell are indicated. The asterisk (∗) indicates clones obtained more than once in the screen.

Protein	Localization	Role
LITAF (Lipopolysaccharide-induced TNF-α factor)	Placenta, Lymphoid organs. (Nucleus)	Binds to human tumour necrosis factor (hTNF)-α promoter and regulates its transcription and thus regulates the inflammatory effect of TNF-α (Tang et al., 2003)
Ror 2 (Retinoic acid orphan receptor 2) ∗	Transmembrane	Involved in skeletal and cardiac development. Mutants lead to septal and respiratory defects resulting to neonatal lethality (Nomi et al., 2001).
Dlxin1∗	Nucleus/Cytoplasm	**Nucleus:** Binds to genes involved in skeletal development and regulate their transcription (Masuda et al., 2001). **Cytoplasm :** sequested by binding to cytoplasmic tail of Ror2 (Matsuda et al., 2003)
TOLLIP (Toll-interacting protein)	Cytoplasm.	Binds to the cytoplasmic tail of Toll-like receptors and may be involved in control of the inflammatory effects of TNF-α. (Moynagh, 2003).

Table 3: List of potential interacting partners of XTcf-4. The corresponding subcellular localization and the roles played by each of these partners in the cell are indicated. The asterisk (∗) indicates clones obtained more than once in the screen.

Protein	Localization	Role
Hic-5 (Hydrogen peroxide.-inducible clone 5) or Androgen Receptor Activator (ARA 55)	Nucleus and at focal adhesion sites of cells.	**Nucleus:** Binds to androgen receptors and participates in hormone-regulated of transcription (Fujimoto et al., 1999). **Focal adhesions:** Binds to focal contacts (Shibanuma et al., 2003).
Dlxin1∗	Nucleus/Cytoplasm.	Mentioned already in table 2
ARC 105 (Activated Recruited cofactor 105)./ TIG-1 / Glutamine-rich transcriptional adaptor protein	Cytoplasm.	Induces axis duplication, morphogenic elongation and mesoderm differentiation. (Induces *Xbra, chordin, mixer, goosecoid, xwnt8* and *xnr4*. but not *Xnr3*. Suppresses ventral markers: vent-1 and vent-2. Binds to smad2/3-4 in response to TGF-β and not samd1-4 of the BMP pathway (Kato et al., 2000).
RNF4	Nucleus and cytoplasm of cells of developing nervous and reproductive systems.	Promotes the formation of intra-cytoplasmic complexes involved in shuttling between the cytoplasm and the nucleus (Galili et al., 2000).
MBP1 Mutant-binding protein-1 (Fibulin4)	Extracellular and nucleus.	Binds to p53 protein and enhances tumour cell growth (Gallagher et al., 1998).

5.4.4 Confirmation of the putative interacting partners by GST pull-down assays

In order to confirm the yeast two-hybrid screen results, GST pull-down assays were carried out. *In vitro* translated ^{35}S-labelled constructs of mouse RNF-4, ARC-105, MBP-1, TOLLIP, ROR2 and DLXIN were incubated with immobilized GST-fusions of the core domains of XLef/Tcfs. Bound proteins were separated on 10 % SDS PAGE gel, exposed to the phosphoimaging plate and analyzed for specific Lef/Tcf sub-type interaction using a phosphoimager (Fig. 19A). Whereas RNF-4 binds neither to the GST control nor the Lef/Tcfs, ARC105 and ROR2 bind to both the GST control and all the Lef/Tcf fusions. TOLLIP and DLXIN discriminated between the GST control and the Lef/Tcfs. Although TOLLIP and DLXIN bind to GST control, the signal with Lef/Tcfs are much more intense. Both proteins seem to bind preferentially to XTcf-3 and XTcf-4A/B and less to XLef-1. However, further tests are required to confirm these two proteins as potential binding partners. MBP-1 does not bind to the GST control but binds to XLef-1 and even better to XTcf-3 and XTcf-4A/B. Interaction of Hic-5 with the Lef/Tcfs was investigated by Western blotting crude lyastes from human kidney epithelial (h293) cells transfected with myc-tagged versions of XLef/Tcfs. The cells were incubated with immobilised recombinant Hic-5. Bound Lef/Tcfs were detected with anti-myc antibody. Hic-5 neither binds to the GST control nor to XLef-1 but binds strongly to XTcf-3 and XTcf-4A/B (Fig. 19B).

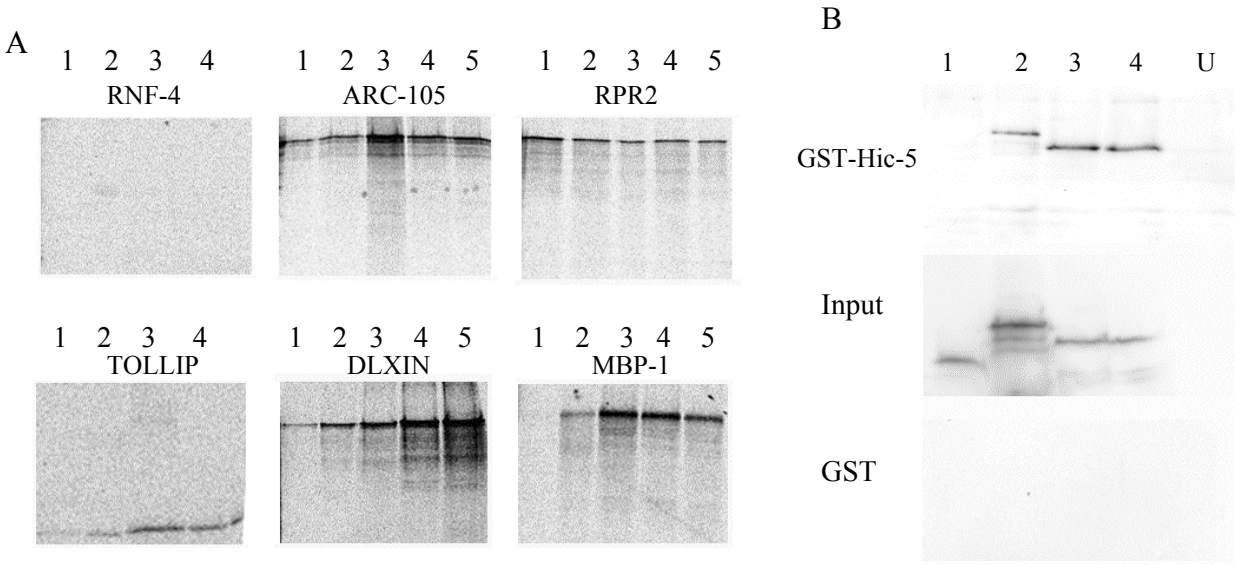

Fig. 19: Analysis of Xenopus Lef/Tcf-subtype-specific interaction with mouse clones isolated from yeast two-hybrid screen. A: *In vitro* translated ^{35}S-labelled constructs (RNF-4, ARC-105, MBP-1, TOLLIP, ROR2, DLXIN) were incubated with immobilized control GST constructs or recombinant core domains of XLef/Tcfs. Bound proteins were separated on 10 % SDS PAGE and analyzed for specific Lef/Tcf sub-type interaction using a phosphoimager. RNF-4 neither binds to GST control (lane 1), XLef-1 (lanes 2), XTcf-3 (lane 3) nor XTcf-4A (lane 4). ARC-105 and ROR 2 bind indiscriminately to both GST control (lane1), XLef-1 (lane 2) XTcf-3 (lane 3), XTcf-4A, (lane 4) and XTcf-4B (lane 5) respectively. TOLLIP and DLXIN bind weakly to GST control (lane 1) and Lef-1(lane 2) but strongly to XTcf-3 (lane 3) and XTcf-4 (lane 4). MBP-1 does not bind to GST control (lane 1), binds weakly to XLef-1 (lane 2), but strongly to XTcf-3 (lane 3), XTcf-4A (lane 4) and XTcf-4B (lane 5). B) Hic-5 interacts with XTcf-3 and XTcf-4 but not with XLef-1. Crude lysates of h293 cells transfected with myc-tagged versions of Lef/Tcfs were incubated with recombinant Hic-5. Bound Lef/Tcfs were detected with anti-myc antibody. Hic-5 does not interact with XLef-1 (lane1) but interacts with XTcf-3, XTcf-4A and XTcf-4B (lanes 2, 3 and 4 respectively). No interaction was observed with GST control. Lysates of transfected cells constitute the input. Untransfected cell lysates (U) were used as control.

In summary, some of the proteins isolated from the library by the yeast two hybrid system were false positives (RNF-4, ARC-105 and ROR2). Some (TOLLIP and DLXIN) have to be further analysed while others (MBP1 and Hic-5) were confirmed as new binding partners. Among all the clones characterized, preliminary results revealed that binding of Hic-5 and MBP-1 to LefTcfs was more discriminatory than the others. Further characterization was focused on MBP1 and Hic-5 but predominantly on Hic-5.

5.5. Isolation of the *Xenopus* orthologue of Hic-5.

Interaction between the mouse Hic-5 and the *Xenopus* Lef/Tcfs could be artificial due to differences in species. Therefore, a *Xenopus* ortholoque of Hic-5 was isolated to

verify this interaction. Using degenerate primers, a 510 bp fragment of XHic-5 was amplified and inserted into the pGEMT cloning vector. This 510 bp fragment was amplified by PCR and labelled with [α-^{32}P]-dCTP and used as a probe to screen a *Xenopus* tadpole stage λ-zap cDNA library. Radioactive signals indicating to hybridisation of the probe to the target DNA were detected as black spots by the phosphoimager (Fig. 20).

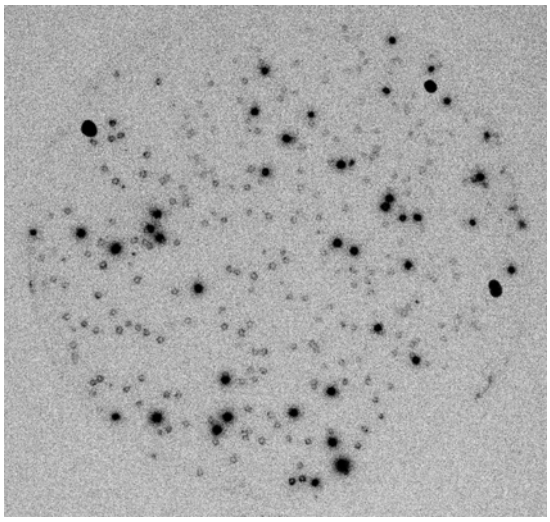

***Fig. 20: Xenopus 510 bp fragment binds to a complementary strand in the Xenopus tad pole stage library*:** *E. coli* XL-1Blue cells were transformed with the *Xenopus* tadpole stage library and plaque lifts were hybridised with a 510 bp *Xenopus* fragment labelled with [α-^{32}P]-dCTP. Radioactive signals corresponding positive clones.

A 3.4 kb XHic-5 cDNA was isolated (Acc. No. AY971603) and this clone consisted of an open reading frame of about 1.3 kbp and a 2.1 kbp 3´untranslated sequence. The deduced amino acid sequence of XHic-5 protein contains 459 aa (Fig. 21) with a predicted molecular weight of about 52 kD.

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ACT AAA GGG AAC AAA AGC TGG AGC TCC ACC GCG GTG GCG GCC GCT CTA GAA CTA GTG GAT CCC CCG GGC TGC AGG AAT TCG GCA
CGA GAT GCA CTA CTC GCT GAC CTC CAA ATC ACG ACC ACT CCA CGT TGT CCA GTT TTA CTA ACA GAT CTC CCT GAG AAA CCT CAG

                                                                  M   S   N   T   T   S   H   H   E   T   F   P
CCT GCT GAG ACT CGA CCT CCT CCA CCG CCC TAC GAT CCC AAG ACT GCA ATG TCG AAT ACG ACG TCC CAC CAT GAG ACC TTT CCA

 V   D   K   D   H   L   Y   S   T   V   Q   K   Y   P   L   P   S   V   S   P   A   L   G   G   G   L   C   E
GTG GAC AAG GAC CAT CTC TAT AGC ACT GTA CAG AAA TAT CCT TTG CCT TCT GTC TCG CCA GCC TTG GGT GGT GGG CTG TGT GAA

 L   D   R   L   L   N   E   L   N   A   T   Q   F   N   I   T   D   E   I   M   S   Q   F   P   T   R   D   P
TTG GAC CGA CTT CTC AAT GAA CTC AAC GCC ACA CAA TTC AAC ATC ACT GAT GAG ATA ATG TCC CAG TTC CCT ACT CGG GAT CCC

 N   E   Q   K   A   E   A   Q   K   E   A   E   K   R   A   L   A   A   S   S   A   T   L   E   L   D   R   L
AAT GAA CAG AAA GCT GAA GCA CAG AAA GAG GCA GAG AAA AGG GCC CTC GCT GCC TCC TCT GCC ACT CTT GAG TTG GAC CGC CTG

 M   A   S   L   S   D   F   H   K   Q   N   T   V   S   Q   E   G   E   V   P   D   S   N   K   G   S   E   E
ATG GCT TCC CTG TCT GAT TTC CAC AAG CAG AAC ACG GTA TCA CAA GAA GGG GAG GTT CCT GAC TCT AAT AAA GGC TCT GAG GAA

 V   F   K   P   R   D   T   E   D   P   S   S   P   R   D   T   L   D   V   P   K   A   L   E   D   I   P   S
GTT TTC AAA CCT AGA GAT ACA GAA GAC CCA TCA AGC CCT AGA GAC ACA CTT GAT GTT CCT AAA GCT TTG GAG GAC ATA CCA AGT

 P   K   S   S   E   V   M   S   T   P   G   H   M   E   V   K   I   D   Q   V   N   S   D   K   V   T   A   S
CCT AAA AGT TCT GAG GTG ATG TCC ACC CCT GGG CAT ATG GAA GTC AAA ATC GAC CAA GTA AAT TCT GAT AAA GTA ACA GCT TCC

 R   L   P   D   S   V   S   G   S   K   V   P   E   A   T   S   V   P   R   S   D   L   D   S   M   L   V   K
CGG TTG CCT GAC TCT GTG TCT GGT TCC AAA GTT CCA GAA GCC ACA AGT GTT CCA AGG AGT GAT CTG GAC TCC ATG CTG GTA AAG

 L   Q   S   G   L   K   Q   Q   G   I   E   T   H   S   K   G   L   C   E   S   C   Q   R   P   I   A   G   Q
CTA CAG TCT GGG CTC AAG CAA CAA GGC ATT GAA ACG CAC AGC AAG GGA CTG TGT GAG AGC TGC CAA CGA CCT ATA GCC GGG CAG

 V   V   T   A   L   G   H   T   W   H   P   E   H   F   V   C   A   H   C   H   A   L   I   G   T   S   N   F
GTG GTT ACT GCT CTT GGA CAT ACC TGG CAC CCG GAG CAC TTT GTG TGT GCC CAC TGC CAT GCC CTG ATT GGC ACT TCC AAC TTC

 F   E   K   D   G   R   P   Y   C   E   K   D   Y   F   M   L   Y   A   P   R   C   A   L   C   D   L   P   I
TTT GAG AAG GAC GGA CGG CCA TAT TGC GAG AAA GAC TAC TTT ATG CTC TAC GCC CCA CGC TGT GCC CTG TGT GAC TTG CCA ATT

 V   Q   N   M   V   T   A   L   G   R   T   W   H   P   E   H   F   C   C   K   I   C   K   K   P   I   G   E
GTG CAG AAC ATG GTT ACG GCA CTT GGT CGC ACT TGG CAC CCA GAG CAT TTT TGT TGT AAA ATC TGC AAA AAA CCG ATA GGG GAA

 E   G   F   H   E   K   D   G   E   Q   Y   C   S   D   D   Y   F   R   L   F   G   A   V   C   A   G   C   T
GAG GGT TTC CAT GAG AAG GAT GGG GAA CAG TAT TGC TCA GAC GAC TAT TTC CGG CTG TTT GGA GCT GTC TGT GCA GGC TGC ACT

 E   A   V   K   E   S   Y   I   S   A   L   G   G   L   W   H   P   Q   C   F   V   C   H   V   C   H   T   P
GAG GCC GTG AAG GAG AGT TAT ATC TCT GCC CTG GGA GGC TTG TGG CAC CCA CAA TGC TTT GTG TGT CAT GTG TGT CAC ACA CCA

 F   I   N   G   S   F   F   E   H   E   G   L   P   L   C   E   T   H   Y   H   S   R   R   G   S   L   C   A
TTC ATC AAT GGA AGT TTC TTT GAA CAT GAA GGC TTA CCT CTA TGT GAG ACT CAT TAC CAC TCT CGT CGT GGC TCG CTG TGT GCT

 G   C   E   Q   P   I   T   G   R   C   V   A   A   M   G   K   K   F   H   P   Q   H   L   S   C   T   F   C
GGG TGT GAG CAG CCA ATA ACA GGG CGC TGC GTG GCT GCA ATG GGC AAG AAG TTC CAC CCC CAA CAC TTG AGT TGC ACT TTT TGC

 L   R   Q   L   N   K   G   T   F   R   E   H   D   G   K   P   Y   C   Q   A   C   Y   A   R   L   Y   G
CTC CGA CAG CTG AAC AAG GGC ACC TTC CGG GAG CAT GAT GGG AAA CCT TAC TGC CAG GCT TGC TAC GCC CGA CTG TAT GGG TAG

GGC AGT TAG ATG GTT TCA GGA AAG GAG GGT CAT CTG AAA TAT CAA GCT TCA CAA AGA CTA TAC AAA GGA TGT TGT GCA AGG CAC
CAG ATT GCA AGG CTC TGC AAG GGA AAG GGC TGG CTG CTC TGA AGG TCA CAC AAA ATG CAC TGC TCA TCC AGC TCT GCA AGA ATC
CAC GTG CAA TTT TTT CTG CAG GGA AAT GGG CTA AAA GCT TTT GGT TTT GTA CGT GAA CAC ATG CAT GTA AGG TCA AAT GGA GAA
GGT GAC ATC TGC TCT GCA AAC CCC TTG GGC ATC ACA TTA ACT GGT CTT ATG GAA AGG AGC CAG CTG CTC TGC AAG CTT TGT GCA
GAG TTC TAC AAT ATA AAA CAG CTG CTG TGC ACA CAC TCC AAG GCT ATG TGC AAA GTT TGT CAA GGA GAC TGA TCT CCA AGC CGT
CTG AGA TCA AGA CAA AGT GCA AAA TTG TGT GGA GGC GTC TGC TCT GCA ACC TTC TGA GAC TAA GTG CAA AAT TGT GCA AGG TTG
GAG TCA GCG AAA CTA TAG AGG AAG CAG ACC GTG CAG CTG CAG GGG GGC CCA GGA AAT ATA GGG GGC CCC AAG AGG CTC TAA TTC
ATA TAC AAT TTC AGT AAA TAT TGG TAA AAC AGG ACA ACC TCT GGA TAT GTT GGG GGC CCT AAA ACT AAT TTG CTG TGG GGC CCA
GTA ACA TCT AGT TAC GCC ACT GAG AGG GGA CAT CTG CTC TGC CAA ACC TTC TGA GGC AAA GTG CAA AGT TCT GCA AGG GAG GAG
CCA GCT GCT CTG TAA ACC TTC TGA GAC AAA GTG CAA AGT TCT GCA GGG AGG AAC CAG CTG CTT TGC AAA CCT TCT GAG ACA AAG
TGC AAA GTT CTG CCA GGG AAG GAG CCA TCT TCT NTG CAA ACC TTC TGA GAC TAA GTG CAA CAT TCT GCA AGG GAG GAA CCA TCT
GTT CTG CAA ACC TTC TGA GAC TAA GTT CAA AGT TCT GCA AGG GAG GAG ACA TCT GCT CTG CAA ACC TTC TGA GAT TAA GTG CAC
AGT TCT ACA AGG GAT GAG CCA GCT GCT CTG CAA ACC TTC TGA GAC AAA GTG CAA AAT TCT GCA AGG GAG GAG ACA TCT GCT CTG
CAA ATC TTC TGA AAC TAA GTA CAA AGT TCA GCA AGG AAG GAG ACA TCT GCT TTG CAA ACC TTC TGA GAC AAA GTG CAA AGT TCT
GCA AGG AAG GAG CCA TCT GCT CTG CAA ACC TTC TGA GAG TAA GTG CAA CAT TCT GCA AGG GAG GAA CCA TCT TTT CTG CAA ACC
TTC TGA GAC TAA GTT CAA AGT TCT GCA AGG GAG GAG ACA TCT GCT CTG CAA ACC TTC TGA GAT TAA GTG CAC AGT TCT ATA AGG
GAT GAG CCA GCT GCT CTG CAA ACC TTC TGA AAC TAA GTA CAA AGT TCT GCA AGG AAG GAG ACA TTG CTC AGC AAA CCT TCT GAG
ACT AAG TGC AAT GTT CTG CAA GGG AGG AAC CAT CTG TTC TGC AAA CCT TCT GAG ACT AAG TGC AAA GTT CTG CAA GGG AGG AAC
CAC CTG TTC TGC AAA CCT TCT GAG ACT AAG TGC AAA GTT CTG CAA GGG AGA AGC CAG CTA CTC TGC AAA CCA TCT GAG ATG GAG
CAA AAA GAG CTA CTA TGC ATT CAT TGG TCA GCT TTC TAA AAC CAA ACA TAT GAT TGG TCA CAG AGT TTC TGC ATA AGC CTG AGA
GTA TTT TTA CAC ACA TCC AAT TAA AAA GGG CAT TGT AAT CAC AGT TGT AGG GTT GCT CCA TGT CTA GTA ACC CAT AGC AAC CAA
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Fig. 21: The isolated Xenopus Hic-5 DNA has an open reading frame of 1.3 kbp and 2.1 kbp 3´ untranslated sequence. The deduced amino acid sequence of XHic-5 protein is represented.

Full length Hic-5 protein is 40% identical to its human orthologue. The similarity in the conserved three LD and four Lim domains ranges from 55% (LIM-2) to 100% (LD-2) (Fig. 22).

Fig. 22: Protein alignment of Xhic-5 with its human and murine orthologues reveals high homology in the LD- and LIM- domains. The three LD motives are indicated in violet and the four LIM domains are illustrated by blue bars.

The most obvious differences between the mammalian and the *Xenopus* Hic-5 proteins is the absence of two proline-rich streches of 24 and 11aa respectively which flank a highly conserved region of of 11aa in the *Xenopus* protein. Conversely, XHic-5 possesses two acidic and serine-rich stretches of 28 and 27 aa between LD-2 and LD-3 which are absent in the human and murine orthologues (Fig. 22).

5.6 *Xenopus* Hic-5 discriminates between XTcf-3 and XTcf-4 and XLef-1

Bacterially expressed GST-Lef/Tcf fusion proteins were incubated with *in vitro*-translated ^{35}S-labelled or transfected myc-tagged Xhic-5 and bound proteins were separated on an SDS PAGE gel.

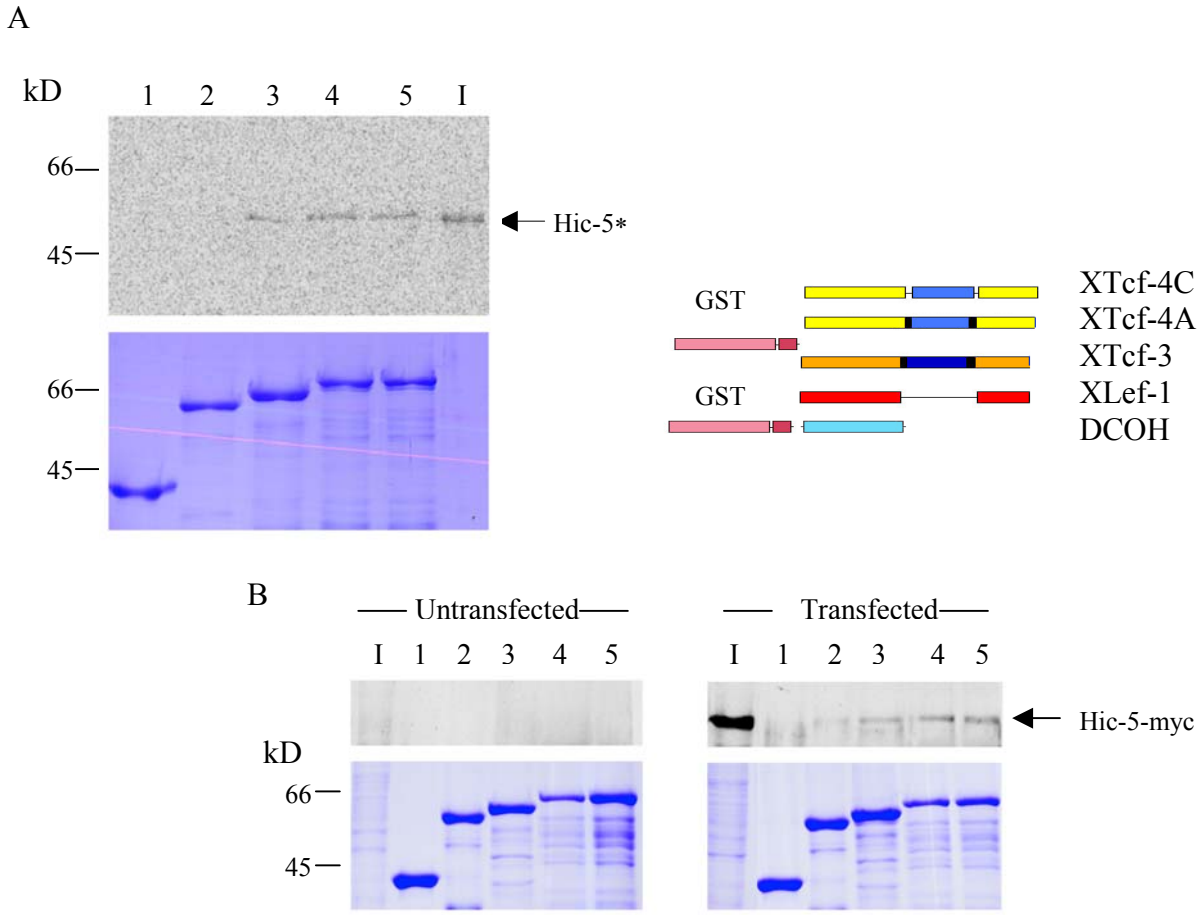

Fig. 23: Xhic-5 binds to Xenopus Tcf-3 and Tcf-4 but not to Lef-1. A) Recombinant Lef/Tcfs were immobilized on glutathione sepharose and incubated with *in vitro* translated ^{35}S-labeled XHic-5. Bound proteins were resolved on an SDS PAGE gel. The autoradiograph (upper panel) shows no binding of ^{35}S-Xhic-5 (represented by Hic-5*) to GST control (lane 1) and XLef-1 (lane 2) while strong binding was observed to the core region of XTcf-3 (lane 3), XTcf-4A (lane 4) and XTcf-4B (lane 5). The lower panel shows the same gel stained with Coomassie blue to demonstrate equal loading. B) Immobilized Lef/Tcf-GST fusions were incubated with crude cell lysates of h293 cells transfected with myc tagged Xhic-5. The upper panel shows the immunoblot stained with anti-myc-epitope specific mAB 9E10. Myc-tagged XHic-5 does not bind to GST control (lane 1), binds very weakly to XLef-1 (lane 2), binds strongly to XTcf-3, XTcf-4A and XTcf-4B (lanes 3,4 and 5). The input (I) is indicated. The lower panel shows the same gel stained with Coomassie blue to demonstrate equal loading. The GST-DCOH fusion and untransfected cell lysates served as negative control. Schematic representation of constructs used is shown.

Exposure of gel revealed that Hic-5 has a molecular weight of 52 kD corresponding to the predicted molecular weight. No binding of ^{35}S-Xhic-5 (represented by Hic-5*) to

GST control and XLef-1 core region was detected while strong binding was observed to XTcf-3 and XTcf-4 core regions (Fig. 23A). Similar results were obtained when bound proteins from kidney epithelial cells transfected with myc-tagged XHic-5 were probed with anti-myc epitope antibody. Whereas no binding of Hic-5 was observed for the GST control, binding to XLef-1 core region was very low while strong binding was detected for XTcf-3, XTcf-4 and XTcf-4B core regions (Fig. 23B).

5.7 Hic-5 binds to the conserved Exon

The most obvious difference between XLef-1 and Tcf-3 and -4 in the core region is the absence of an alternatively spliced activating Exon in XLef-1. Since Hic-5 binds to XTcf-3 and XTcf-4 but not XLef-1, this Exon might be the domain of interaction with Hic-5. To confirm this, a myc-tagged version of XLef-1 containing the Exon excised from XTcf-3 was used in GST pull-down assays. Whereas Hic-5 did not interact with XLef-1, it bound to XLef-1+Exon Fig. 24A).

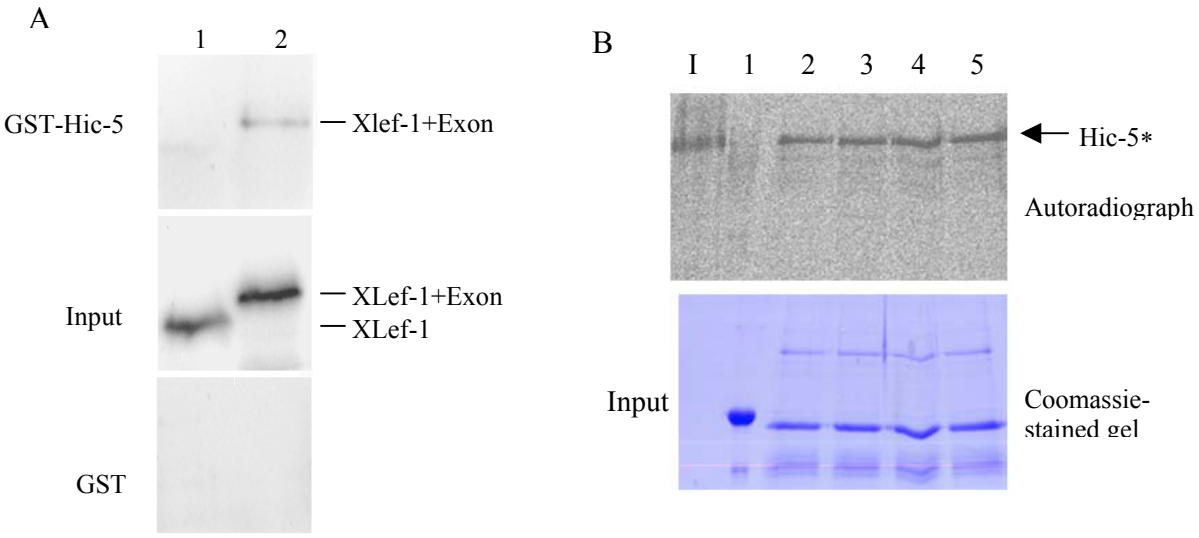

Fig. 24: Hic-5 binds to the activating Exon. A) Myc-tagged version of XLef-1 containing the Exon excised from XTcf-3 was used in GST pull-down assays. Hic-5 does not bind to XLef-1 (lane 1) but binds to XLef-1+Exon (lane 2). B) Pull-down assays with ^{35}S-labelled Hic-5 and bacterially expressed GST fusions of Exons from XTcf-3 and XTcf-4. Hic-5 neither interacts with GST (lane 1) nor does it discriminate in binding between the Exons from XTcf-3 (lane 2) and XTcf-4A, 4B and 4C (lanes 3, 4 and 5 respectively). The input (I) is indicated.

Similarly, pull-down assays with ^{35}S-labelled Hic-5 and bacterially expressed GST fusions of Exons from XTcf-3 and XTcf-4 were carried out. Hic-5 does not discriminate in binding between the Exons from XTcf-3 and XTcf-4A and XTcf-4B (Fig. 24B).

5.8 The LIM domain-containing C-terminal half of Hic-5 binds to Lef/Tcfs

To map the binding domain in Hic-5, GST fusions of the N-terminal half (GST-mHic5ΔC) containing the LD domains (aa_1-aa_{202}) and the C-terminal half (GST-mHic5ΔN) containing the LIM domains (aa_{212}-aa_{430}) were used in GST pull-down assays. Autoradiograph results revealed that all Tcfs that contain the conserved Exon (mLef-1, hTcf-3, hTcf-4, hTcf-1 and mTcf-3) but not XLef-1 interacted with the LIM domain-containing C-terminal half. Whereas binding of mLef-1 was very strong, the Tcfs interacted only weakly with the C-terminal half of Hic-5. In one case (hTcf-4) a very weak binding of the LD domain-containing N-terminal half was observed. In summary, Lef/Tcfs bind *via* a conserved Exon preferentially to the Lim domain-containing C-terminal half of Hic-5 (Fig. 25).

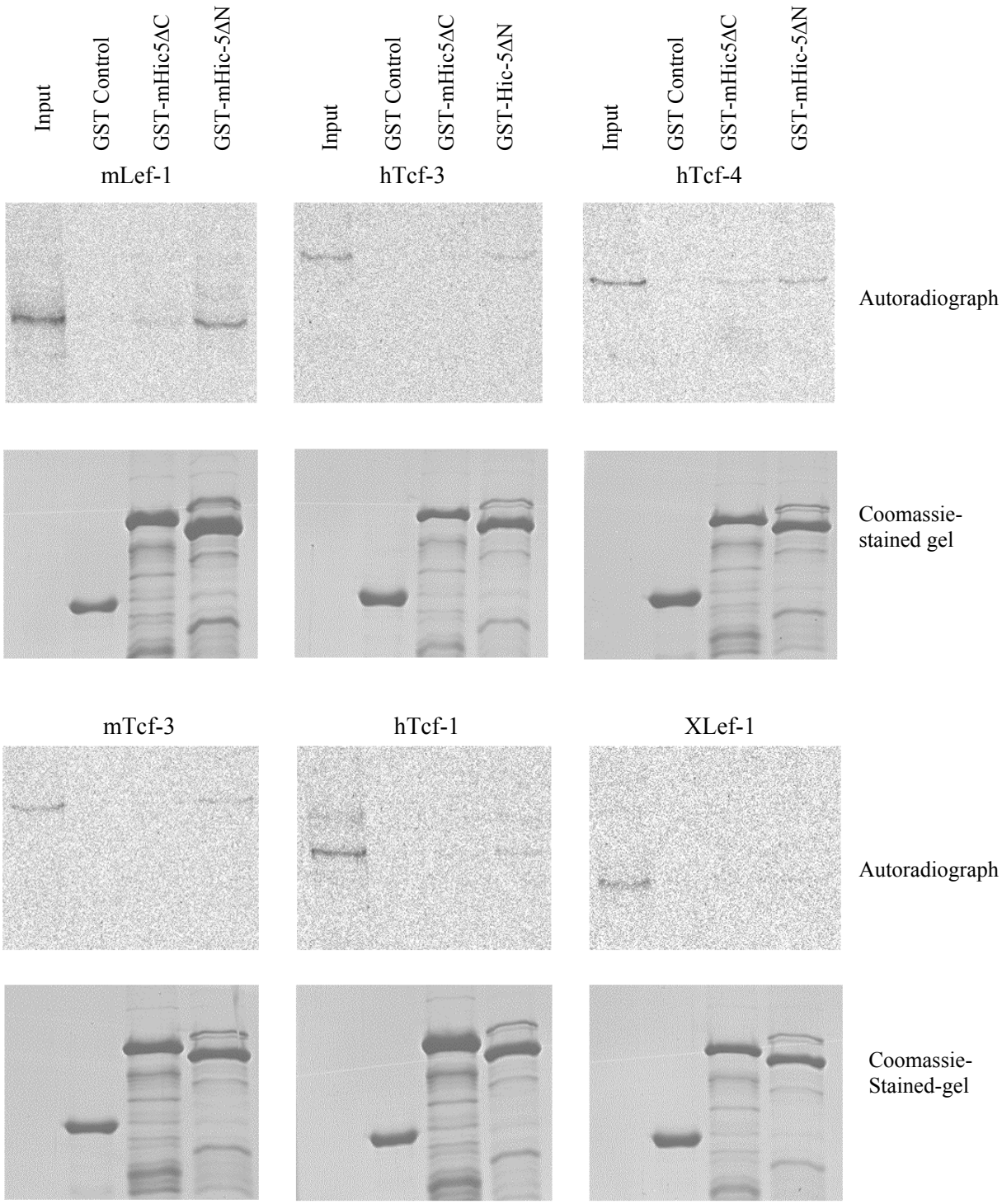

Fig. 25: Lef/Tcfs weakly bind to the LIM domain-containing C-terminal half of mHic-5. Recombinant C-terminal half (GST-Hic-5ΔN, LIM domains, aa_{212}-aa_{430}) and N-terminal half (GST-Hic5ΔC, LD-domains, aa_1-aa_{202}) of mHic-5 were immobilized on glutathione sepharose and incubated with *in vitro* translated ^{35}S-labeled murine and human Lef/Tcfs. The upper panels show the autoradiograph. XLef1 served as a negative control and neither bound to the C-terminal half nor the N-terminal half of mHic-5. The lower panels show the same gels stained with Coomassie blue to demonstrate equal loading.

5.9 Hic-5 represses Lef/Tcf-induced target gene promoter activation

To investigate if the physical interaction between Lef/Tcfs and Hic-5 could modulate transcription of Wnt/β-catenin target genes, reporter gene assays were carried out in h293 cell co-transfected with Lef/Tcf reporter constructs, Hic-5, CMV-β-galactosidase (for normalization) and different Lef/Tcf expression constructs.

5.9.1 Hic-5 represses TOPFlash promoter activation

Mouse Hic-5 and *Xenopus* Hic-5 regulate Tcf optimal binding (TOPFlash) promoter in a similar manner. In the absence of Lef/Tcfs both murine and the *Xenopus* Hic-5 had insignificant effect on the activity of the TOPFlash promoter. XLef-1, XLef-1+Exon and XTcf-4C but not XTcf-4A and XTcf-3 significantly activate the TOPFlash promoter in h293 cells in the absence of Hic-5 (Fig. 26A) consistent with the fact that XLef-1 and XTcf-4C are general activators while XTcf-3 and XTcf-4 are repressors (Pukrop et al, 2001, Gradl et al., 2002). In the presence of Hic-5, however, XTcf-4C did not activate the TOPFlash promoter and the activation by the chimeric XLef-1+Exon construct was drastically reduced. In the case of XTcf-4C, the promoter activation dropped from 2.1 fold to 1.2 (murine Hic-5) or 0.8 fold (Xhic-5), while for XLef-1+Exon fold activation dropped from 4.1 to 1.9 or 1.5, respectively. Activation *via* Hic-5 had no effect on TOPFlash activation *via* XLef-1. The promoter was activated 1.5 to 2 fold, irrespective to whether Hic-5 was co-transfected or not (Fig. 26A). The mutated TOPFlash control promoter (FOPFlash) was neither activated by XLef-1+Exon nor repressed by Hic-5 (Fig. 26B).

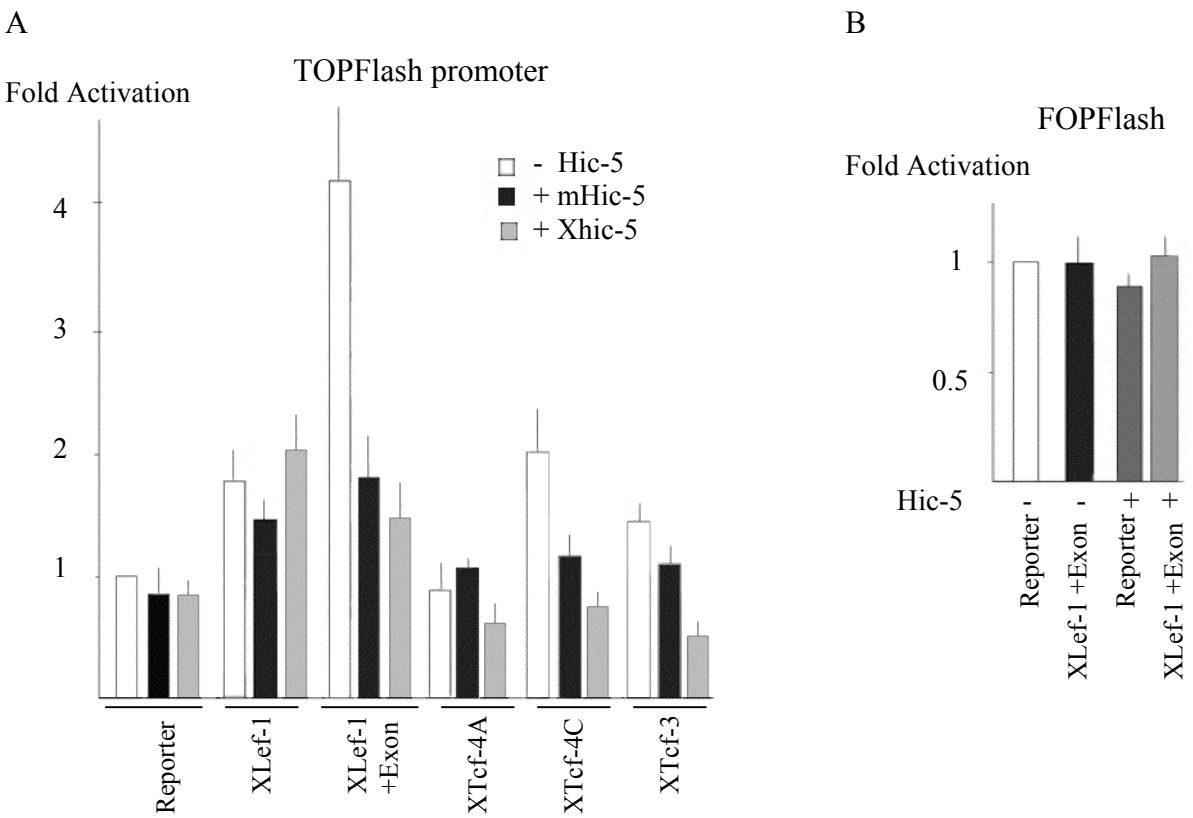

Fig. 26: Hic-5 represses Lef/Tcf-induced TOPFlash promoter activation. A) H293 cells were co-transfected with TOPFlash, CMV-β-Galactosidase (for normalization) and the indicated Lef/Tcf constructs in the absence of Hic-5 (-Hic-5, white bars) or in the presence of murine Hic-5 (+mHic-5, black bars) or *Xenopus* Hic-5 (+XHic-5, grey bars). B) Hic-5 does not regulate the FOPFLash promoter, in the presence or in the absence of XLef-1+Exon. H293 cells were co-transfected with FOPFlash, CMV-β-Galactosidase for normalization and XLef-1+Exon in the presence or absence of murine Hic-5. Each bar represents the average of 6 to 14 transfections. The error bars indicate the standard error.

5.9.2 Hic-5 represses *fibronectin* promoter activation.

Regulation of Wnt target genes by Hic-5 was not restricted to the artificial TOPFlash promoter. Similar responses were observed with the *Xenopus fibronectin* promoter. Again, co-transfection of Hic-5 prevented promoter activation by XLef-1+Exon (1.7 fold versus 3.2 fold) and XTcf-4C (1.6 fold versus 2.3 fold) but not by XLef-1 (1.9 versus 1.9) (Fig. 27).

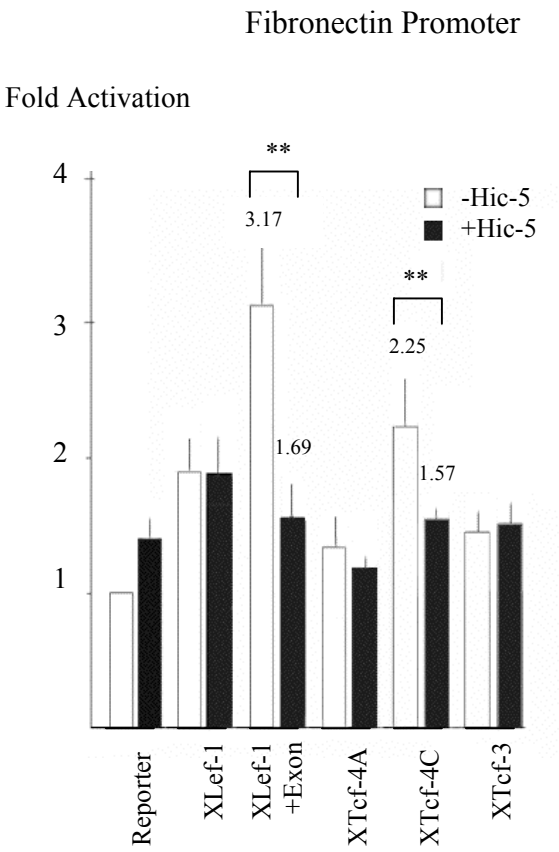

Fig. 27: Hic-5 represses Lef/Tcf induced-fibronectin promoter activation. H293 cells were co-transfected with the *Xenopus fibronectin* promoter, CMV-β-galactosidase and the indicated Lef/Tcfs, either in the absence (-Hic-5, white bars) or in the presence (+Hic-5, black bars) of murine Hic-5. Each bar represents the average of 6 to 14 transfections. The error bars indicate the standard error. Asterisks (**) indicate significant differences ($p < 0.05$ in student t-test).

5.9.3 Hic-5 repression of Lef/Tcf-induced target promoter activation is conserved in vertebrates.

In addition to binding to XLef/Tcfs, Hic-5 also binds to murine and human Lef/Tcfs in reporter gene assays. In the absence of Hic-5, mLef-1, hTcf-1 and h-Tcf-4 activated the TOPFlash promoter over 3 fold but following co-transfection of Hic-5, the mammalian Lef/Tcf-induced promoter activation was inhibited (Fig. 28). Although Tcf-3 contains the conserved Exon it did not activate the TOPFlash promoter consistent with published data that XTcf-3 is a strong repressor of Wnt/β-catenin target promoters (Pukrop et al, 2001, Gradl et al., 2002). Hic-5 could not further repress it.

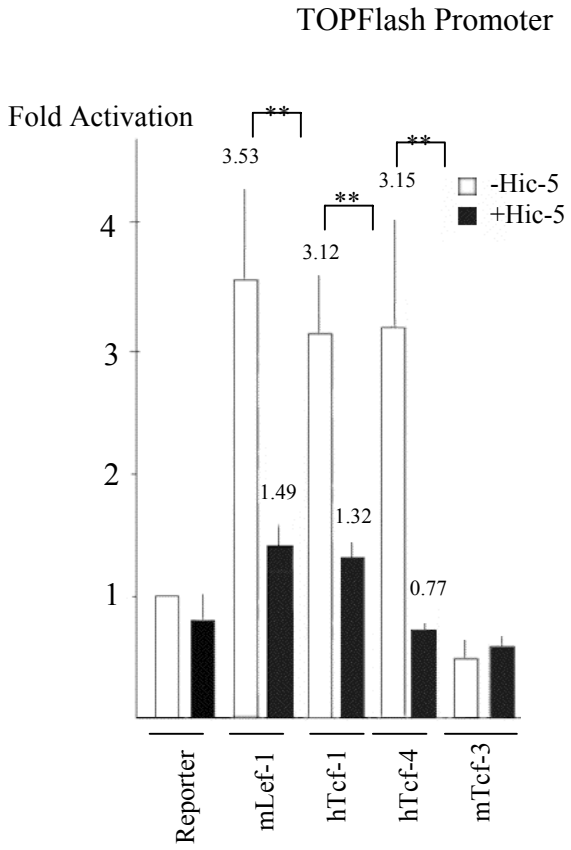

Fig. 28: Hic-5 repression of Lef/Tcf-induced target promoter activation is conserved in vertebrates. H293 cells were co-transfected with TOP-Flash, CMV-β-galactosidase and the indicated human and murine Lef/Tcfs, either in absence (-Hic-5, white bars) or in the presence (+Hic-5, black bars) of murine Hic-5. Each bar represents the average of 6 to 14 transfections. The error bars indicate the standard error. Asterisks (**) indicate significant differences ($p < 0.05$ in student t-test).

5.10 Hic-5 represses Lef/Tcf-induced target gene activation

If Hic-5 is really binding to and repressing Lef/Tcf function, then it should repress the transcription of direct down stream Lef/Tcf target genes. In *Xenopus*, *siamois* and *Xnr-3* are well characterized down stream targets genes of Lef/Tcfs. Activation of the canonical Wnt pathway at the ventral side of the embryo leads to the formation of secondary axes and at higher doses to dorso-anteriorisation of the embryo. Injection of 70 pg *XWnt 8* mRNA into the ventral blastomers of 4-cell stage *Xenopus* embryos resulted in 98% (n=80) dorso-anteriorised embryos characterized by a ring-shaped cement gland (Fig. 29A. After co-injection of 500pg Hic-5 mRNA only 3 % of the injected embryos (n = 104) showed this complete dorso-anteriorization while 58 % of the embryos now showed a partial rescue as seen by the appearance of a secondary axis and 39 % revealed a complete rescue as seen by the appearance of a single axis (Fig. 29A). Consistently, Wnt/β-catenin target genes, *siamois* and *Xnr-3*, were

down-regulated following co-transfection of Hic-5 as revealed by RT-PCR analysis (Fig. 29B).

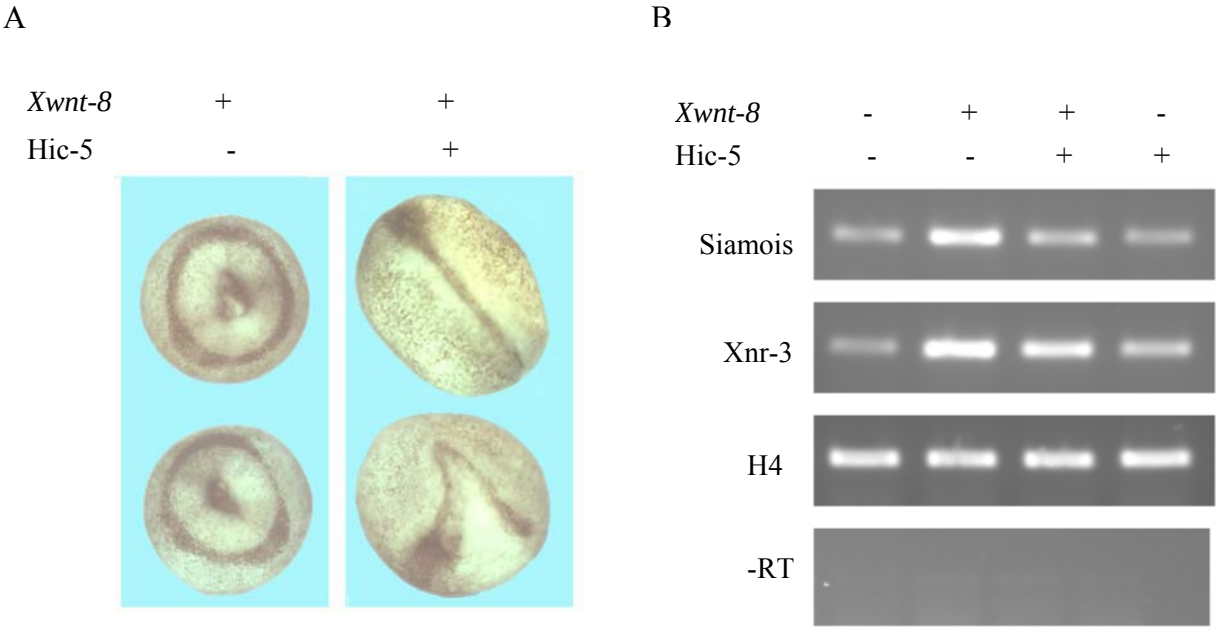

Fig. 29: Hic-5 represses Lef/Tcf-induced target gene activation. A) Embryos injected ventrally with 70 pg *Xwnt-8* RNA revealed complete dorso-anteriorization which is best seen by the appearance of a ring shaped cement gland. Co-injection of 500 pg Hic-5 mRNA resulted in a partial rescue and the appearance of secondary axis or in a complete rescue and the appearance of a single axis. B) Expression analysis of the Wnt-target genes *siamois* and *Xnr-3* by RT-PCR in injected and uninjected stage 10.5 *Xenopus* embryos. Histone (H)4 shows the amplification of the housekeeping gene histone 4, -RT shows the control reaction without reverse transcriptase.

5.11 Lef/Tcfs regulate steroid receptors in a Hic-5 dependent manner

Hic-5 is known to be a co-activator of glucocorticoid receptors (Guerrero-Santoro et al., 2004) and androgen receptors (Fujimoto et al, 1997). It is hypothesised that Hic-5 might be a key player in a cross-talk to Wnt/β-catenin signalling. To investigate whether Lef/Tcfs regulate Hic-5-mediated activation of glucocorticoid-receptor driven transcription, reporter gene assays were carried out using the mouse mammary tumour virus (MMTV) long terminal repeat (LTR) in human cervix carcinoma (Hela) cells. These cells endogenously express Hic-5 and contain substantial amounts of Hic-5 protein in the nucleus, where it co-localizes with Lef/Tcfs as revealed by double staining of cells with polyclonal anti-Hic-5 and monoclonal anti-Lef-1 or Tcf-3/-4 (Fig. 30).

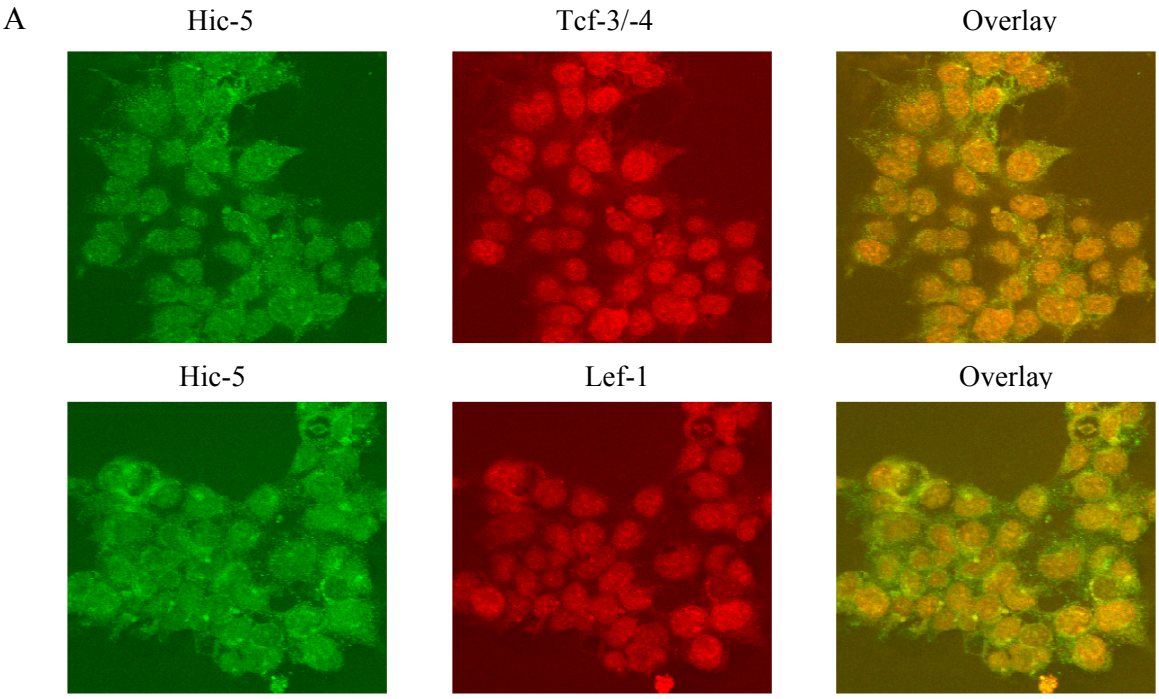

Fig. 30: Hic-5 protein co-localizes with Lef/Tcfs in the nucleus. Double staining of HeLa cells with a polyclonal antibody against Hic-5 (green) and a monoclonal antibody against Tcf3/4 or Lef-1 (red). Overlay of the Hic-5 signal with Lef/Tcf signals reveals that HeLa cells contain substantial amounts of Hic-5 protein in the nucleus, where it co-localizes with Lef/Tcf proteins.

The MMTV is a model promoter for studying steroid receptor activation, because it regulates target gene expression in response to signals from steroid receptors (glucocorticoid, mineralcorticoid, progesterone and androgen receptors).

5.11.1. MMTV reporter responses to Dexamethaxone in a dose dependent manner.

Before studying the influence of Lef/Tcf interaction with Hic-5 on MMTV response it is necessary to determine the appropriate hormone concentration at which the glucocorticoid receptor (GR) is sufficiently activated so that both activation and repression can be observed. Hela cells were therefore co-transfected with MMTV-luciferase and CMV-β-galactosidase (for normalization) and treated with different concentrations of dexamethasone.

Reporter gene assay results reveal that MMTV promoter responds to the hormone in a dose dependent manner. Up to 1 nM dexamethasone activated the promoter about 3 fold, 10 nM approximately 9 fold and further increase to 20 nM resulted in over 15-fold activation (Fig. 31A). RT-PCR analysis revealed that this dose dependency was also observed at the level of the endogenous glucocorticoid target gene transcription.

Transcription of *p21*^{cip1} and *p27*^{kip1} genes was almost undetectable at 0 to 1 nM dexamethasone concentration but robustly increased at higher concentrations (Fig. 31B). The moderate concentration of10 nM dexamethasone was chosen for subsequent assays.

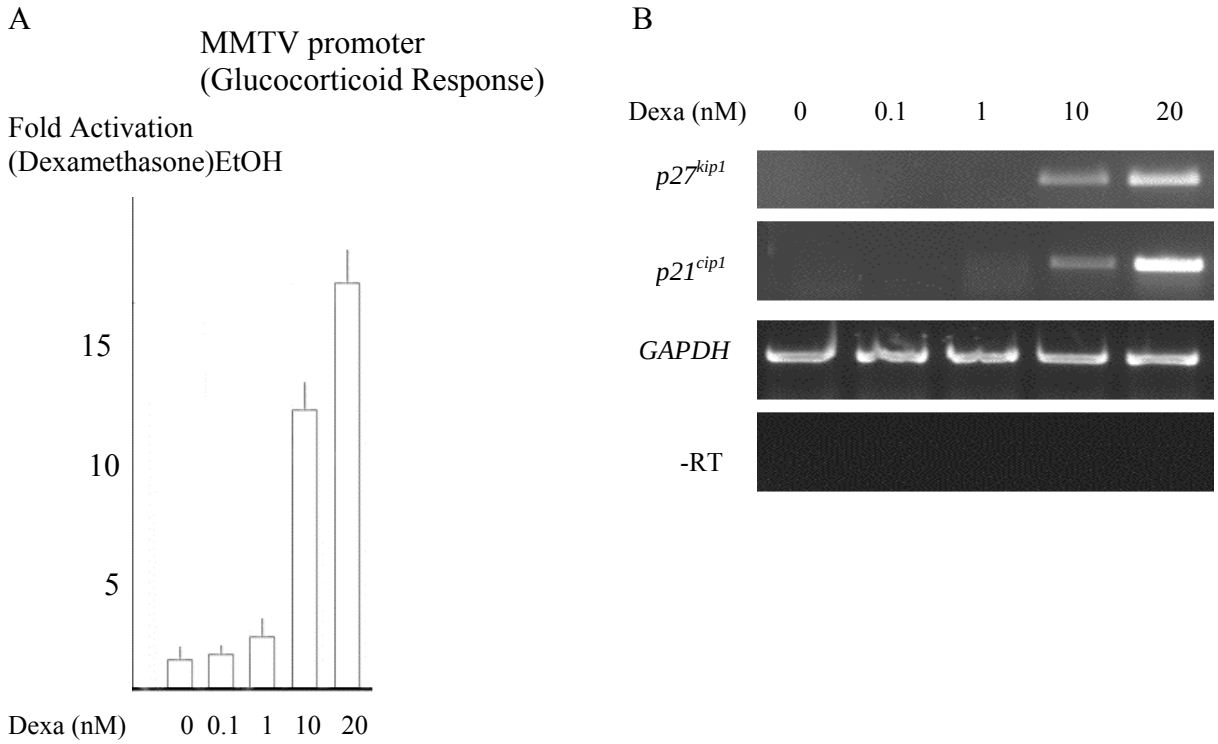

Fig. 31: MMTV reporter responses to dexamethaxone (Dexa) in a dose dependent manner. A) Hela cells were co-transfected with MMTV-Luciferase and CMV-β-galactosidase (for normalization) and treated with the indicated amounts of dexamethasone. Each bar represents the mean value of at least 7 transfections. The error bars indicate the standard error. B) RT-PCR-analysis reveals that the expression of the glucocorticoid receptor target genes *p21*^{cip1} and *p27*^{kip1} depends on the hormone concentration. GAPDH shows the amplification of a house keeping gene, -RT indicates the control amplification of the housekeeping gene without reverse transcription.

As expected for a steroid recepto-coactivator, Hic-5 enhances reporter activation from 9 to about 20 fold in the absence of Lef/Tcfs, (Fig. 32A). Over expression of XLef-1 and XLef-1+ Exon did not affect reporter activation in the absence of Hic-5. Overexpression of XLef-1 also had no significant effect on promoter activation (fold activation remains at approximately 20) in the presence of Hic-5. Conversely, overexpression of XLef-1+ Exon resulted in a repression of promoter activity (fold activation drops from about 20 to 14.5) in the presence of Hic-5.

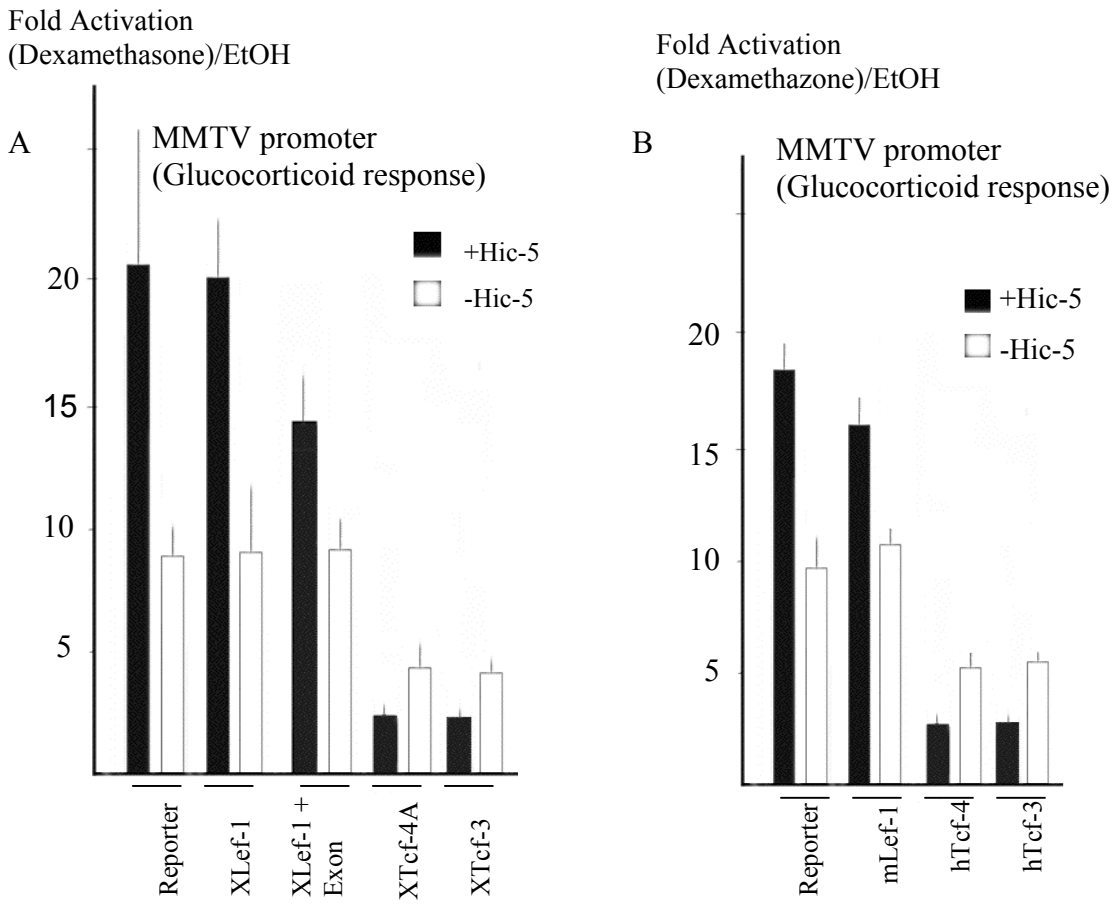

Fig. 32: Lef/Tcfs repress glucocorticoid receptor target promoters in a Hic-5-dependent manner. A) Hela cells were co-transfected with MMTV-Luciferase, CMV-β-galactosidase and the indicated *Xenopus* Lef/Tcf constructs either in the presence (+Hic-5, black bars) or absence (-Hic-5, white bars) of mHic-5. The glucocorticoid receptor was activated by adding 10 nM dexamethasone dissolved in ethanol or by adding the solvent alone. Shown is the fold activation by dexamethasone treatment normalized to ethanol treatment. B) Same as in A) but with human and murine Lef/Tcfs.

XTcf-3 and XTcf-4 regulate the MMTV promoter in a different manner. Both Tcfs repress the GR response even in the absence of co-transfected Hic-5 from 9 fold to about 4 fold respectively. This repression was even more pronounced in the presence of Hic-5.

Similar results were obtained when human and murine Lef/Tcfs were tested for their influence on Hic-5 induced glucocorticoid response (Fig. 32B). In the absence of Lef/Tcfs, Hic-5 enhances reporter activation from 9 fold to about 18.5. Overexpression of mLef-1 did not affect reporter activation in the absence of Hic-5. In the presence of Hic-5 overexpression of mLef-1 had little effect on promoter activation (fold activation falls from 18.5 to approximately 16). In the absence of Hic-5, overexpression of hTcf-3 and hTcf-4 inhibited dexamethasone-induced activation

from 9 fold to about 5 fold. Again, this repression was more pronounced in the presence of Hic-5.

The influence of the interaction between Hic-5 and Lef/Tcfs on the activity of Hic-5-regulated glucocorticoid target gene transcription was investigated by RT-PCR. Results reveal that both *p21^{cip1}* and *p27^{kip1}* target genes which remain at endogenous levels are activated by Hic-5 but not by Tcf-4. In the presence of both hTcf-4 and mHic-5, the target genes are down-regulated (Fig. 33).

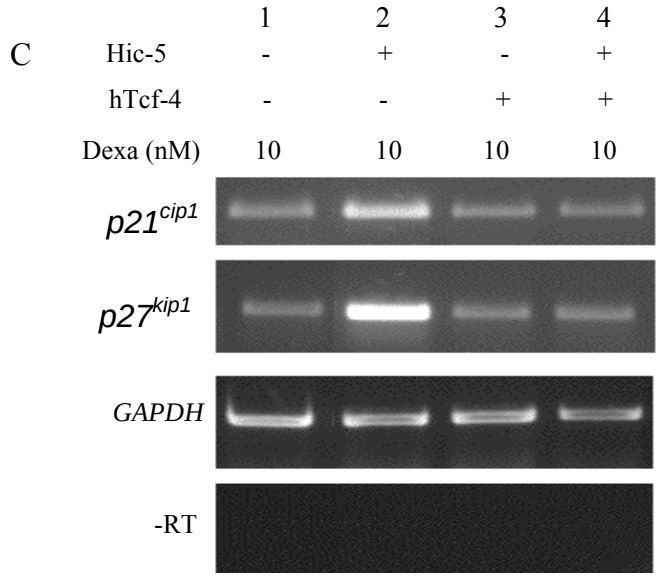

Fig. 33: RT-PCR-analysis: expression of the glucocorticoid receptor target genes p21^{cip1} and p27^{kip1} is down-regulated by the interaction between Hic-5 and Tcf-4. GAPDH shows the amplification of a house keeping gene, -RT the control amplification of the housekeeping gene without reverse transcription.

5.11.2 Lef/Tcfs repress androgen receptor target genes in a Hic-5 dependent manner.

To figure out if the repression of the MMTV promoter by Lef/Tcfs is specific for the glucocorticoid repression or is a general mode of repression of steroid receptor activity, Hela cells were co-transfected with human androgen receptor (AR) since these cells do not endogenously express the androgen receptor. The androgen response was stimulated by dihydroxytestosterone treatment. This hormone activated the promoter to about 5 fold while co-transfection of Hic-5 lead to a 10 fold activation. Similarly, Hic-5-indepenedent activation was observed for XLef-1 and the chimeric construct, XLef-1+Exon. Co-transfection of Hic-5 however discriminated between the two XLef-1 constructs (Fig. 34).

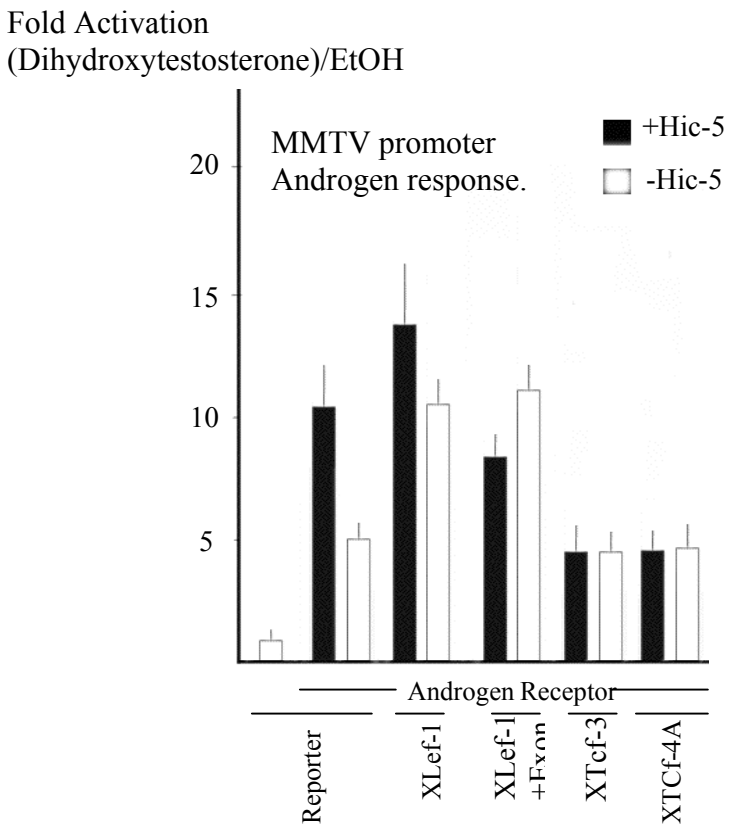

Fig. 34: Lef/Tcfs repress androgen receptor target genes in a Hic-5 dependent manner. Hela cells were co-transfected with MMTV-Luciferase, androgen receptor and CMV-β-galactosidase for normalization and the indicated *Xenopus* Lef/Tcf constructs either in the presence (+ Hic-5, black bars) or absence (- Hic-5, white bars) of Hic-5. The androgen receptor was activated by adding 10 nM dihydroxytstosterone (DHT) dissolved in ethanol or by adding the solvent alone. Shown is the fold activation by DHT treatment normalized to ethanol treatment.

Whereas the combination of XLef-1 and Hic-5 resulted into an additive activation, the combination of XLef-1+Exon and Hic-5 activated the promoter less than each of the constructs alone. Over expression of XTcf-3 and XTcf-4 prevented AR activation *via* Hic-5.

As a summary, Hic-5 binding to the conserved activating and alternatively spliced Exon of XTcfs lead to repression of Hic-5-mediated activation of both steroid receptors and steroid receptor target genes. This repression is not observed with the Xlef-1 which lacks the Hic-5 binding site.

5.12.1 MBP1 binds strongly to Domain A of XTcf-3 and XTcf-4 but weakly to XLef-1

Mutant p53 binding protein (MBP)-1 also known as fibulin 4 is one of the XTcf-4 binding partners isolated from the yeast two-hybrid screen (Table 3 page 64). Its binding to mutant p53 protein and enhancing tumour cell growth (Gallagher et al.,

1998) and its isolation as a binding partner of XTcf-4 (present study) suggests that it might play a central role in Wnt/β-catenin-mediated tumour growth. It was confirmed by GST pull-down assays to bind strongly to the core domains of XTcf-3 and XTcf-4 but weakly to XLef-1 (Fig. 19, page 66). Since binding was observed for both XLef-1 and XTcfs, it was evident that the Exon alone could not account for the recruitment of MBP1. Therefore, in order to further characterize this binding partner, bacterially expressed domain A of *Xenopus* Lef/Tcf proteins fused to GST were incubated with *in vitro*-translated ^{35}S-labelled MBP1 and bound proteins were separated on an SDS PAGE gel. Following exposure of gel to phosphoimaging plate, phosphoimager results revealed that mMBP1 has a molecular weight of 49 kD corresponding to the predicted molecular weight. Binding of ^{35}S-MBP1 to GST control was reduced to traces, while weak binding was observed to XLef-1. Strong binding was detected for XTcf-3 and XTcf-4 (Fig. 35). A faint band is also observed for MBP1 binding to GST but might correspond to a higher amount of GST input as observed on the Coomassie-stained gel.

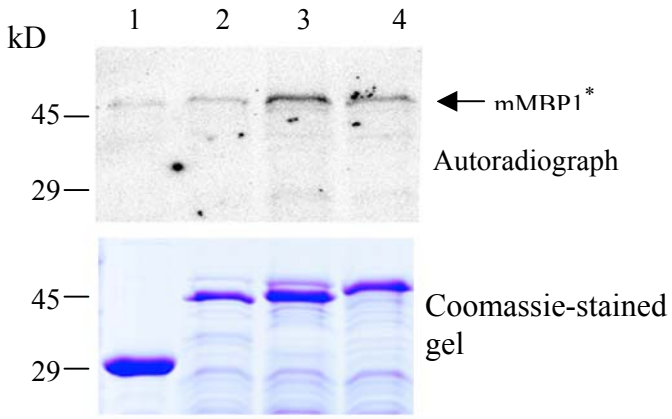

Fig. 35: MBP1 binds to Xenopus Tcf-3 and Tcf-4 but weakly to Lef-1. A) Recombinant Lef/Tcf-GST fusions of domain A were immobilized on glutathione sepharose and incubated with *in vitro* translated ^{35}S-labeled mMBP1. Bound proteins were resolved on an SDS PAGE gel. The autoradiograph (upper panel) shows that binding of ^{35}S-MBP1 (represented by MBP*) to GST control was faint (lane 1), binding of XLef-1 was weak (lane 2) while strong binding was observed to XTcf-3(lane 3) and XTcf-4 (lane4). The lower panel shows the same gel stained with Coomassie to demonstrate equal loading.

Using degenerate primers, the *Xenopus* homologue of MBP1 was isolated from *Xenopus* tadpole stage cDNA library. It comprises of an open reading frame of about 900 bp (Fig. 36). The deduced amino acid sequence reveals that the molecular weight of the XMBP1 is about 35 kD. Alignment of the protein sequence of the

isolated XMBP1 with the murine orthologue revealed 80% amino acid identity (Fig. 37). The XMBP1 sequence however lacked the 5´ leader sequence.

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                                                        M   L   G   S   Y   H   C   K   C   P   E   G   Y
TCG CTG CTC CGG CCG CCA TGG CCG CGG GAT TGG CCT CTC GAG ATG CTT GGT AGC TAT CAC TGC AAG TGT CCA GAG GGC TAC

 R   K   I   G   N   E   C   V   D   I   D   E   C   R   Y   R   Y   C   Q   H   R   C   V   N   Y   P   G
AGG AAG ATT GGG AAT GAG TGT GTA GAC ATA GAT GAG TGC CGA TAT CGA TAC TGC CAA CAC CGA TGC GTT AAC TAC CCA GGT

 S   F   V   C   Q   C   E   P   G   F   Q   L   A   S   N   N   R   S   C   V   D   V   N   E   C   S   M
TCT TTT GTT TGT CAG TGC GAG CCT GGA TTT CAG CTG GCA TCA AAT AAC CGC TCC TGT GTG GAT GTT AAT GAG TGC TCA ATG

 G   T   P   C   Q   Q   R   C   Y   N   T   Y   G   T   F   L   C   R   C   N   Q   G   Y   E   L   S   H
GGC ACT CCC TGC CAG CAA AGG TGT TAT AAC ACC TAT GGG ACA TTC CTG TGC CGC TGC AAT CAG GGC TAC GAA CTG AGC CAT

 D   G   Y   T   C   T   D   I   D   E   C   S   Y   S   S   Y   L   C   Q   H   Q   C   V   N   E   P   G
GAT GGA TAT ACC TGC ACT GAT ATT GAT GAG TGC AGT TAT TCC AGT TAC CTT TGC CAG CAT CAG TGT GTG AAT GAG CCC GGA

 S   F   S   C   S   C   P   E   G   Y   Q   L   L   G   T   R   L   C   Q   D   I   N   E   C   E   L   G
AGC TTC TCC TGT AGT TGT CCC GAA GGA TAC CAG CTT TTA GGC ACC CGA CTG TGC CAA GAT ATC AAT GAG TGT GAA CTT GGG

 T   H   S   C   S   E   Q   Q   T   C   V   N   F   H   G   G   Y   Q   C   V   E   K   N   R   C   Q   E
ACC CAT AGC TGC TCA GAA CAG CAG ACC TGT GTC AAC TTT CAT GGG GGT TAC CAA TGC GTG GAA AAG AAT CGC TGC CAG GAA

 P   F   I   Q   V   S   E   N   R   C   M   C   P   G   T   N   P   L   C   H   D   E   P   G   S   V   V
CCT TTT ATC CAA GTG TCT GAA AAC CGC TGT ATG TGT CCT GGG ACC AAT CCC CTG TGC CAT GAT GAA CCG GGC TCT GTG GTG

 Y   R   Y   M   S   I   T   S   D   R   S   V   P   S   E   I   F   Q   I   Q   A   T   S   V   Y   P   G
TAT AGA TAC ATG AGC ATC ACA TCA GAC AGA AGT GTG CCC TCC GAA ATA TTC CAG ATA CAG GCA ACC AGC GTC TAC CCT GGA

 A   Y   N   I   F   Q   I   R   S   G   N   E   Q   G   E   F   F   I   R   Q   I   N   N   L   T   A   M
GCT TAT AAC ATA TTC CAA ATC CGC TCA GGA AAT GAG CAA GGA GAG TTC TTC ATC AGG CAA ATC AAC AAC CTC ACA GCT ATG

 L   V   L   A   R   P   V   I   G   P   K   E   Y   V   L   D   L   E   M   L   T   T   N   S   L   L   S
CTT GTC CTG GCC CGC CCT GTC ATT GGC CCC AAA GAG TAT GTA CTG GAT CTG GAA ATG CTA ACC ACC AAT TCT CTG CTG AGT

 Y   R   S   S   S   V   L   R   L   T   V   F   V   G   A   H   S   F
TAC CGC TCC AGC TCT GTG CTT AGA CTC ACC GTG TTT GTG GGA GCT CAT TCC TTC TAG CTC GAG AGG CCA ATC ACT AGT GCG

GCC GCC TGC AGG TCG ACC ATA TGG GAG AGC TCC CAA CGC GTG GAG CAA
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Fig. 36: ***Xenopus MBP1 DNA has an open reading frame of 900 bp.*** The deduced amino acid sequence ofMBP1 protein is indicated.

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mMBP-1 MLPFASCLPG SLLLWAFLLL LLGAASPQDP EEPDSYTECT DGYEWDADSQ HCRDVNECLT

mMBP-1 IPEACKGEMK CINHYGGYLC LPRSAAVISD LHGEGPPPPA AHAQQPNPCP QGYEPDEQES

mMBP-1 CVDVDECTQA LHDCRPSQDC HNLPGSYQCT CPDGYRKIGP ECVDIDECRY RYCQHRCVNL
XMBP-1                    MLGSYHCK CPEGYRKIGN ECVDIDECRY RYCQHRCVNY

mMBP-1 PGSFRCQCEP GFQLGPNNRS CVDVNECDMG APCEQRCFNS YGTFLCRCNQ GYELHRDGFS
XMBP-1 PGSFVCQCEP GFQLASNNRS CVDVNECSMG TPCQQRCYNT YGTFLCRCNQ GYELSHDGYT

mMBP-1 CSDIDECGYS SYLCQYRCVN EPGRFSCHCP QGYQLLATRL CQDIDECETG AHQCSEAQTC
XMBP-1 CTDIDECSYS SYLCQHQCVN EPGSFSCSCP EGYQLLGTRL CQDINECELG THSCSEQQTC

mMBP-1 VNFHGGYRCV DTNRCVEPYV QVSDNRCLCP ASNPLCREQP SSIVHRYMSI TSERSVPADV
XMBP-1 VNFHGGYQCV EKNRCQEPFI QVSENRCMCP GTNPLCHDEP GSVVYRYMSI TSDRSVPSEI

mMBP-1 FQIQATSVYP GAYNAFQIRS GNTQGDFYIR QINNVSAMLV LARPVTGPRE YVLDLEMVTM
XMBP-1 FQIQATSVYP GAYNIFQIRS GNEQGEFFIR QINNLTAMLV LARPVIGPKE YVLDLEMLTT

mMBP-1 NSLMSYRASS VLRLTVFVGA YTF
XMBP-1 NSLLSYRSSS VLRLTVFVGA HSF
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Fig. 37: Protein alignment of XMBP1 protein sequence with its murine orthologue reveals about 80% identity. This sequence however lacks the 5´ leader sequence.

5.12.2 MBP1 activates Wnt/β-catenin target promoters

To study the influence of the MBP1 interaction with Lef/Tcfs, human epithelial kidney cells (h293) cells were co-transfected with *siamois*, CMV-β-galactosidase (for normalization), Lef/Tcf and MBP1constructs. Reporter gene assay results reveal that

in the absence of MBP1, XLef-1, XLef-1+Exon and XTcf4-C activate the *siamois* promoter while Tcf-4A and XTcf-3 has little effect on promoter activity (Fig. 38A).

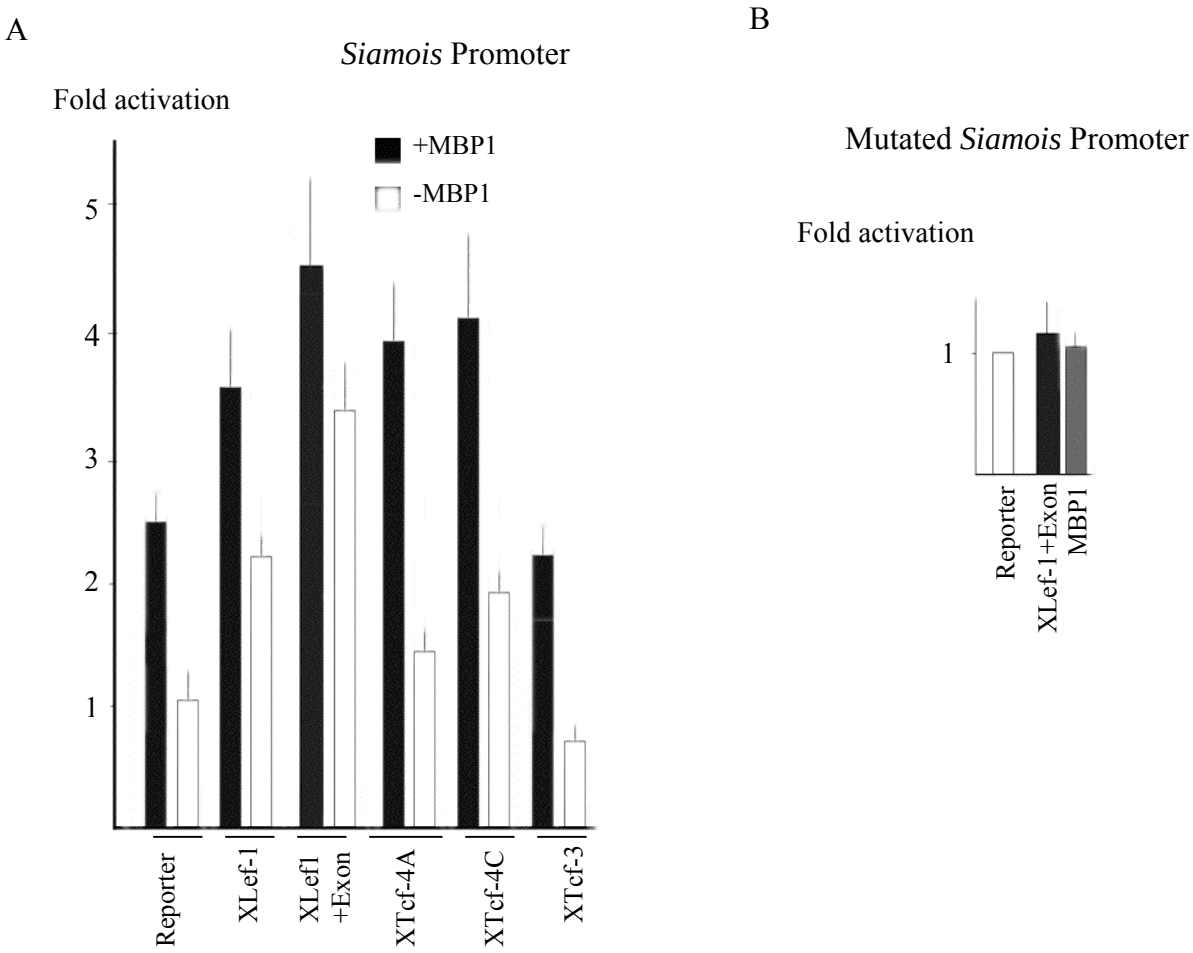

Fig. 38: MBP1 activates Lef/Tcf induced siamois promoter activation. A) H293 cells were co-transfected with *siamois*, CMV-β-galactosidase (for normalization) and the indicated Lef/Tcf constructs in the absence of MBP1 (- MBP1, white bars) or in the presence of murine MBP1 (+ MBP1, black bars). B) MBP1 does not regulate the mutated *siamois* promoter either in the presence or absence of XLef-1+Exon. H293 cells were co-transfected with FOPFlash, CMV-β-galactosidase for normalization and XLef-1+Exon in the presence or absence of murine MBP1. Each bar represents the average of 8 to 16 transfections. The error bars indicate the standard error.

This is consistent with published data that XLef-1, XLef-1 + Exon and XTcf-4C are activators of Wnt/β-catenin target promoters while XTcf-3 and XTcf-4A are not (Pukrop et al., 2001, Gradl et al., 2002). In the presence of MBP1, promoter activation is generally enhanced. Enhancement is observed for the promoter alone (from 1 to 2.5), XLef-1 (from 2.1 to 3.6), XLef-1+Exon (3.5 to 4.5), XTcf-4A (1.5 to 3.4), XTcf-4C (2.0 to 4.0) and XTcf-3 (0.8 to 2.2) (Fig. 38A). As negative control, the

mutated *siamois* promoter which lacks the Lef/Tcf binding sites was neither regulated by MBP1 not XLef-1+Exon (Fig. 38B).

Similar results were obtained with the *fibronectin* promoter. In the absence of MBP1, only XLef-1 and XTcf-4C activate the promoter but in the presence of MBP1, there is a general enhancement of the promoter in the absence as well as in the presence of Lef/Tcfs (Fig. 39).

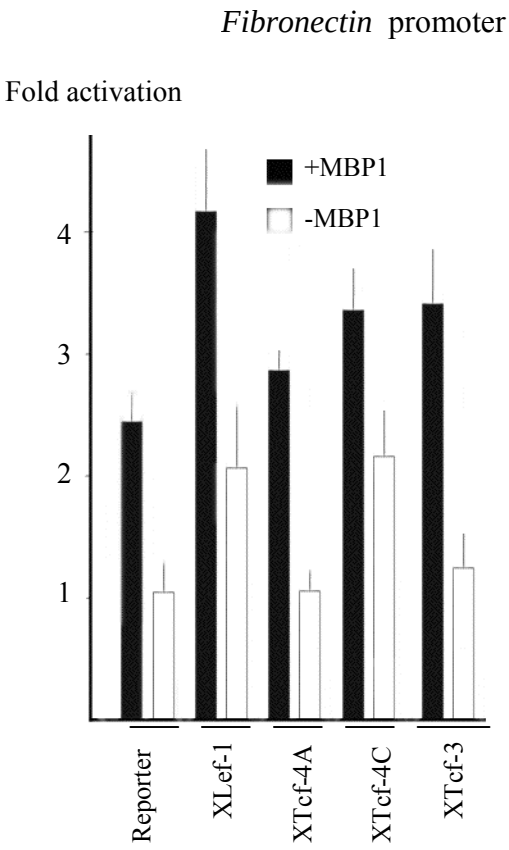

Fig. 39: MBP1 activates fibronectin promoter. H293 cells were co-transfected with the *fibronectin* promoter, CMV-β-galactosidase and the indicated Lef/Tcfs, either in the absence of MBP1 (- MBP1, white bars) or in the presence of murine MBP1 (+ MBP1, black bars). Each bar represents the average of 8 to 16 transfections. The error bars indicate the standard error.

As a summary, MBP1 is a general co-activator of XLef and XTcfs. The alternatively spliced activating Exon and Domain A (region between the β-catenin-binding site and the first flanking motif of the activation Exon) are the putative binding regions of MBP1 to Lef/Tcfs.

6. Discussion

6.1 Identification of putative binding partners of the XLef/Tcfs

The functional diversity between the different XLef/Tcf family members (Gradl et al., 2002, Roel et al., 2002) implies that the individual family members recruit specific binding partners which lead to their characteristic functions. Analysis of various XTcf mutants and XLef/Tcf chimeric constructs indicate that the region responsible for the individual characteristics is the less conserved and poorly characterised region (core region) spanning from the β-catenin binding site to the HMG box (Gradl et al., 2002).
Putative Lef/Tcf binding partners of the Lef/Tcfs were identified using this core region as bait in a two-hybrid. GST pull-down assays to confirm the yeast two-hybrid results revealed that Hydrogen peroxide-inducible clone 5 (Hic-5) and Mutant-binding protein-1 (MBP-1) were true positives while Melanoma-associated antigen D1 (MAGED1 or DLXIN) and Toll-interacting protein (TOLLIP) still need to be verified. TOLLIP is reported to interact with Toll-like receptors thereby blocking the Toll-Like receptor signalling pathway (Zhang and Ghosh, 2002). This pathway is responsible for accumulation of tumour necrosis factor (TNF)-α and interleukin (IL)-β in inflammatory processes (Moynagh, 2003). The binding of TOLLIP to Tcf-3 and Tcf-4 might reveal a cross-talk between Wnt/β-catenin signalling and Toll-like receptor signalling pathways in which TOLLIP serves as an adaptor protein. This might therefore link the Wnt/β-catenin signalling to the modulation of inflammatory processes.
Like β-catenin, the stability of DLXIN-1 is under the control of an ubiquitin-dependent degradation pathway (Masuda et al., 2001). It is a transcriptional regulator of DLXIN-5, which is involved in osteoblast formation. Dlx5 is a Bone morphogenic protein (BMP)-inducible gene (Miyama et al., 1999) expressed in most developing skeletal elements. It is induced during the process of bone development and post-fracture healing (Yaoita et al., 1999). The interaction of DLXIN-1 with Tcf-3 and Tcf-4 might suggest an additional level of cross-talk between the BMP pathway and the Wnt/β-catenin signalling. However, this cross-talk needs to be verified.
The fact that none of the known Lef/Tcf binding partners was got in the yeast two-hybrid screen is not unexpected since most of the known Lef/Tcf binding partners bind to the β-catenin binding site or the HMG box. The region of Lef/Tcfs used as bait in the screen was the core region of these transcription factors. Groucho which binds

to this core domain of Lef/Tcfs (Fig. 40) is a strong repressor (Brantjes et al., 2002) and might have repressed the VP16 activation domain in the library vector such that target gene activation is impossible. Hence, Groucho is not necessarily expected to be got from the screen.

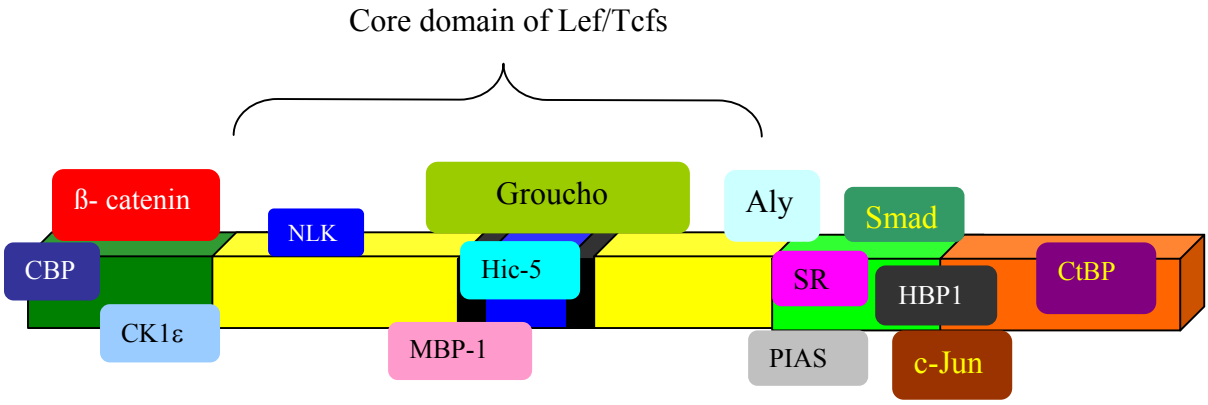

Fig. 40: In addition to Groucho, ALY and Nemo-like kinase (NLK), Hic-5 and MBP1 bind to the core domain of Lef/Tcfs. Hic-5 binds to the alternatively spliced activating and conserved Exon of XTcf-3 and XTcf-4. MBP1 binds to the same Exon as well as well as to the poorly characterised domain A of all XLef/Tcfs. All other known binding partners bind outside the core region.

Another protein, ALY, binds specifically to part of the core domain (Fig. 40) corresponding to the context dependent activation domain (CAD) of Lef-1 (Bruhn et al., 1997). This CAD is absent in Tcf-3 and Tcf-4. Since only XTcf-3 and XTcf-4 and not XLef-1 was used in the screen, the isolation of ALY was not expected. The Nemo-like kinase (NLK) has also been described to bind and phosphorylate Lef/Tcfs at their core domain (Ishitani et al., 2003) (Fig. 40). However, considering the transient nature of kinase binding the chance of isolating NLK is very low.

All new identified potential binding partners did not discriminate in binding between XTcf-3 and XTcf-4 in GST pull-down assays thereby confirming the specificity of the bait-prey interaction observed in the yeast two-hybrid screen. This inability to discriminate between XTcf-3 and XTcf-4 also implies that the binding site does not represent the XTcf-4-specific region comprised of 20 amino acids located at a position spanning from aa_{127}- aa_{147} (König et al., 2000). However, differences in Lef-1 and Tcf-3/-4 binding was observed for Hic-5 and to a lesser extent also for MBP1.

6.1.1 Hic-5, A Novel Repressor of Lef/Tcf-Driven Transcription

Hydrogen peroxide induced clone 5 (Hic-5) is a dual location protein found both at focal adhesion sites and in the nucleus (Shibanuma et al., 2003). It is a LIM domain protein and together with leupaxin, paxB and paxillin (Lipsky et al., 1998) forms a subfamily of type 3 LIM proteins. In addition to having four contiguous LIM domains in their C-terminal half, they have common LD motives in the N-terminal half (Brown et al., 1998). Both the LD motives and LIM domains potentially serve as an interface for interactions with other proteins (Dawid et al., 1995) or with DNA (Nishita et al., 2000). Because interaction between the mouse Hic-5 and the *Xenopus* Lef/Tcfs could be artificial as a result of differences in species, a *Xenopus* ortholoque of Hic-5 was isolated and used with the *Xenopus* Lef/Tcfs in GST pull-down assays.

6.1.1.1 Hic-5 binds to XTcf-3 and XTcf-4 but not to XLef-1

Hic-5 was identified to bind preferentially to XTcf-3 and XTcf-4 but not to XLef-1. This subtype-specific binding suggests that Hic-5 might regulate XTcf but not XLef-1 function. The binding site of Hic-5 to XTcf-3 and XTcf-4 was mapped to the conserved and alternatively spliced activating exon which is absent in all XLef variants reported so far (Levanon et al., 1998, Pukrop et al., 2001). This Exon shares 70% amino acid identity between mLef-1 and XTcf-3 and XTcf-4. The conserved nature of this Exon indicates to a common function and a common set of binding partners.

The Hic-5 binding domain in XTcfs overlaps with the Groucho-binding domain, which has been shown to span the region between the β-catenin binding site and the HMG-box (Brantjes et al., 2001). However, the binding domains for Groucho and Hic-5 are not identical because, despite the presence of the Groucho binding site in all *Xenopus* Lef/Tcfs (Pukrop et al., 2001) only XTcf-3 and XTcf-4 could bind Hic-5.

The binding region in Hic-5 was mapped to the Lim domain-containing C-terminus although this binding appeared weak. These LIM domains in Hic-5 have been found to be responsible for binding to a multitude of proteins as well as nucleic acids. Spectroscopic analysis demonstrates that the LIM domains in general define a specific zinc binding structure in which zinc coordination renders it functional in binding to other proteins and DNA (Michelsen et al., 1993). The fact that mouse and *Xenopus* Hic-5 bound to all human and murine and *Xenopus* Lef/Tcfs tested indicates that this binding is conserved within and across the vertebrate species.

The binding of Hic-5 to Lef/Tcfs might indicate that it is an adaptor protein mediating the cross-talk between different signalling cascades. Because Hic-5 binds both Lef/Tcfs (present study) and smad 3 (Shibanuma et al., 2003) it might likely also link the transforming growth factor (TGF)-β and Wnt/β-catenin signalling pathways. Since Hic-5 is also known to bind to and activate steroid receptors (Fujimoto et al., 1999) its binding to Lef/Tcfs couples the Wnt/β-catenin signalling with steroid signalling. The localization of Hic-5 at focal contacts and the binding to the focal adhesion kinase (FAK) (Nishiya et al., 2002) might indicate that in addition to integrin-linked kinase (ILK) Hic-5 might regulate Wnt/β-catenin signalling and thus provide an additional mechanism that integrates integrin-mediated outside-in signalling with Wnt/β-catenin signalling.

6.1.1.2 Hic-5 represses Wnt/β-catenin target genes

Although Hic-5 has been described as an activator of androgen receptors (Fujimoto et al., 1999) it turned out in this study that the interaction with Lef/Tcfs results into a repressive complex. The repressive nature of this complex was not only observed in reporter gene assays where target gene promoters such as TOPFlash and *Xenopus fibronectin* were down regulated but was also observed in injected *Xenopus* embryos where transcription of Lef/Tcf target genes such as *siamois* and *Xnr3* were repressed. The fact that this repression was demonstrated not only with *Xenopus* Tcfs but also with murine and human Lef/Tcfs indicates to what extent this regulatory step is conserved in vertebrates.

Hic-5 was mapped to bind to the conserved Exon previously described to represent the activating motifs of the Lef/Tcfs (Gradl et al., 2002). Although the mode of activation *via* the conserved Exon is still unknown, the specific binding of Hic-5 to this Exon constitutes a novel repressive mechanism of Lef/Tcf function. The presence of this Exon in *Xenopus* Tcfs but not in XLef-1 explains the importance of this regulation: it favours Hic-5-mediated repression of all XTcf-driven genes and avoids inappropriate repression of XLef-1. Numerous experiments have revealed that Lef/Tcfs do not only function as activators of transcription when activated by Wntβ-catenin signals. They have been described to actively interact with co-repressors to inhibit target gene expression thereby preventing inappropriate Wnt/β-catenin signalling. Many mechanisms have been proposed on how this interaction inhibits target genes. Alteration of the chromatin structure has been described for interactions

between Lef/Tcfs and Groucho (Brantjes et al., 2002) or CtBP (Brannon et al., 1999). Lef/Tcf function is also inhibited by masking of their conserved domains by proteins such as CBP (Waltzer and Bienz, 1998), HBP1 (Sampson et al., 2001), PIAS1 (Sachdev et al., 2000) and steroid receptors (Amir et al., 2003) while phosphorylation of Lef/Tcfs by NLK constitute an unusual means by which Wnt/β-catenin signalling is inhibited (Ishitani et al., 2003). Whether Hic-5 binding to the activating Exon uses one of these mechanisms or an alternative mode to inhibit Lef/Tcf-induced transcription remains a subject of controversy.

XHic-5 has been found to display both timely and spatially restricted expression patterns. From RT-PCR results, XHic-5 is maternally expressed, down regulated at blastula, peaks at gastrula and continues to be expressed at low levels in the neurula and tail bud stages (Stephanie van Venrooy, Diploma thesis, 2004). *In situ* hybridisation reveals that at the blastula stage XHic-5 is expressed in the animal hemisphere, at neurula stage it is expressed at the dorsal side and at later stages its expression is observed around the eye, in the branchial arch, somites and in the pituitary gland (Martin Ritthaler, Diploma thesis, 2005). Despite the evidence from the present study that XHic-5 binds to the XTcfs, only partial overlapping patterns of expression of both XHic-5 and some of the Lef/Tcf transcription factors were observed in *Xenopus*. Like XHic-5, XTcf-3 is expressed maternally and transcripts have also been detected around the eyes and the branchial arch. This co-expression might suggest their involvement in the regulation of the canonical Wnt signalling pathway in these organs.

Expression of Tcf-4 in the mouse is detected in the crypts where it plays a role in the maintenance of stem cells (Korinek et al., 1998) and in the maturation of Paneth cells in the mouse intestine (vanEs et al., 2005). On the contrary, Hic-5 and its interaction partner, the orphan nuclear receptor, peroxisome proliferator-activated receptor (PPAR) gamma (PPARγ), induce an epithelial program in the mouse intestinal microvilli (Drori et al., 2005). Whereas PPARγ expression is restricted to the microvilli, Tcf-4 expression is uniquely in the crypts while Hic-5 expression extends from the crypts to the microvilli. These overlapping patterns between mHic-5 and Tcf-4 on one hand and mHic-5 and PPARγ on the other hand give evidence that the interaction of Hic-5 with PPARγ (Drori et al., 2005) and Lef/Tcfs (present study) is involved in the decision whether the intestinal cells continue to proliferate (high Tcf-4,

low Hic-5, low PPARγ) or whether they start to differentiate to epithelial cells (Low Tcf-4, high Hic-5, high PPARγ).

Although XHic-5 was found to bind to XTcf-4, during development XHic-5 and XTcf-4 are not co-expressed in the developing brain. In both mouse and *Xenopus*, Tcf-4 is expressed in the developing midbrain (König et al., 2000, Barker and Clevers, 2000) but XHic-5 expression has not been detected in this region (Yuminamochia et al., 2003, Martin Ritthaler, Diploma thesis, 2005).

6.1.2 Lef/Tcfs repress Hic-5-induced steroid receptor target gene activation

Hic-5 has been described as a co-activator of a subset of steroid receptors (SRs). It binds to and activates androgen receptors (ARs) (Fujimoto et al., 1999) and glucocorticoid receptors (GRs), (Yang et al, 2000). Although it does not directly bind to estrogen receptors (ERs), it potentiates ER activity in the presence of a glutamate receptor interacting protein (GRIP)-1 (Yang et al., 2000). SRs belong to the family of ligand-activated Zinc-finger transcription factors and consist of an N-terminal transactivation domain, two zinc-fingers and a hormone-binding site. Upon ligand binding, the steroid receptor changes its conformation, which reduces the binding affinity of inhibitors (e.g. hsp90). After dimerization it enters the nucleus and activates target genes (Roy *et al*. 2001).

Consistent with the fact that Hic-5 binds to the Lef/Tcf transcription factors resulting into a repressive complex, Hic-5-induced SR activation was repressed in the presence of Lef/Tcfs. This repression was also mediated through recruitment of Hic-5 to the conserved Exon of Lef/Tcfs since XLef-1 lacking this Exon had no effect on Hic-5-mediated SR activation. Interestingly, all other tested Lef/Tcf family members from mouse and human species possessing the conserved Exon down-regulated Hic-5-induced SR activation indicating to a highly conserved mechanism in vertebrates. This repression was also observed at the level of SR target gene activation where glucocorticoid target genes, $p21^{cip1}$ *and* $p27^{kip1}$ were up-regulated by Hic-5 and this up-regulation was repressed by co-transfection of Tcf-4. In this study Hic-5 binding to the Lef/Tcfs was preferentially through their LIM domains suggesting that this repression is mediated through the LIM domains of Hic-5. LIM domains of Hic-5 interact with SRs. The fact that deletion of these domains abolishes activation of ARs (Fujimoto et al., 1999) and GRs (Yang et al, 2000) points to the indispensable role they play in Hic-5-induced SR activation. The binding of the LIM domains of Hic-

5 to the Lef/Tcfs may mask and make them unavailable for SR activation. The interaction between Hic-5 and Lef/Tcfs also exposes a novel mechanism by which the Wnt/β-catenin signalling regulates SR signalling.

Tcf-4 has been described to pay a repressive role in signalling through AR in CV1 epithelial cells (Amir et al. 2003) and ER in rat mammary cells (Eltanini et al., 2001). Although this repression has been found to be mediated by direct interaction between these SRs and Tcf-4, the present study which reveals a physical interaction between Hic-5 and the Lef/Tcfs adds a further mechanism to the present view of the cross-talk between SR signalling and Wnt/β-catenin signalling. The recruitment of Hic-5 by the Lef/Tcfs might represent a means by which the Wnt/β-catenin pathway represses and thus controls steroid receptor signalling.

6.1.3 MBP1, a Novel Activator of Wnt/β-catenin target genes

MBP1 (mutant p53 binding protein)-1 or fibulin-4 belongs to a versatile family of glycoproteins known as fibulins. They are characterised by an epidermal growth factor (EGF)-like repeat at the N-terminus and a fibulin-type module in their C-terminus (Argraves et al., 1989). Although fibulins are described as extracelluar matrix proteins, a cytosolic/nuclear role has been ascribed to MBP1. It binds to an oncogenic mutant of p53 protein, preferentially to an Arg175His mutant and promotes neoplastic transformation and tumour growth (Gallagher et al., 1998).

6.1.3.1 Mouse MBP1 binds to XLef-1, XTcf-4 and XTcf-3

Mouse MBP1 was identified to bind to XTcf-3 and XTcf-4 and to XLef-1. Whereas strong binding to core domains of XTcf-3 and XTcf-4 was observed, the binding to XLef-1 was weak. Since the major difference between these transcription factors is the absence of the alternatively spliced activating Exon in XLef-1, this Exon alone might not account for the difference in affinity of Lef/Tcfs for binding mMBP1. The fact that similar results were obtained in GST pull-down assays with domain A (region between the β-catenin-binding site and the left flanking motive of the activating Exon) of XLef/Tcfs and mMBP1 suggests that this domain might collaborate with the Exon in binding to MBP1.

The identification of MBP1 as a binding partner of Lef/Tcfs is tempting to implicate MBP1 in p53 signalling. P53 has been found to activate glycogen synthase kinase (GSK)-3β thereby reducing β-catenin levels and consequently limiting Lef/Tcf-

induced target gene activation. However, the preferential binding of MBP1 to mutated forms and not the wild type form of p53 (Gallagher et al., 1998) suggests that its endogenous function might not be the control of p53/β-catenin/Lef/Tcf signalling. The interaction of MBP1 and Lef/Tcfs might instead suggest a cross-talk between the Wnt/β-catenin and mutated p53 signalling in neoplastic transformation and tumour growth. This crosstalk could be exposed by either investigating the influence of MBP1 and Lef/Tcfs on mutant p53 target gene promoters in reporter gene assays or knocking out MBP1 in cancer cell lines to observe if the two oncogenic pathways (mutant p53 and the Wnt/β-catenin signalling) share a common binding partner.

6.1.3.2 Mouse MBP1 activates Wnt target gene promoters

In contrast to Hic-5, MBP1 was observed to enhance the Lef/Tcf-induced transcription. This enhancement was not specific to any Lef/Tcf family member suggesting that MBP1 is a general Lef/Tcf co-activator and might not contribute to the functional specificity observed in Lef/Tcfs. This indiscriminate activation of Lef/Tcfs by MBP1 is similar to that of other activators that synergistically enhance Lef/Tcf transcriptional potential such as the CREB-binding protein (CBP) and the smads. The mechanisms of Lef/Tcf activation involve modelling of the chromatin structure by CBP (Takemaru and Moon, 2000) and recruitment of co-activators to target promoters for smads (Deynck et al., 1998). The mechanism by which MBP1 activates the Lef/Tcf target promoters is not yet known. It is speculative that MBP1 might either use the same mechanism like CBP and smads or an alternate mechanism to activate the Lef/Tcfs.

Activation of Wnt/β-catenin target gene promoters (*siamois* and *fibronectin)* by MBP1 might suggest that MBP1 required in endogenous axis specification. This is excluded because in *Xenopus*, XMBP1 expression starts at the end of gastrulation and increases in the neural tissue (D. Gradl, personal communication). β-catenin/Lef/Tcf-induced endogenous axis specification however takes place before gastrulation. Instead MBP1 might be involved in later developmental processes such as Wnt-triggered neural patterning. XMBP1 expression is localized in the early neural tissue (D. Gradl, Personal communication) overlapping with the expression pattern of XTcf-1 (Roel et al., 2003), XLef-1, and XTcf-3 (Molenaar et al., 1998) thereby indicating that it might play a role in Wnt/β-catenin-regulated patterning of the neuroectoderm. If this is the case then MBP1 should be involved in posteriorizing the neural tissue

which is mediated by Wnt proteins. Expression of XWnt-3a has been found to induce posterior and suppresses anterior neural marker genes in neuralized animal explants (McGrew et al., 1995). Conversely, *in vivo* blockage of Wnt ligands by expression of a dominant negative Xwnt-8 results in loss of posterior neural fates (McGrew et al., 1997). Overexpression studies and morpholino knockdown of XMBP1 might therefore be necessary to investigate its precise role in neural patterning. A *Xenopus* homologue of mouse MBP1 has been isolated from the *Xenopus* tadpole stage library. Although the 5´ leader sequence is missing in the isolated XMBP1, the deduced amino acid sequence shows that it is about 80% identical to the mouse orthologue therefore suggesting a conservation in function between these fibulins. However, the absence of a leader sequence suggests that further screening is necessary to verify if a longer clone exists for XMBP1 or not.

In summary, MBP1 binds to part of the core region and the alternatively spliced conserved and activating Exon. This binding does not only reveal that the two oncogenic pathways, Wnt/β-catenin and mutant p53 signalling, might share a common co-activator but also that MBP1 might be involved in Wnt/β-catenin mediated neural patterning.

6.2 Proposed model by which recruitment of Hic-5 and MBP1 by Lef/Tcfs establishes cross-talks with other signalling pathways

In addition to cross talks between the Delta/notch, p53, TGF-β, steroid receptors and Wnt/β-catenin signalling, the recruitment of Hic-5 and MBP1 exposes novel cross-talks between the Wnt/β-catenin ant other signalling pathways which suggests that communication could be bidirectional. Hic-5 establishes a cross talk between the Wnt/β-catenin signalling and steroid signalling pathways. In this cross-talk, the Wnt/β-catenin represses steroid signalling through the recruitment of the steroid receptor co-activator, Hic-5 by the Lef/Tcfs. Reciprocally, steroid receptors are shown to regulate Wnt/β-catenin signalling (Smith and Frankel, 2005) thereby suggesting a complex cross- regulatory network between these pathways (Fig. 41).

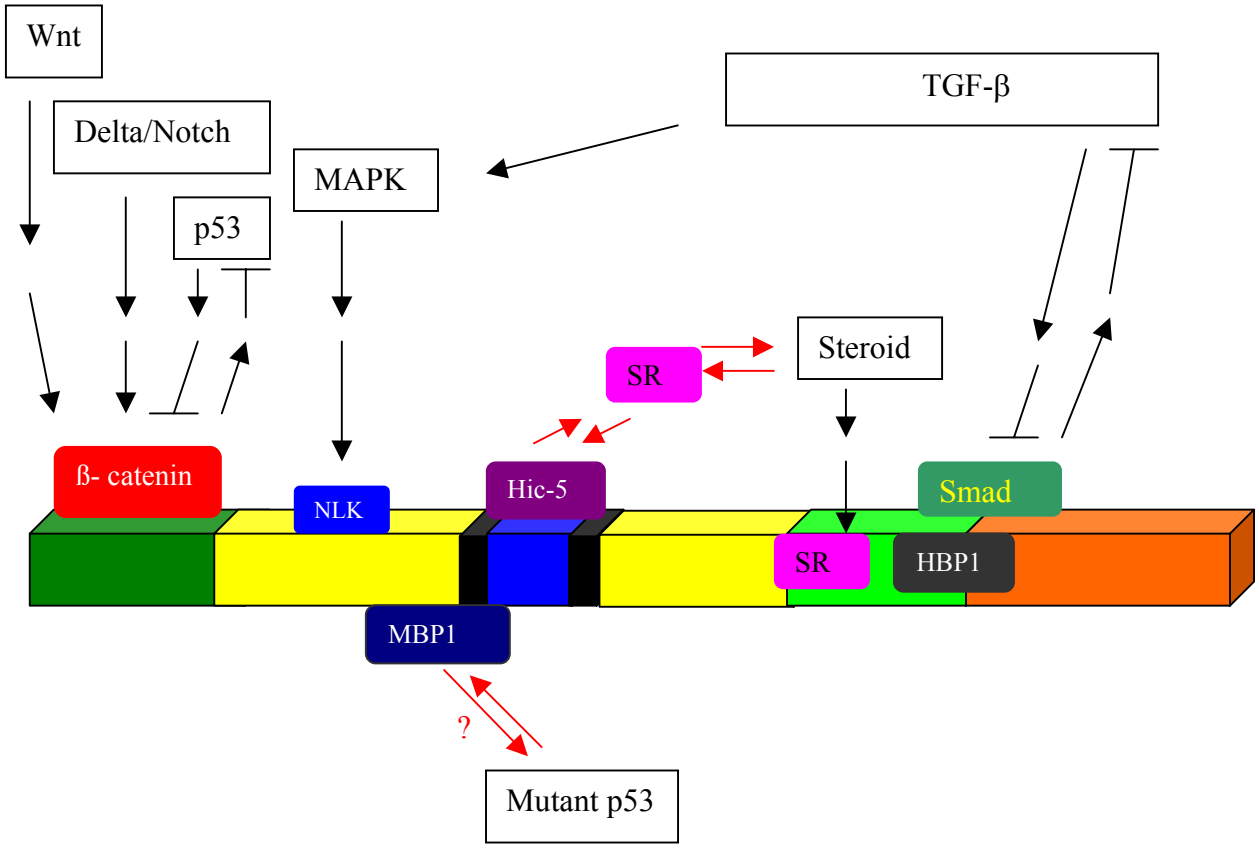

Fig. 41: Hic-5 and MP1 bind to Lef/Tcfs and establishes novel cross-talks between Wnt/β-catenin signalling and other signalling pathways. In addition to cross talks established between the Wnt/β-catenin pathway and the Delta/Notch, p53, Transforming growth factor (TGF)-β and steroid receptors (SR), Hic-5 binding to Lef/Tcfs establishes a novel link between Wnt/β-catenin signalling and steroid signalling through SRs. MBP1 binding to Lef/Tcfs might also establish a co-operation between Wnt/β-catenin signalling and mutant p53 signalling. These two prominent oncogenic pathways might share the same co-activator, MBP1. Red arrows correspond to novel signalling pathways.The question mark coreespond to a pathway that needs further investigation.

MBP1 also establishes a cross-talk between the Wnt/β-catenin signalling and the mutant p53 signalling through the mutant p53 suggesting that these two oncogenic pathways might use the same co-activator. (Fig. 41).

7. Summary

As nuclear transducers of the Wnt/β-catenin signalling cascade, Lef/Tcf transcription factors are involved in many important developmental decisions and in the development of cancer. The four vertebrate members: Tcf-1, Tcf-3, Tcf-4 and Lef-1 are characterized by a highly conserved N-terminal β-catenin binding site and an even more conserved DNA-binding site, the HMG-box. The region in between these two conserved domains is less conserved and is thought to provide the individual Lef/Tcfs with their characteristic functional properties. Although many binding partners of Lef/Tcfs have been isolated over the past years, the molecular nature of the functional diversity of Lef/Tcfs is not yet known. In order to identify novel Lef/Tcf-subtype specific binding partners, a mouse embryonic day 10 yeast-two hybrid library was screened using the core-region of XTcf-3 and XTf-4 as bait. Among the isolated clones, Hic-5 and MBP-1 were confirmed in GST-pulldown experiments and further characterized.

The *Xenopus* homologue of Hic-5 was isolated and the predicted protein revealed an overall identity of 40 % to its murine and human orthologues. Protein binding assays revealed that the interaction between Hic-5 and Lef/Tcfs is conserved in vertebrates. Hic-5 binds *via* its Lim-domains containing C-terminus to an alternatively spliced Exon in Lef/Tcfs, resulting in a complex that represses both Tcf-driven transcription and Hic-5 mediated steroid receptor activation. Thus, Hic-5 is a novel Lef/Tcf-subtype specific binding partner that mediates the dialogue between Wnt/β-catenin and steroid signalling.

The *Xenopus* homologue of MBP1 was isolated and the predicted protein revealed an overall identity of 80% to its murine orthologue. The most obvious difference between the Xenopus and murine orthologues is, that XMBP1 is 142 amino acids shorter at the N-terminus. This might indicate that the isolated cDNA does not encode the entire open reading frame.

In contrast to Hic-5, MBP1 activates Lef/Tcf-sensitive promoters. MBP1 is reported to bind to mutated p53 leading to an enhancement of its activity in neoplastic transformation and tumour growth. It is therefore likely that the two most prominent oncogenic pathways (Wnt/β-catenin and mutated p53 signalling pathways) do not only share the same co-activator (MBP1) but also communicate through MBP1.

8. Zusammenfassung

Als nukleäre Mediatoren der Wnt/β-catenin Signalkaskade sind die Transkriptionsfaktoren der Lef/Tcf Familie an zahlreichen entwicklungsbiologischen Prozessen und in der Entstehung einiger Krebsarten entscheidend beteiligt. Die vier Vertebraten-Vertreter der Lef/Tcfs zeichnen sich durch eine hoch konservierte β-catenin-Bindestelle am N-Terminus und eine noch höher konservierte DNA-Bindedomäne, die HMG-box, aus. Die Region zwischen diesen hoch konservierten Bereichen ist weniger konserviert und stattet die einzelnen Lef/Tcfs mit ihren individuellen Charakteristika aus. Obwohl in den letzten Jahren zahlreiche Interaktionspartner identifiziert wurden, ist der molekulare Mechanismus, welcher der funktionalen Diversität der Lef/Tcfs zugrunde liegt, noch weitgehend unbekannt. Um neue Tcf-Subtyp spezifische Interaktionspartner zu identifizieren wurde im Hefe „two-hybrid“ System eine murine embryonale Bibliothek mit der zentralen Region von XTcf-3 und -4 durchgemustert. Von den dabei erhaltenen putativen neuen Interaktionpartnern ließen sich *hydrogenperoxide inducible clone 5* (Hic-5) und *mutant p53 binding protein* (MBP1) in GST-pulldown Expermenten bestätigen.

Das *Xenopus* Homologe zu Hic-5 wurde isoliert und ist auf Aminosäureebene zu 40% identisch zu seinem murinen und humanem Orthologen. Protein-Bindungstudien zeigen, dass in Vertebraten die Interaktion von Hic-5 und Lef/Tcfs konserviert ist. Hic-5 bindet mit seinem LIM-Domänen-enthaltenden C-Terminus an ein konserviertes, alternativ gespleißtes Exon der Lef/Tcfs. Dies resultiert in einem repressiven Komplex, der sowohl Tcf-abhängige Trankription unterdrückt, als auch die Hic-5 vermittelte Aktivierung von Steroidrezeptoren reprimiert. Somit wurde Hic-5 als neuer Tcf-Subtyp-spezifischer Interaktionspartner identifiziert, der im Dialog zwischen der Wnt/β-Catenin Signalkaskade und Steroidrezeptoren involviert ist.

Das *Xenopus* Homologe zu MBP1 wurde isoliert und ist auf Aminosäureebene zu 80% identisch zu seinem murinen Orthologen. Auffallend ist, dass das *Xenopus* Homologe im N-Terminus um 142 Aminosäuren kürzer ist, was darauf hindeutet, dass die isolierte cDNA nicht den vollständigen offenen Leserahmen umfasst. Im Gegensatz zu Hic-5 aktiviert MBP1 Lef/Tcf sensitive Promotoren. Da MBP1 bekannter weise mit mutiertem p53 interagiert und dadurch neoplastische Transformation und Tumorwachstum fördert und, wie in der vorliegenden Arbeit gezeigt, Lef/Tcf sensitive Promotoren aktiviert, scheint MBP1 als Mediator in zwei der

wichtigsten onkogenen Signalwegen beteiligt zu sein und könnte die Kommunikation zwischen diesen beiden Signalwegen regulieren.

9. References

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10. Miscellenous.

List of abbreviations:

AR	Androgen Receptor
ARA-55	Androgen Receptor co Activator
BMP	Bone Morphogenic Protein
CAD	Context-dependent Activation Domain
CBD	β-Catenin Binding Domain
CBP	Cyclic AMP Receptor-Binding (CREB) Binding Proteins
CtBP	C-terminal Binding Protein
DBD	DNA-Binding Domain
Dexa	Dexamethasone
DHT	Dihydoroxytestosterone
dNTP	Deoxyribonucleotides
dTcf	*Drosophila* Tcf
ER	Estrogen Receptor
GR	Glucocorticoid Repressor
GST	Glutathione-S-Transferase
Hic-5	Hydrogen peroxide-Inducible Clone-5
HMG	High Mobility Group
Kbp	Kilo Base Pair
KD	Kilo Dalton
Lef	Lymphoid Enhancer Factor
MALDI TOF	Matrix-Assisted Laser- Desorption Ionization Time Of Flight
MBP1	Mutant-p53-Binding Protein-1
MMTV	Mouse Mammary Tumour Virus
PPARγ	Peroxisome Proliferator-Activated Receptor (PPAR) gamma
SR	Steroid Receptors
Tcf	T-Cel Factor
TGF	Transforming Growth Factor
TOLLIP	Toll-Interacting Protein
X. laevis	*Xenopus laevis*

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List of Publications.

Scientific Articles :

- **Ghogomu, S. M., Souopgui, J., Pelle, R. and Titanji, V. P. K.** (2002). Expession and Characterization of Ov-47, A Dominant Antigen of *Onchocerca volvulus*. *Exp. Parasitology*, 100, 143-149.

Poster Presentations:

- **Ghogomu, S. M, vanVenrooy, S., Ritthaler, M., Wedlich, D. and Gradl, D.** Hic-5, a novel repressor of Lef/Tcf driven transcription. (Gesellschaft für Entwicklungsbiologie) (GFE) meeting, University of Münster, April 6th-9th 2005.

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