WNT SIGNALING:

ACTIVATION, REPRESSION AND FINE-TUNING OF TCF TRANSCRIPTION FACTORS

Helen Mechteld Brantjes

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ACTIVATION, REPRESSION AND FINE-TUNING OF TCF TRANSCRIPTION FACTORS

WNT signalering: activatie, repressie en fine-tuning van TCF transcriptiefactoren

(met een samenvatting in het Nederlands)

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Contents

CHAPTER 1

AN INTRODUCTION TO

THE WNT SIGNALING CASCADE

Adapted from

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Helen Brantjes, Nick Barker, Johan van Es and Hans Clevers
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CHAPTER 1

1. WNT SIGNALING AN OVERVIEW

The gene to which this signaling pathway owes its name was initially characterized as Int-1, later called Wnt-1, by Nusse et al. in mouse mammary tumours. They showed that this gene was frequently activated by retroviral insertion of the mouse mammary tumour virus (MMTV) (1). This oncogene is highly conserved throughout evolution and is an orthologue of the *Drosophila* segment polarity gene Wingless (2). The similarity in sequence and function between Int-1 and Wingless lead to the re-naming of Int-1 as Wnt-1.

The Wingless protein is essential for segmentation in *Drosophila*. It is a secreted factor that influences cell fate of nearby cells in several organs, like in the cuticle and imaginal discs. The *Drosophila* cuticle comprises a repeated pattern of segments, with the anterior part consisting of denticles and the posterior part made of naked cuticle (Figure 1A). Mutant flies deficient for the Wingless gene product show highly disrupted patterning of the larval cuticle, resulting in a lawn of uniform denticles. Many components that have a role in this signal transduction pathway have been identified in extensive mutagenesis screens in *Drosophila*, based on the phenotype of disturbed segmental patterning. From these experiments a picture emerged of two types of proteins implicated in this cascade. Activating components, comprising proteins that upon mutation give rise to flies with a cuticle consisting of a uniform lawn of denticles (Figure 1B), include Dishevelled (3), Armadillo (4) and Tcf (5). Negative regulators for the pathway were also identified in this way, by virtue of their mutants giving rise to a cuticle lacking denticles (Figure 1C). Examples of these include Shaggy (6) and Axin (7).

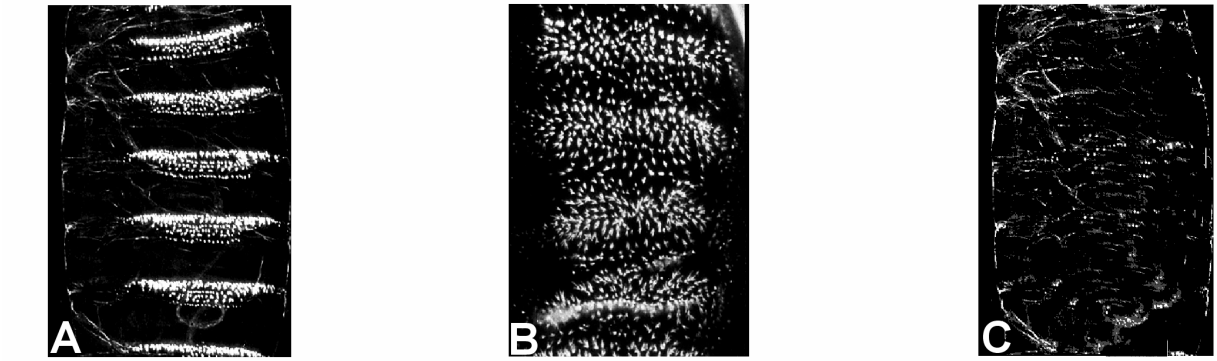

Figure 1. Cuticle phenotype of *Drosophila* mutant flies.
Depicted are the cuticle phenotypes of wild type (A), and flies mutant for either activating components of the Wingless signaling cascade, like Wingless, Dishevelled, Armadillo and Tcf (B), or negative regulators, like Axin and Shaggy (C). (This figure was kindly provided by R. Nusse)

From genetic epistasis experiments the order of events in the signaling cascade was determined and an initial picture of the Wnt signaling cascade emerged: Wingless activates Dishevelled, which in turn inhibits the kinase Shaggy, resulting in activation of Armadillo.

The identified *Drosophila* proteins are conserved throughout evolutions, with orthologs present in many species e.g. frogs, mouse and humans. The first vertebrate assay to determine the role of a protein in the Wnt signaling cascade came from Xenopus. Ectopic expression of Int-1 in Xenopus leads to duplication of the body axis, resulting in a two headed tadpole (8). The sequence homology of different components and their effect in the *Xenopus* double axis assay showed the striking conservation between the Drosophila and vertebrate Wnt signaling cascade. Moreover, in vertebrates Wnt activates Dishevelled, Dishevelled inactivates GSK-3 (the vertebrate ortholog of Shaggy), resulting in activation of β-catenin, the mammalian Armadillo ortholog.

For a long period, β-catenin was the most downstream component identified in the Wnt signaling pathway. It was known to have a dual function in cell adhesion and in Wnt signaling, with separate domains in these different roles. The N-terminus is required for cell-cell adhesion, in which β-catenin is a component of the adherens junctions, and the C-terminus is essential for its role in Wnt signaling (reviewed in (9)). It only became clear how a Wnt signal can affect the downstream genetic program upon the identification of the family of TCF/LEF/Pangolin transcription factors as binding partners of β-catenin (10, 5, 11, 12). Many other components have now been identified, delineating the Wnt signaling cascade as we now know it (Figure 2). Some of the steps in the pathway will be discussed in more detail below.

In the absence of Wnt signaling, β-catenin levels are kept low by the activity of a complex containing the serine/threonine kinase GSK3-β, the scaffold protein Axin (13, 14, 15) and the tumour suppressor protein APC (reviewed in (16)). This so called degradation complex phosphorylates β-catenin. The F-box protein β-TRCP recognises phosphorylated β-catenin and primes it for destruction by the proteasome (17, 18). Without free β-catenin, Tcfs bind members of the Groucho family and actively repress transcription of target genes (19, 20).

When a Wnt protein reaches a cell, it is bound by Frizzled and its co-receptor LRP (21, 22, 23). Accumulation of β-catenin results in its translocation to the nucleus (reviewed in (24, 25)), by an as yet unidentified mechanism. In the nucleus, β-catenin associates with transcription factors of the TCF family to generate a functional transcription factor complex, resulting in the transactivation of target genes, such as *c-Myc* (26), *Tcf-1* (27), and EPHB-2 (122), marking the onset of a defined genetic program.

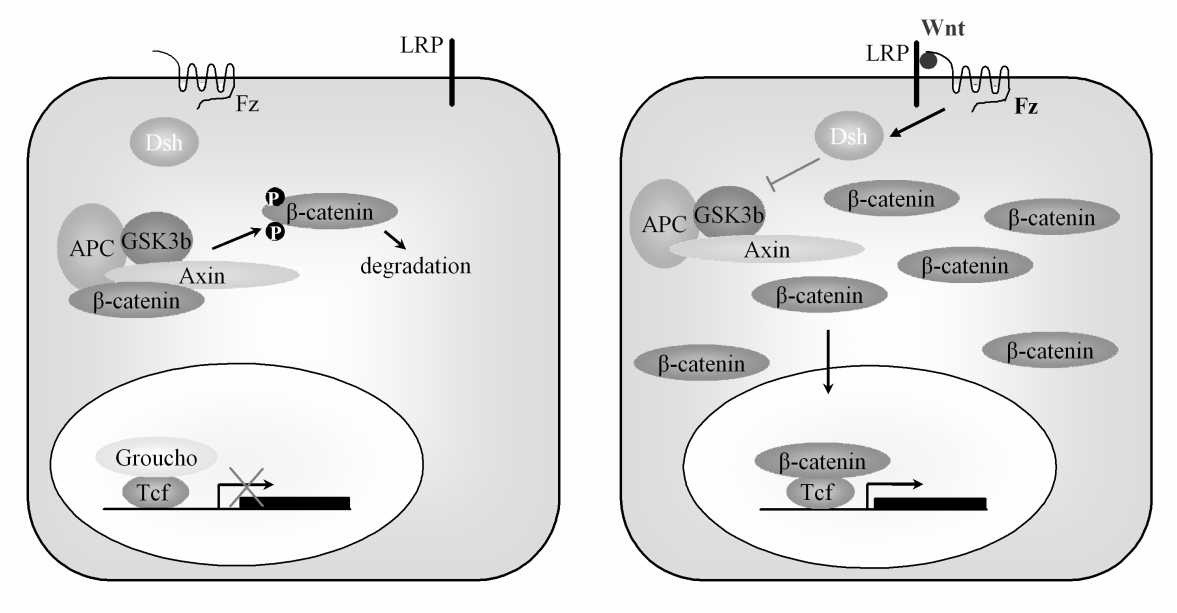

Figure 2. A schematic overview of the Wnt signaling pathway

In the absence of a Wnt signal (left) β-catenin is phosphorylated by GSK3-β, in a complex also containing Axin and APC. Upon phosphorylation, β-catenin is primed for ubiquitination and subsequent degradation by the proteasome. In the nucleus, Tcf proteins bind Groucho family members and repress target genes.

When a Wnt protein reaches the cell (right), it associates with the transmembrane receptors Frizzled and LRP. The destruction complex of GSK3-β, APC and Axin is subsequently inactivated via Dishevelled, resulting in the accumulation of β-catenin and its translocation to the nucleus (25). Here, β-catenin associates with transcription factors of the TCF family to generate a functional transcription factor complex, resulting in transactivation of target genes.

2. WNT SIGNALING AT THE CELL MEMBRANE

The receptors for Wnt proteins, Frizzleds have also been implicated in other signaling pathways (figure 3). Initially they were identified because of their role in hair polarity in the *Drosophila* wing (29). A number of genes have been identified in screens for flies with mutant polarisation of cuticle structures, like *strabismus*, *RhoA*, *Frizzled* and *Dishevelled*. These and many other genes make up the pathway determining the polarity of hairs, bristle, ommatidia etc, referred to as the planar polarity pathway. This is also called the non-canonical Wnt pathway to make the distinction from the canonical pathway, which makes use of the same Wnt, Frizzled and Dishevelled proteins to mediate up-regulation of β-catenin and Tcf activation.

In *Xenopus*, Wnts and Frizzleds have been shown to be implicated in yet another signaling route, the Wnt/Ca^{2+} pathway (reviewed in (30)). Some Wnts stimulate intracellular Ca^{2+} release and activate two kinases, CamKII and PKC.

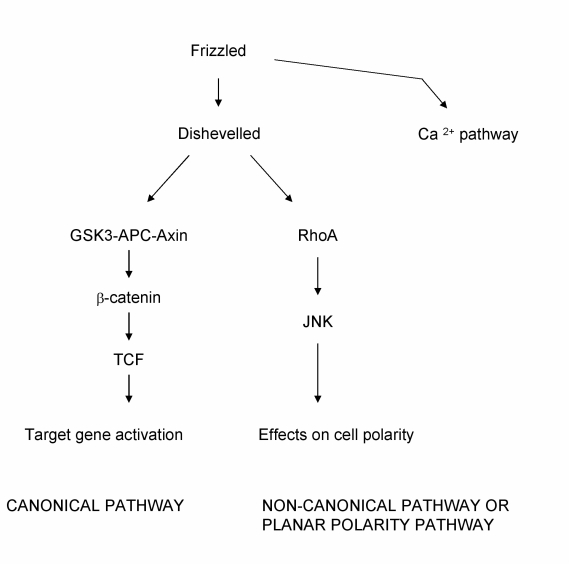

Figure 3. Frizzled receptors are implicated in different signaling cascades.
Frizzled can signal via Dishevelled to β-catenin and Tcf, using the canonical pathway, and to RhoA using the planar polarity pathway. Frizzled can signal to the Ca^{2+} pathway independent of Dishevelled.

Regulation of the transduction of a Wnt signal to intracellular responses occurs at the cell membrane. A number of mechanisms are known to fine-tune this first step in the Wnt signaling cascade (Figure 4). Several secreted molecules have been identified, which interfere with the association of Wnts with their receptors. Frizbees (FRZB) were identified in *Xenopus*, which are secreted proteins that consist of the ligand binding domain of Frizzled receptors (31, 32, 33). These FRZBs compete for binding to Wnt proteins, thereby preventing Wnt Frizzled interaction. A structurally unrelated Wnt inhibitory factor (WIF) also encodes a secreted protein able to bind to Wnts directly and thereby inhibit signaling (34). Additionally, the protein Cerberus was shown to be a pluripotent antagonist for several growth factors; it can inhibit Wnt BMP and nodal signaling (35).

The mechanism governing how the distinction is made between canonical and non canonical signal transduction is not yet entirely clear. One way to selectively signal to one route is conferred by specific types of Wnt proteins binding to specific Frizzleds. Wnt proteins are classified as two groups based on their ability to signal through either of the pathways. Wnts such as Wnt-1, -3A and 8A, which induce a secondary axis in *Xenopus* embryos following ectopic expression and posses the ability to transform an epithelial cell line are classified to the canonical pathway. Wnt proteins such as Wnt-5a, -4 and –11, which can reduce cell adhesion and induce cell movement in *Xenopus* embryos, are assigned as signaling through the non-canonical pathways of RhoA or Ca^{2+}.

With the recent identification of the Frizzled co-receptor LRP (23, 22, 21) and its inhibitory complex Dickkopf and Kremen, it has become more clear how the planar polarity and canonical Wnt signaling cascade are intertwined. A Wnt protein requires binding to both Frizzled and its co-receptor LRP to transduce the signal to the canonical pathway. This can be inhibited by

the Dickkopf protein, and its receptor Kremen (36, 37). Dickkopf binds simultaneously to Kremen and LRP, resulting in internalisation of this ternary complex (38). LRP is thus depleted from the cell membrane and the Wnt-Frizzled complex can no longer signal to the canonical pathway. Signaling to the non-canonical pathway is not, however, inhibited by Dickkopf and Kremen.

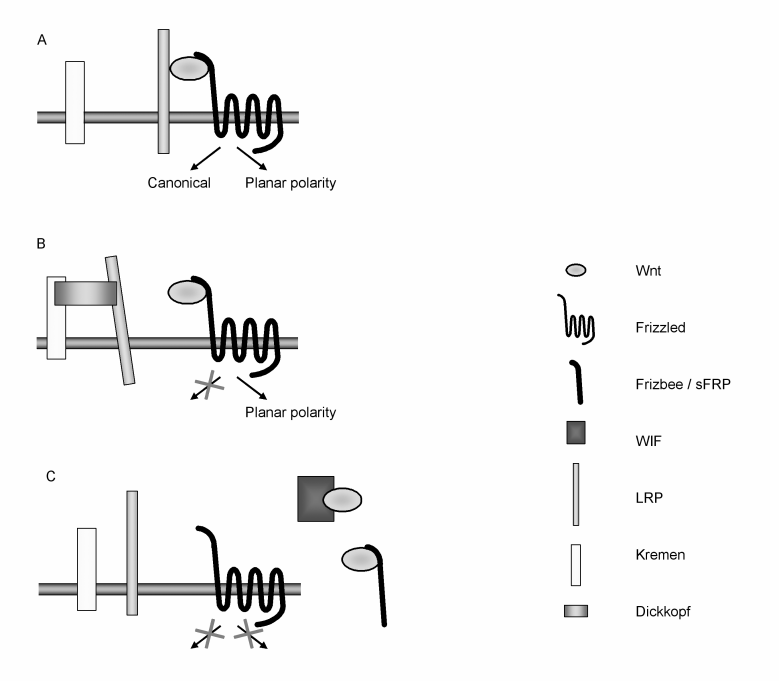

Figure 4. Wnt signaling at the membrane.
A. A Wnt ligand binds its receptors Fz and LRP, initiating signaling through both canonical and non-canonical pathways.
B. Dickkopf prevents LRP from associating with Wnt and Fz, ensuring that only signaling through the planar polarity pathway can occur.
C. Soluble factors like FZB and WIF, bind Wnt directly, inhibiting binding to the Fz receptor, and thereby blocking any signaling through this receptor.

3. THE DESTRUCTION COMPLEX AND NUCLEAR TRANSPORT

In the normal cell, β-catenin is mainly present in adherens junctions, where it mediated cell adhesion as a component of the E-cadherin complex. Any free β-catenin in the cytosol will be immediately degraded by the proteasome in the cytoplasm. This degradation is initiated by the phosphorylation of β-catenin which occurs in a multiprotein complex of the serine/threonine kinase GSK-3β, the tumour suppressor APC, the scaffold protein Axin and Casein Kinase I (CKI). β-catenin contains four highly conserved phosphorylation sites, S33, S37, T41 and S45. Phosphorylation of these sites is a two step process. Initially CKI, or 'priming kinase',

phosphorylates S45, which triggers phosphorylation of the other sites by GSK3β (39, 40). Phosphorylated S33 and S37 are the recognition site for β-TRCP, which ubiquitinates β-catenin and targets it for destruction by the proteasome (17, 18).

The function of APC in this complex is not yet entirely clear. The protein was first identified as the gene defective in familial adenomatous polyposis (FAP), a dominantly inherited disease that predisposes to colorectal tumours (41, 42). It has become clear that >80 % of all colon cancers have mutations in this gene (43, 44). The APC mutations found in the tumours are usually located in a central region of the gene, referred to as the mutation cluster region (MCR), and cause truncation of the protein (Figure 5). Apparently, there is a strong selection against domains downstream of the MCR, and a positive selection for upstream sequences, that may be implicated in functions essential for cell survival. Wnt signaling and cancer will be discussed in more detail later in this report. APC is implicated in a large variety of cellular processes such as regulation of cell proliferation, cell adhesion, cell migration and chromosome stability (reviewed in (16) and (45)). Many of these functions have been assigned following identification of interacting proteins. Only the role of APC in β-catenin regulation will be described in detail here.

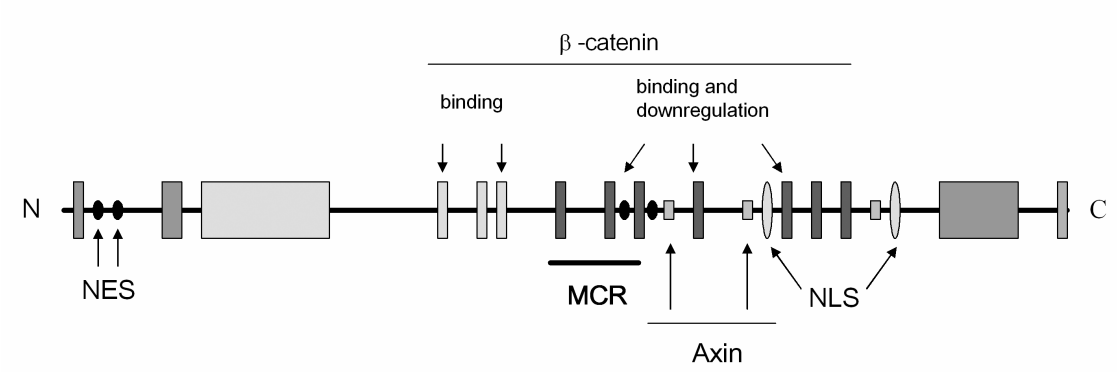

Figure 5. Schematic representation of the APC protein.
The APC protein contains many structural domains. Here are only indicated the mutation cluster region, the domains for nuclear import (NLS) export (NES) and the domains implicated in interaction with β-catenin and Axin.

APC is a 300 kDa highly conserved protein that is localised both in the cytoplasm and nucleus. Several functional domains have been identified in this protein (Figure 5). The N-terminal part of the protein contains an oligomerization domain, two nuclear export signals and armadillo repeats. The central region of the protein contains the β-catenin (46, 47) and Axin (48, 13, 49) interaction domains and another two nuclear export signals (50). The c-terminal part of the protein contains a nuclear localization domain and a basic domain. Analysis of mouse models with different truncations of the APC gene indicated that the Axin interaction domains of APC, but not the more C-terminal domains, were essential for APC function as tumour suppressor. This implies that the down-regulation of β-catenin by APC is essential for tumour suppression (6). This is also indicated by mutations found in several types of tumours in β-catenin phosphorylation sites (reviewed by (51)).

APC has also been shown to shuttle between the nucleus and the cytoplasm (52, 53, 50). APC shifts from cytoplasmic to nuclear localization upon treatment with leptomycine B (LMB), a CRM-1 specific nuclear export inhibitor. Also β-catenin localization can be regulated by LMB, but in an APC dependent manner. Wild-type APC can restore β-catenin degradation in an APC mutant cell line, but this is dependent on functional NES sequences. This data suggests that wild type APC controls β-catenin levels by a combination of nuclear export and subsequent cytoplasmic degradation.

4. TCF TRANSCRIPTION FACTORS

The TCF/LEF transcription factors are the most downstream components of the Wnt signaling cascade. The mammalian genome harbours 4 family members, TCF-1, LEF, TCF-3 and TCF-4, which share homology in their DNA binding domain with members of the HMG box transcription factor family (Figure 6). TCFs bind their target DNA sequences within gene promotors via the HMG box DNA binding domain. The TCF consensus recognition sequence is remarkably conserved, and comprises AGATCAAAGGG (54, 55, 56). The HMG box does not only mediate DNA sequence recognition, but also induces a dramatic bend in the DNA (57, 58). By doing so, HMG boxes may coordinate the binding of other transcription factors (59, 60, 61). Because of this bending ability and the observation that TCF factors cannot directly activate transcription in reporter assays, it has been proposed that TCF/LEF family members primarily serve an architectural function. LEF-1 appears unique in that it contains a context-dependent activation domain (CAD) (62), which can activate transcription in the presence of the co-activator ALY (51). The other TCF family members do not appear to contain a CAD domain (63).

The TCF proteins show extensive alternative splicing, leading to a multitude of TCF proteins in the mammalian system. Splice variants have been identified for Tcf-1, Lef and Tcf-4. The *Tcf-1* gene has two different promotors, four alternative exons and two splice acceptor sites in the last exon, giving rise to proteins with and without the n-terminal β-catenin interaction domain, with and without a central small exon and with a short or long c-terminal tail (64, 63). For Tcf-4 three c-terminal alternative splice variants were identified (65) and the LEF gene also contains three alternatively spliced exons (66).

In general, TCF proteins do not function as classical transcription factors, in that DNA binding alone is not sufficient to cause transcriptional activation. Promotor activation is only achieved after complex formation of TCF with β-catenin to generate a functional bi-partite transcription factor. Within this complex TCF provides the DNA binding moiety and β-catenin the transcription activation domain (reviewed in (67)).

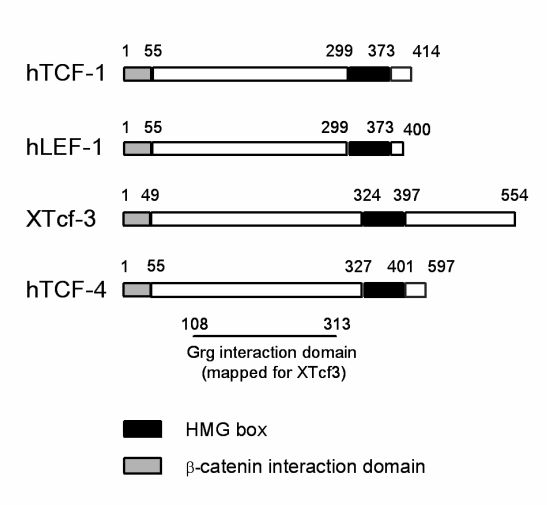

Figure 6. Structure of TCF family members

The mammalian genome harbours four TCF family members. They all contain three distinct conserved domains: a N-terminal β-catenin interaction domain, a HMG box DNA binding domain and a Groucho interaction domain.

4.1 TCF AS A TRANSCRIPTIONAL ACTIVATOR

Following association of TCF and β-catenin to form a bipartite transcription factor, target gene promotors are activated. Although the mechanism is not fully elucidated, some recent progress has been made to describe the mechanisms involved in this process. β-catenin is composed of several domains. Its N terminal domain contains putative GSK3-β sites, regulating its priming for ubiquitination (68, 69). The central domain consists of 12 armadillo repeats that mediate binding to α-catenin, E-cadherin, APC, Axin and TCF. The C-terminus of β-catenin contains a potent transactivation domain (reviewed in (70)).

Several mechanisms are described through which β-catenin can stimulate transcription. β-catenin may directly interact with TATA binding protein (TBP) (71), or indirectly via Pontin52 (72). This would provide a direct interaction between TCF-β-catenin and the basal transcription machinery, resulting in transactivation of target genes. Another mechanism through which β-catenin stimulates transcription is through interaction with the histone acetyl transferase CBP (CREB binding protein), that binds to a region C-terminal of the armadillo repeat 10. In *Xenopus* embryos β-catenin synergises with CBP to stimulate transactivation of the target genes *Siamois* and *Xnr3* (73, 74, 75). This transactivation is sensitive to E1A, an antagonist of CBP function. Through the interaction with the β-catenin-TCF complex, CBP is tethered to the DNA and may locally acetylate nucleosomal histones. This acetylation will result in a more accessible or 'open' conformation of the chromatin. This conformation might promote binding of other transcription factors and allow the general transcription machinery to gain access to the promotor to activate transcription (reviewed (76, 77)).

Several lines of evidence, however, indicate that there are also CBP-independent mechanisms through which β-catenin can mediate transactivation. A β-catenin mutant lacking the CBP binding domain still had residual transactivation activity correlating well with the results of a previous study, which demonstrated the presence of additional transactivation elements in the N-terminus of β-catenin (5). Moreover, the cooperative effect of CBP on β-catenin-mediated transactivation is only present for a subset of TCF target genes (74). A recent study has shown that β-catenin also interacts functionally with Brg-1, a core component of the SWI/SNF chromatin remodelling complex (78). This SWI/SNF complex has been shown to participate in the displacement of histone octamers from DNA fragments (79) and to promote the sliding of histone octamers along the DNA (80). In this way, the SWI/SNF complex is thought to participate in the formation of regions with altered nucleosomes at the promotor of specific genes. This could then increase the accessibility of the DNA for other transcriptional regulators, resulting in more efficient transactivation of target genes. The enhancing effects of CBP or Brg-1 on β-catenin mediated transactivation are, however limited, on average inducing an increase of only 2-3 fold. This indicates that it is likely that all these factors are acting in concert to achieve efficient target gene activation.

On the other hand, in *Drosophila* was shown that over-expression of another SWI/SNF component, Osa, results in repression of the Wingless target genes Dpp and Distal-less (81). Moreover, loss of Osa function induces ectopic expression of these Wg target genes. This indicates that chromatin remodelling is also involved in negative regulation of Wingless target genes. Clearly, multiple determinants are affecting the final outcome of β-catenin-SWI/SNF interaction on Wg/Wnt target genes and further research is needed to elucidate this.

4.2. TCF AS A TRANSCRIPTIONAL REPRESSOR

4.2.1. ASSOCIATION WITH CO-REPRESSORS

Experiments in *Drosophila* and *Xenopus* have shown that TCF does not only function as a transcriptional activator, but that it can also actively repress Wingless target genes. This was for instance shown for the expression of *Ubx* in the visceral mesoderm of the fly embryo. Upon mutation of TCF sites in the *Ubx* enhancer it was shown that Ubx expression was decreased near the Wingless source, but enhanced in cells further away from the source (82). This effect was also reported for *Dpp* (83) and the *Xenopus Siamois* gene (84). Apparently, loss of TCF mediated regulation can result in increased target gene expression, implying that TCF can also function as a transcriptional repressor. Several other observations have confirmed that TCF has this dual function (reviewed in (85)). On one side of the balance, TCF will transactivate transcription when activated by a Wnt signal, following its association with β-catenin. On the other side, however, it can associate with several different transcriptional co-repressors, resulting in inhibition of target gene expression in the absence of Wnt signaling.

Groucho

Drosophila Groucho is the founding member of an evolutionary conserved family of transcriptional co-repressors (reviewed in (86)). The murine and human genomes harbour four full-length homologues of Groucho in addition to a gene that encodes a truncated Groucho protein, comprising only the two amino terminal domains. The human Groucho homologues are termed TLE-1, -2, -3, -4 (for Transducin- Like Enhancer of Split) and the truncated variant is named hAES (for Amino terminal Enhancer of Split). The homologous mouse Groucho family consists of the Groucho-related genes *Grg-1, -2, -3, and -4* and the *Grg-5* gene, which encodes a shorter variant (Figure 7).

The Grg/TLE proteins contain 5 protein domains: an amino terminal Q (Glutamine rich) domain, followed by a GP (glycine/proline rich) domain, a CCN domain (containing putative casein kinase II/cdc2 phosphorylation sites and a nuclear localization signal), an SP (serine/proline rich) domain and four WD40 repeats (protein interaction domain). Of these, the Q and WD40 domain sequences are most highly conserved. Groucho-related proteins have been described to tetramerize through a leucine zipper-like structure in the amino-terminal Q domain (87).

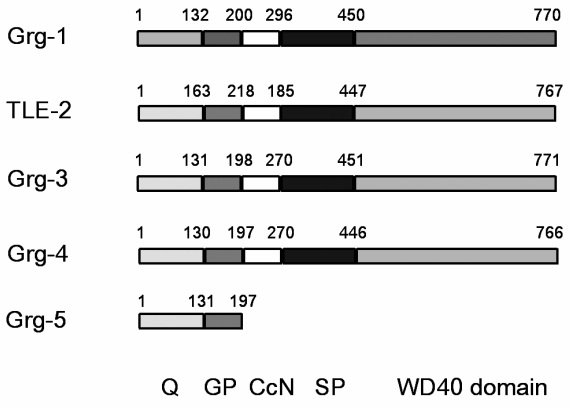

Figure 7. Domain structure of Groucho related proteins

The family of Groucho-related-genes in the mammalian genome consists of four full length homologues of Groucho, *Grg-1, -2, -3,* and *-4* and a gene that encodes a truncated Groucho protein*, Grg-5*. The Grg proteins 1-4 contain five protein domains*:* an amino-terminal Q (glutamine-rich) domain, followed by a GP (Glycine/proline rich domain), a CCN domain (containing a putative casein kinase phosphorylation sites and a nuclear localization signal), a SP (serine/proline-rich) domain and four WD40 repeats. Grg-5 only contains the two amino-terminal domains, the Q and the GP domains.

Groucho proteins function as co-repressors for a multitude of DNA binding transcription factors of distinct families. Some of these transcription factors, such as Hairy (88), HES (89) and Blimp-1 (90), specifically act to repress target gene expression, a function that requires their interaction with Groucho. Others, like Runt (91) and Dorsal (92) are activators and convert to repressors upon association with Groucho-related proteins.

The *Drosophila* histone deacetylase Rpd3 has been shown to interact with Groucho (93). Recruitment of Rpd3 to the promotor of target genes promotes modulation of the local chromatin structure. By mechanisms that are not yet fully understood, this appears to result in a more compact chromatin structure that is associated with a repressed transcriptional state. Grg-5 cannot bind RPD3, which could explain the lack of repression observed with this protein. Grg-5 does, however, interact with other Grgs and may in this way decrease the amount of HDAC present in the Grg tetramer, thereby lowering the net repressive effect (94).

Histone deacetylation by RPD3/HDAC is likely to be the predominant mechanism through which Groucho-related proteins exert their function, although some data indicate that an additional mechanism of repression may exist (88, 93, 93). When Groucho-mediated repression is assayed in the presence of the HDAC inhibitor TSA (Trichostatin A) some residual repression activity remains. When the histone deacetylase RPD3 in *Drosophila* is mutated, only a mild phenotype is observed (95). These studies suggest that HDAC is an important, but not the sole, mediator of transcriptional repression. Further study of the mechanism of Groucho-mediated repression will undoubtedly address these questions in the future.

Several groups have reported that TCFs interact with members of the Groucho family of transcriptional co-repressors (19, 20, 96). We found in a yeast two hybrid assay that TCF can interact with members of the Groucho family of transcriptional repressors. Using a synthetic reporter construct containing TCF binding sites that can be activated by TCF/β–catenin complexes, mammalian Groucho family members were demonstrated to repress this TCF-mediated transactivation. When Grg is injected into *Xenopus* embryos, it represses endogenous target genes such as *Xnr3* and *Siamois*. Another study showed that LEF interacts with TLE-1 and inhibits LEF-β-catenin mediated transactivation (96). It was demonstrated that the activity of the T cell receptor (TCR) enhancer α, which contains functional LEF and AML binding sites, can be inhibited by TLE1. Independently, genetic evidence was obtained in *Drosophila* confirming a functional interaction between Groucho and dTcf (20). Reduction of dTcf activity, suppresses *wingless* and *armadillo* mutant phenotypes, resulting in de-repression of Wingless responsive genes, like *engrailed*. This indicated a repressive role of dTcf when the Wingless signaling pathway is not active. This repression was shown to be Groucho-dependent since mutations in the *Drosophila Groucho* gene also suppress *wingless* and *armadillo* phenotypes.

In contrast to the long Grg variants, Grg-5 functions as a de-repressor for TCF, alleviating endogenous Grg mediated repression both in the described reporter assay, as well as *in vivo* in *Xenopus* injection experiments (19). Every mammalian TCF family member can functionally interact with each of the five Grg proteins (94).

Remarkably, it should be noted that expression of splice variants of TCF proteins lacking the β-catenin interaction domain, would result in the presence of a 'repressor' TCF, since these proteins can still interact with the ubiquitously expressed Groucho proteins.

C-terminal binding protein

Another study addressing the repressive function of XTcf-3 in *Xenopus* embryos identified the transcriptional co-repressor CtBP as an interaction partner for TCF (97). Two consensus motifs for interaction with CtBP, P*X*DLS*X*(K/R), are present in the C-terminus of XTcf-3. These domains are indeed required for repression mediated by the C-terminal domain of XTcf-3. The CtBP interaction motifs are present in TCF-3 and the alternative splice-isoform TCF-4E, but not in the other TCF family members. In *Drosophila,* CtBP mediates short-range repression through a

number of unrelated transcription factors, acting over distances of approximately 100 bp (98). In contrast, the co-repressor Groucho is classified as a long-range repressor, capable of silencing transcription over long distances of approximately 1 kilobase pair (99). Groucho also differs from CtBp in its sensitivity to the histone deacetylase inhibitor, TSA. CtBP is not sensitive to the drug, whereas Groucho is sensitive to TSA (100).

4.2.2. ACETYLATION OF TCF

The histone acetylase dCBP was shown to directly interact with TCF in *Drosophila* (101), resulting in acetylation of a conserved lysine in the β-catenin-binding domain of TCF. This acetylation interferes with its binding to β-catenin. dCBP mutants show mild overactivation of Wingless targets genes. This activity of dCBP primarily antagonises Wnt signaling in cells that are weakly stimulated. Thus, CBP can apparently play a dual role. On one hand it can activate transcription by binding to β-catenin, acetylating nucleosomes and opening the chromatin. But CBP can also acetylate TCF, reducing its affinity for β-catenin and resulting in a decrease of transcriptional activation. The acetylation of TCF by CBP has so far only been described in *Drosophila*. Therefore, it is not known if this is a general function of CBP or whether this is specific for *Drosophila*.

5. WNT SIGNALING IN CELL FATE AND CARCINOGENESIS

Wnt signaling and cell fate.

Wnt signaling results in the transcriptional stimulation of genes controlling many differentiation events in development, both during embryogenesis and adult life. A broad variety of processes are regulated by Wnt signaling as was concluded from the expression pattern of the different components of the cascade and the phenotype of mice or flies when these proteins are mutated. For example Wnt signaling in *Drosophila* is implicated in the embryonic segmentation process and in development of the eye and limbs (reviewed in (102)). In mouse development, Wnt signaling was shown to be involved in development of hair follicles (103, 104), thymocyte differentiation (105) and gut development (106).

The role of Wnt signaling in the development of the intestine has been studied in great detail. The mammalian intestine is made up of crypts, the proliferative unit containing stem cells, and villi, consisting of migrating and terminally differentiating cells, that will be shed into the lumen in a process taking no longer than two to three days. The stem cells in the crypts produce a population of highly proliferative cells that are rapidly renewed and migrate up the crypt-villus axis. During their migration they differentiate to different cell lineages, namely goblet, enteroendocrine and absorptive cells. TCF-4 is the most prominent expressed TCF family member

in the intestinal epithelium (107). Targeted disruption of this gene in mice results in loss of the proliferative compartment of the intestine during embryonic development (51). The neonatal epithelium is composed of differentiated villus cells, resulting in death of the mice shortly after birth. This indicates that Tcf-4 controls the genetic program maintaining the crypt stem cells of the intestine (106).

There are several lines of evidence for tissue-specific responses of the different Tcf/Lef proteins during development. In the intestinal epithelium also Tcf-1 is also expressed, predominantly as a dominant-negative form lacking the β-catenin interaction domain (27). Genetic evidence suggests that Tcf-1 acts as an antagonist of Tcf-4 (27). Also the skin epithelium is derived from multipotent stem cells that differentiate into different lineages, the epidermis, sebaceous gland and hair follicle. Lef and Tcf-3 were shown to control these differentiation lineages (108). Lef requires stabilized β-catenin to express the specific genes and control hair differentiation, whereas Tcf-3 can act independently of β-catenin interaction, to suppress epidermal differentiation and promotes follicle outer root sheat and multipotent stem cell compartments (108). Additionally it was shown in *Xenopus* was shown that Tcf proteins have different, specific functions during embryonic development. Wnt signaling in very early embryos leads to a dorsalizing response, which establishes the endogenous dorsal axis. Only a few hours later in development, almost the opposite happens: Xwnt-8 functions to pattern the embryonic mesoderm by promoting ventral and lateral mesoderm (109). XTcf3 is required for establishment of the dorsal body axis by maternally encoded Wnt signaling components, whereas XLef is required for dorsoventral patterning of the mesoderm by Xwnt-8 signaling after onset of zygotic transcription, a process that is independent of XTcf3 (110).

Additionally, specific functions are being attributed to different splice variants of Tcf family members. Initially the difference in function of the full length Tcf family members and the variants lacking the N-terminal β-catenin interaction domain, was demonstrated, showing that the short variants were unable to transactivate transcription, whereas the long versions can (10). Recently, also different functions were assigned to the Tcf splice variants with different C-terminal domains, the longer E variant and shorter B variant. The *Lef* gene has been shown to be aberrantly activated in colon cancer and the promoter of this gene is activated by Tcf-β-catenin complexes in transient transfection assays (111). This promoter was selectively activated by the isoforms with the C-terminal ‘E’ tail, but not with the ‘B’ tail. The CRARF box in the E-tail is essential for this specific activity (112). Correspondingly, the Cdx-1 gene was found to be activated by Tcf-4E, but not by Lef, lacking the N-terminus (113).

Wnt signaling and carcinogenesis

APC was initially identified as a tumour suppressor gene implicated in colorectal cancer, both in hereditary disease and in sporadic tumours (41, 42, 114). The observation that APC binds β-catenin was the first link between Wnt signaling and carcinogenesis (47, 46). Mutations in APC have been identified in more than 80% of primary colon carcinomas and colon carcinoma cell lines. These mutations result in a truncated version of the APC protein, which can no longer bind Axin, disrupting the destruction complex. β-catenin is no longer down regulated resulting in aberrant activation of target genes. Colon cell lines with mutated APC contain a β-catenin-TCF-4 complex, which is constitutively active, as measured by a TCF reporter gene (107). However, also

colon carcinoma cell lines were analysed that do not have mutations in the *APC* gene, but still display activated TCF-β-catenin signaling. In these cell lines mutations were found in the phosphorylation site of β-catenin, rendering it insensitive to phosphorylation and subsequent degradation and thereby stabilizing the protein (115). Mutations in Axin2 have also been identified in colon carcinoma cell lines resulting in aberrant TCF-β-catenin activation (116). These data indicate that constitutive activation of TCF target genes, caused by loss of function of the destruction complex, may be a crucial event in transformation of colonic epithelium. Deregulation of β-catenin activation is known to be implicated in a large number of other malignancies, such as melanoma (117), hepatocellular carcinoma (118, 119), ovarian cancer (120), endometrial cancer (119) and pilomatricoma (121).

Mutational activation of Wnt signaling in tumourigenesis implied that inappropriate activation of the TCF-β-catenin complex would result in the aberrant activation of a genetic program leading to carcinogenesis. The relationship between the aberrantly stimulated program in colorectal cancer and the physiological program as active during the development of the colon has been studied in more detail by Van de Wetering et al (122). Colon cancer cell lines with activated TCF-β-catenin complexes were stably transfected with an inducible dominant negative version of TCF-4 (DN Tcf-4). Upon induction, endogenous β-catenin-TCF complex were inhibited as was shown by TCF reporter assay. Induction of the dominant negative TCF also resulted in a G1 cell cycle arrest and cell proliferation was halted. By DNA array analysis it was determined which genes were affected upon blocking the endogenous active TCF-β-catenin complex. Two different sets of genes were identified. Several genes were down regulated upon induction of DN TCF-4, including CD44, c-Myc, BMP4, EPHB-2, -3 and ETS. A group of genes was up regulated upon blocking TCF-β-catenin signaling, many of which represented differentiation markers of the mucosecretory or absorbtive intestinal cells, like carbonic anhydrase and fatty acid binding protein. The induction of several of the identified genes was confirmed by Northern blotting and expression patterns were determined in normal common and early neoplastic lesions. All tested genes that were down regulated in the array were expressed in early neoplastic lesions. This indicates that the constitutive activity of TCF-4 in APC-deficient human epithelial cells may contribute to their malignant transformation. Moreover, these same genes were also up regulated specifically in the proliferative compartment of normal colon crypts. A complementary expression pattern was observed for the genes that were up regulated in the array as they were expressed in the top of the villus, in the differentiated compartment. Thus, the changes in gene expression induced by blocking TCF-β-catenin activity in colon carcinoma cell lines reflects the physiological differentiation of crypt progenitor cells (Figure 8).

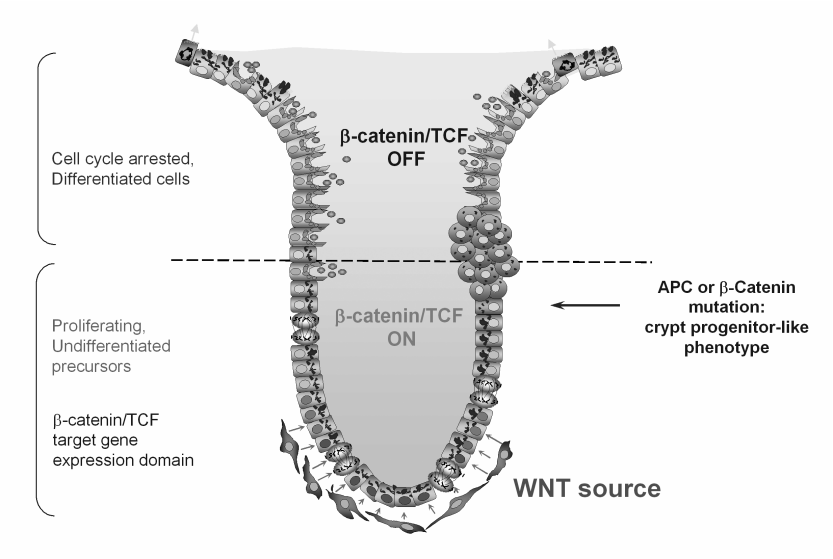

Figure 8. Wnt signaling regulates proliferation and differentiation in the intestine.
The bottom third of the colon crypt contains the proliferative cells. Here active Wnt signaling takes place and Tcf-β-catenin target genes are expressed. As the cells migrate up the crypt-villus axis, Tcf-β-catenin activity is down regulated, resulting in cell cycle arrest and differentiation. Upon APC or β-catenin mutation, cells become subject to active Tcf-β-catenin signaling and take on a crypt-like undifferentiated phenotype.

Of course many questions remain, such as how the border between differentiated cells and proliferative cells is achieved and which mechanism ensures differentiated cell migrating only in one direction. This has been addressed by Battle et al., where the role of the Tcf-β-catenin target genes of the Ephrin receptors family and their ligands are studied in more detail (123). These proteins have been implicated in guidance and sorting of different cell types (reviewed in (124)). Interaction between Eph receptors and Ephrin ligands involves direct cell to cell contact and frequently results in repulsion. The EPH receptors B2 and B3 and their ligand Ephrin B1 have been identified as targets in the array experiment, but with inverse effects. The EPH receptors are down regulated upon inhibition of Tcf-β-catenin signaling, whereas the Ephrin ligand is up regulated upon inhibition of Tcf-β-catenin, indicative of this ligands being an indirect target. Expression of the Ephrin receptors was essential for the correct positioning of the epithelial cells along the crypt villus axis, as was shown in EphB2 and B3 knock out mice. Ephrins ligands and EphB receptors show a graded expression pattern in the crypts of the small intestine. EphB receptor expression is highest in the bottom of the small intestine crypts and Ephrin ligand expression peaks at the surface epithelium. This inverse gradient could be a mechanism in which it is ensured that the proliferative cells are sorted away from the differentiated cells.

Additional questions still remaining are: how do stem cells obtain and maintain their phenotype? Where does the activating Wnt signal come from? Is it produced in an autocrine fashion by the stem cells or by mesenchymal cells? Moreover, it would be interesting to see if this role for Wnt signaling in regulating proliferation and differentiation also occurs in other differentiation processes of other adult tissues, including breast development and the continuous differentiation within the hematopoetic system. Future research is required to shed more light on these intriguing questions.

6. REFERENCE LIST

1. Ichii, S., Horii, A., Nakatsuru, S., Furuyama, J., Utsunomiya, J., and Nakamura, Y. (1992). Inactivation of both APC alleles in an early stage of colon adenomas in a patient with familial adenomatous polyposis (FAP). *Hum.Mol.Genet.* 1: 387-390.

2. Rijsewijk, F., Schuermann, M., Wagenaar, E., Parren, P., Weigel, D., and Nusse, R. (1987). The Drosophila homolog of the mouse mammary oncogene int-1 is identical to the segment polarity gene wingless. *Cell* 50: 649-657.

3. Perrimon, N. and Mahowald, A. P. (1987). Multiple functions of segment polarity genes in Drosophila. *Dev.Biol.* 119: 587-600.

4. Peifer, M. and Wieschaus, E. (12-21-1990). The segment polarity gene armadillo encodes a functionally modular protein that is the Drosophila homolog of human plakoglobin. *Cell* 63: 1167-1176.

5. Van de Wetering, M., Cavallo, R., Dooijes, D., van Beest, M., van Es, J., Loureiro, J., Ypma, A., Hursh, D., Jones, T., Bejsovec, A., Peifer, M., Mortin, M., and Clevers, H. (1997). Armadillo coactivates transcription driven by the product of the Drosophila segment polarity gene dTCF. *Cell* 88: 789-799.

6. Hamada, F., Tomoyasu, Y., Takatsu, Y., Nakamura, M., Nagai, S., Suzuki, A., Fujita, F., Shibuya, H., Toyoshima, K., Ueno, N., and Akiyama, T. (3-12-1999). Negative regulation of Wingless signaling by D-axin, a Drosophila homolog of axin. *Science* 283: 1739-1742.

7. Willert, K., Logan, C. Y., Arora, A., Fish, M., and Nusse, R. (1999). A Drosophila Axin homolog, Daxin, inhibits Wnt signaling. *Development* 126: 4165-4173.

8. McMahon, A. P. and Moon, R. T. (1989). Ectopic expression of the proto-oncogene int-1 in Xenopus embryos leads to duplication of the embryonic axis. *Cell* 58: 1075-1084.

9. Willert, K. and Nusse, R. (1998). Beta-catenin: a key mediator of Wnt signaling. *Curr.Opin.Genet.Dev.* 8: 95-102.

10. Molenaar, M., van de Wetering, M., Oosterwegel, M., Peterson Maduro, J., Godsave, S., Korinek, V., Roose, J., Destree, O., and Clevers, H. (1996). XTcf-3 transcription factor mediates beta-catenin-induced axis formation in Xenopus embryos. *Cell* 86: 391-399.

11. Huber, O., Korn, R., McLaughlin, J., Ohsugi, M., Herrmann, B. G., and Kemler, R. (1996). Nuclear localization of beta-catenin by interaction with transcription factor LEF-1. *Mech.Dev.* 59: 3-10.

12. Behrens, J., von Kries, J. P., Kuhl, M., Bruhn, L., Wedlich, D., Grosschedl, R., and Birchmeier, W. (1996). Functional interaction of beta-catenin with the transcription factor LEF-1. *Nature* 382: 638-642.

13. Behrens, J., Jerchow, B. A., Wurtele, M., Grimm, J., Asbrand, C., Wirtz, R., Kuhl, M., Wedlich, D., and Birchmeier, W. (1998). Functional interaction of an axin homolog, conductin, with beta- catenin, APC, and GSK3beta. *Science* 280: 596-599.

14. Nakamura, T., Hamada, F., Ishidate, T., Anai, K., Kawahara, K., Toyoshima, and Akiyama, T. (1998). Axin, an inhibitor of the Wnt signaling pathway, interacts with beta-catenin, GSK-3beta and APC and reduces the beta-catenin level. *Genes Cells* 3: 395-403.

15. Rubinfeld, B., Albert, I., Porfiri, E., Fiol, C., Munemitsu, S., and Polakis, P. (1996). Binding of GSK3beta to the APC-beta-catenin complex and regulation of complex assembly [see comments]. *Science* 272: 1023-1026.

16. Van Es, J. H., Giles, R. H., and Clevers, H. C. (3-10-2001). The many faces of the tumor suppressor gene APC. *Exp.Cell Res.* 264: 126-134.

17. Aberle, H., Bauer, A., Stappert, J., Kispert, A., and Kemler, R. (1997). beta-catenin is a target for the ubiquitin-proteasome pathway. *EMBO J.* 16: 3797-3804.

18. Kitagawa, M., Hatakeyama, S., Shirane, M., Matsumoto, M., Ishida, N., Hattori, K., Nakamichi, I., Kikuchi, A., and Nakayama, K. (5-4-1999). An F-box protein, FWD1, mediates ubiquitin-dependent proteolysis of beta-catenin. *EMBO J.* 18: 2401-2410.

19. Roose, J., Molenaar, M., Peterson, J., Hurenkamp, J., Brantjes, H., Moerer, P., van de Wetering, M., Destree, O., and Clevers, H. (1998). The *Xenopus* Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors. *Nature* 395: 608-612.

20. Cavallo, R., Cox, R., Moline, M., Roose, J., Polevoy, G., Clevers, H., Peifer, M., and Bejsovec, A. (1998). *Drosophila* Tcf and Groucho interact to repress Wingless signaling activiy. *Nature* 395: 604-608.

21. Mao, J., Wang, J., Liu, B., Pan, W., Farr, G. H., III, Flynn, C., Yuan, H., Takada, S., Kimelman, D., Li, L., and Wu, D. (2001). Low-density lipoprotein receptor-related protein-5 binds to Axin and regulates the canonical Wnt signaling pathway. *Mol.Cell* 7: 801-809.

22. Wehrli, M., Dougan, S. T., Caldwell, K., O'Keefe, L., Schwartz, S., Vaizel-Ohayon, D., Schejter, E., Tomlinson, A., and DiNardo, S. (9-28-2000). arrow encodes an LDL-receptor-related protein essential for Wingless signaling. *Nature* 407: 527-530.

23. Tamai, K., Semenov, M., Kato, Y., Spokony, R., Liu, C., Katsuyama, Y., Hess, F., Saint-Jeannet, J. P., and He, X. (9-28-2000). LDL-receptor-related proteins in Wnt signal transduction. *Nature* 407: 530-535.

24. Polakis, P. (8-1-2000). Wnt signaling and cancer. *Genes Dev* 14: 1837-1851.

25. Bienz, M. and Clevers, H. (10-13-2000). Linking colorectal cancer to Wnt signaling. *Cell* 103: 311-320.

26. He, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., da Costa, L. T., Morin, P. J., Vogelstein, B., and Kinzler, K. W. (1998). Identification of c-MYC as a target of the APC pathway [see comments]. *Science* 281: 1509-1512.

27. Roose, J., Huls, G., van Beest, M., Moerer, P., van der, Horn K., Goldschmeding, R., Logtenberg, T., and Clevers, H. (9-17-1999). Synergy between tumor suppressor APC and the beta-catenin-Tcf4 target Tcf1. *Science* 285: 1923-1926.

28. He, T. C., Chan, T. A., Vogelstein, B., and Kinzler, K. W. (10-29-1999). PPARdelta is an APC-regulated target of nonsteroidal anti-inflammatory drugs. *Cell* 99: 335-345.

29. Vinson, C. R. and Adler, P. N. (10-8-1987). Directional non-cell autonomy and the transmission of polarity information by the frizzled gene of Drosophila. *Nature* 329: 549-551.

30. Kuhl, M., Sheldahl, L. C., Park, M., Miller, J. R., and Moon, R. T. (2000). The Wnt/Ca2+ pathway: a new vertebrate Wnt signaling pathway takes shape. *Trends Genet.* 16: 279-283.

31. Hoang, B., Moos, M., Jr., Vukicevic, S., and Luyten, F. P. (10-18-1996). Primary structure and tissue distribution of FRZB, a novel protein related to Drosophila frizzled, suggest a role in skeletal morphogenesis. *J.Biol.Chem.* 271: 26131-26137.

32. Leyns, L., Bouwmeester, T., Kim, S. H., Piccolo, S., and De Robertis, E. M. (1997). Frzb-1 is a secreted antagonist of Wnt signaling expressed in the Spemann organizer. *Cell* 88: 747-756.

33. Wang, S., Krinks, M., Lin, K., Luyten, F. P., and Moos, M., Jr. (3-21-1997). Frzb, a secreted protein expressed in the Spemann organizer, binds and inhibits Wnt-8. *Cell* 88: 757-766.

34. Hsieh, J. C., Kodjabachian, L., Rebbert, M. L., Rattner, A., Smallwood, P. M., Samos, C. H., Nusse, R., Dawid, I. B., and Nathans, J. (4-1-1999). A new secreted protein that binds to Wnt proteins and inhibits their activities. *Nature* 398: 431-436.

35. Piccolo, S., Agius, E., Leyns, L., Bhattacharyya, S., Grunz, H., Bouwmeester, T., and De Robertis, E. M. (2-25-1999). The head inducer Cerberus is a multifunctional antagonist of Nodal, BMP and Wnt signals. *Nature* 397: 707-710.

36. Bafico, A., Liu, G., Yaniv, A., Gazit, A., and Aaronson, S. A. (2001). Novel mechanism of Wnt signaling inhibition mediated by Dickkopf-1 interaction with LRP6/Arrow. *Nat.Cell Biol.* 3: 683-686.

37. Mao, B., Wu, W., Li, Y., Hoppe, D., Stannek, P., Glinka, A., and Niehrs, C. (5-17-2001). LDL-receptor-related protein 6 is a receptor for Dickkopf proteins. *Nature* 411: 321-325.

38. Mao, B., Wu, W., Davidson, G., Marhold, J., Li, M., Mechler, B. M., Delius, H., Hoppe, D., Stannek, P., Walter, C., Glinka, A., and Niehrs, C. (6-6-2002). Kremen proteins are Dickkopf receptors that regulate Wnt/beta-catenin signaling. *Nature* 417: 664-667.

39. Liu, C., Li, Y., Semenov, M., Han, C., Baeg, G. H., Tan, Y., Zhang, Z., Lin, X., and He, X. (3-22-2002). Control of beta-catenin phosphorylation/degradation by a dual-kinase mechanism. *Cell* 108: 837-847.

40. Amit, S., Hatzubai, A., Birman, Y., Andersen, J. S., Ben Shushan, E., Mann, M., Ben Neriah, Y., and Alkalay, I. (5-1-2002). Axin-mediated CKI phosphorylation of beta-catenin at Ser 45: a molecular switch for the Wnt pathway. *Genes Dev.* 16: 1066-1076.

41. Groden, J., Thliveris, A., Samowitz, W., Carlson, M., Gelbert, L., Albertsen, H., Joslyn, G., Stevens, J., Spirio, L., Robertson, M., and et al. (1991). Identification and characterization of the familial adenomatous polyposis coli gene. *Cell* 66: 589-600.

42. Kinzler, K. W., Nilbert, M. C., Su, L. K., Vogelstein, B., Bryan, T. M., Levy, D. B., Smith, K. J., Preisinger, A. C., Hedge, P., McKechnie, D., and . (8-9-1991). Identification of FAP locus genes from chromosome 5q21. *Science* 253: 661-665.

43. Kinzler, K. W. and Vogelstein, B. (1996). Lessons from hereditary colorectal cancer. *Cell* 87: 159-170.

44. Nagase, H. and Nakamura, Y. (1993). Mutations of the APC (adenomatous polyposis coli) gene. *Hum.Mutat.* 2: 425-434.

45. Bienz, M. (2002). The subcellular destinations of APC proteins. *Nat.Rev.Mol.Cell Biol.* 3: 328-338.

46. Su, L. K., Vogelstein, B., and Kinzler, K. W. (1993). Association of the APC tumor suppressor protein with catenins. *Science* 262: 1734-1737.

47. Rubinfeld, B., Souza, B., Albert, I., Muller, O., Chamberlain, S. H., Masiarz, F. R., Munemitsu, S., and Polakis, P. (1993). Association of the APC gene product with beta-catenin. *Science* 262: 1731-1734.

48. Hart, M. J., de los Santos, R., Albert, I. N., Rubinfeld, B., and Polakis, P. (1998). Down-regulation of beta-catenin by human Axin and its association with the APC tumor suppressor, beta-catenin and GSK3 beta. *Curr.Biol.* 8: 573-581.

49. Zeng, L., Fagotto, F., Zhang, T., Hsu, W., Vasicek, T. J., Perry, W. L-3rd, Lee, J. J., Tilghman, S. M., Gumbiner, B. M., and Costantini, F. (1997). The mouse Fused locus encodes Axin, an inhibitor of the Wnt signaling pathway that regulates embryonic axis formation. *Cell* 90: 181-192.

50. Rosin-Arbesfeld, R., Townsley, F., and Bienz, M. (8-31-2000). The APC tumour suppressor has a nuclear export function. *Nature* 406: 1009-1012.

51. Bruhn, L., Munnerlyn, A., and Grosschedl, R. (1997). ALY, a context-dependent coactivator of LEF-1 and AML-1, is required for TCRalpha enhancer function. *Genes Dev.* 11: 640-653.

52. Henderson, B. R. (2000). Nuclear-cytoplasmic shuttling of APC regulates beta-catenin subcellular localization and turnover. *Nat.Cell Biol.* 2: 653-660.

53. Neufeld, K. L., Zhang, F., Cullen, B. R., and White, R. L. (2000). APC-mediated down-regulation of beta-catenin activity involves nuclear sequestration and nuclear export. *EMBO Rep.* 1: 519-523.

54. Van de Wetering, M., Oosterwegel, M., Dooijes, D., and Clevers, H. (1991). Identification and cloning of TCF-1, a T lymphocyte-specific transcription factor containing a sequence-specific HMG box. *EMBO J.* 10: 123-132.

55. Giese, K., Amsterdam, A., and Grosschedl, R. (1991). DNA-binding properties of the HMG domain of the lymphoid-specific transcriptional regulator LEF-1. *Genes Dev.* 5: 2567-2578.

56. Van Beest, M., Dooijes, D., van De, Wetering M., Kjaerulff, S., Bonvin, A., Nielsen, O., and Clevers, H. (9-1-2000). Sequence-specific high mobility group box factors recognize 10-12-base pair minor groove motifs. *J.Biol.Chem.* 275: 27266-27273.

57. Giese, K., Cox, J., and Grosschedl, R. (1992). The HMG domain of lymphoid enhancer factor 1 bends DNA and facilitates assembly of functional nucleoprotein structures. *Cell* 69: 185-195.

58. Dooijes, D., van de Wetering, M., Knippels, L., and Clevers, H. (1993). The Schizosaccharomyces pombe mating-type gene mat-Mc encodes a sequence-specific DNA-binding high mobility group box protein. *J.Biol.Chem.* 268: 24813-24817.

59. Grosschedl, R., Giese, K., and Pagel, J. (1994). HMG domain proteins: architectural elements in the assembly of nucleoprotein structures. *Trends Genet.* 10: 94-100.

60. Giese, K., Kingsley, C., Kirshner, J., and Grosschedl, R. (1995). Assembly and function of a TCR alpha enhancer complex is dependent on LEF-1-induced DNA bending and multiple protein- protein interactions. *Genes-Dev.* 9: 995-1008.

61. Bianchi, M. E. and Beltrame, M. (1998). Flexing DNA: HMG-box proteins and their partners. *Am.J.Hum.Genet.* 63: 1573-1577.

62. Carlsson, P., Waterman, M. L., and Jones, K. A. (1993). The hLEF/TCF-1 alpha HMG protein contains a context-dependent transcriptional activation domain that induces the TCR alpha enhancer in T cells. *Genes Dev.* 7: 2418-2430.

63. Van de Wetering, M., Castrop, J., Korinek, V., and Clevers, H. (1996). Extensive alternative splicing and dual promoter usage generate Tcf-1 protein isoforms with differential transcription control properties. *Mol.Cell Biol.* 16: 745-752.

64. Van de Wetering, M., Oosterwegel, M., Holstege, F., Dooyes, D., Suijkerbuijk, R., Geurts van Kessel, A., and Clevers, H. (1992). The human T cell transcription factor-1 gene. Structure, localization, and promoter characterization. *J.Biol.Chem.* 267: 8530-8536.

65. Duval, A., Rolland, S., Tubacher, E., Bui, H., Thomas, G., and Hamelin, R. (7-15-2000). The human T-cell transcription factor-4 gene: structure, extensive characterization of alternative splicings, and mutational analysis in colorectal cancer cell lines. *Cancer Res.* 60: 3872-3879.

66. Tjian, R. and Maniatis, T. (1994). Transcriptional activation: a complex puzzle with few easy pieces. *Cell* 77: 5-8.

67. Barker, N., Morin, P. J., and Clevers, H. (2000). The Yin-Yang of TCF/beta-catenin signaling. *Adv.Cancer Res.* 77: 1-24.

68. Harwood, A. J. (6-29-2001). Regulation of gsk-3. a cellular multiprocessor. *Cell* 105: 821-824.

69. Dajani, R., Fraser, E., Roe, S. M., Young, N., Good, V., Dale, T. C., and Pearl, L. H. (6-15-2001). Crystal structure of glycogen synthase kinase 3beta. structural basis for phosphate-primed substrate specificity and autoinhibition. *Cell* 105: 721-732.

70. Barker, N. and Clevers, H. (2000). Catenins, Wnt signaling and cancer. *Bioessays* 22: 961-965.

71. Hecht, A., Litterst, C. M., Huber, O., and Kemler, R. (6-18-1999). Functional characterization of multiple transactivating elements in beta-catenin, some of which interact with the TATA-binding protein in vitro. *J.Biol.Chem.* 274: 18017-18025.

72. Bauer, A., Huber, O., and Kemler, R. (12-8-1998). Pontin52, an interaction partner of beta-catenin, binds to the TATA box binding protein. *Proc.Natl.Acad.Sci.U.S.A* 95: 14787-14792.

73. Takemaru, K. I. and Moon, R. T. (4-17-2000). The transcriptional coactivator CBP interacts with beta-catenin to activate gene expression. *J.Cell Biol.* 149: 249-254.

74. Hecht, A., Vleminckx, K., Stemmler, M. P., van Roy, F., and Kemler, R. (4-17-2000). The p300/CBP acetyltransferases function as transcriptional coactivators of beta-catenin in vertebrates. *EMBO J.* 19: 1839-1850.

75. Vleminckx, K., Kemler, R., and Hecht, A. (1999). The C-terminal transactivation domain of beta-catenin is necessary and sufficient for signaling by the LEF-1/beta-catenin complex in Xenopus laevis. *Mech.Dev* 81: 65-74.

76. Goodman, R. H. and Smolik, S. (7-1-2000). CBP/p300 in cell growth, transformation, and development. *Genes Dev* 14: 1553-1577.

77. Struhl, K. (3-1-1998). Histone acetylation and transcriptional regulatory mechanisms. *Genes Dev* 12: 599-606.

78. Barker, N., Hurlstone, A., Musisi, H., Miles, A., Bienz, M., and Clevers, H. (9-3-2001). The chromatin remodelling factor Brg-1 interacts with beta-catenin to promote target gene activation. *EMBO J.* 20: 4935-4943.

79. Owen-Hughes, T. and Workman, J. L. (9-2-1996). Remodeling the chromatin structure of a nucleosome array by transcription factor-targeted trans-displacement of histones. *EMBO J.* 15: 4702-4712.

80. Whitehouse, I., Flaus, A., Cairns, B. R., White, M. F., Workman, J. L., and Owen-Hughes, T. (8-19-1999). Nucleosome mobilization catalysed by the yeast SWI/SNF complex. *Nature* 400: 784-787.

81. Collins, R. T. and Treisman, J. E. (12-15-2000). Osa-containing Brahma chromatin remodeling complexes are required for the repression of wingless target genes. *Genes Dev* 14: 3140-3152.

82. Yu, X., Riese, J., Eresh, S., and Bienz, M. (12-1-1998). Transcriptional repression due to high levels of Wingless signaling. *EMBO J.* 17: 7021-7032.

83. Yang, X., van Beest, M., Clevers, H., Jones, T., Hursh, D. A., and Mortin, M. A. (2000). decapentaplegic is a direct target of dTcf repression in the Drosophila visceral mesoderm. *Development* 127: 3695-3702.

84. Brannon, M., Gomperts, M., Sumoy, L., Moon, R. T., and Kimelman, D. (1997). A beta-catenin/XTcf-3 complex binds to the siamois promoter to regulate dorsal axis specification in Xenopus. *Genes Dev.* 11: 2359-2370.

85. Bienz, M. (1998). TCF: transcriptional activator or repressor? *Curr.Opin.Cell Biol.* 10: 366-372.

86. Chen, G. and Courey, A. J. (5-16-2000). Groucho/TLE family proteins and transcriptional repression. *Gene* 249: 1-16.

87. Chen, G., Nguyen, P. H., and Courey, A. J. (1998). A role for Groucho tetramerization in transcriptional repression. *Mol.Cell Biol.* 18: 7259-7268.

88. Fisher, A. L., Ohsako, S., and Caudy, M. (1996). The WRPW motif of the hairy-related basic helix-loop-helix repressor proteins acts as a 4-amino-acid transcription repression and protein-protein interaction domain. *Mol.Cell Biol.* 16: 2670-2677.

89. Jimenez, G., Paroush, Z., and Ish Horowicz, D. (1997). Groucho acts as a corepressor for a subset of negative regulators, including Hairy and Engrailed. *Genes Dev.* 11: 3072-3082.

90. Ren, B., Chee, K. J., Kim, T. H., and Maniatis, T. (1-1-1999). PRDI-BF1/Blimp-1 repression is mediated by corepressors of the Groucho family of proteins. *Genes Dev.* 13: 125-137.

91. Aronson, B. D., Fisher, A. L., Blechman, K., Caudy, M., and Gergen, J. P. (1997). Groucho-dependent and -independent repression activities of Runt domain proteins. *Mol.Cell Biol.* 17: 5581-5587.

92. Dubnicoff, T., Valentine, S. A., Chen, G., Shi, T., Lengyel, J. A., Paroush, Z., and Courey, A. J. (1997). Conversion of dorsal from an activator to a repressor by the global corepressor Groucho. *Genes Dev.* 11: 2952-2957.

93. Chen, G., Fernandez, J., Mische, S., and Courey, A. J. (9-1-1999). A functional interaction between the histone deacetylase Rpd3 and the corepressor groucho in Drosophila development. *Genes Dev.* 13: 2218-2230.

94. Brantjes, H., Roose, J., van De, Wetering M., and Clevers, H. (4-1-2001). All Tcf HMG box transcription factors interact with Groucho-related co- repressors. *Nucleic Acids Res.* 29: 1410-1419.

95. Mannervik, M. and Levine, M. (6-8-1999). The Rpd3 histone deacetylase is required for segmentation of the Drosophila embryo. *Proc.Natl.Acad.Sci.U.S.A* 96: 6797-6801.

96. Levanon, D., Goldstein, R. E., Bernstein, Y., Tang, H., Goldenberg, D., Stifani, S., Paroush, Z., and Groner, Y. (9-29-1998). Transcriptional repression by AML1 and LEF-1 is mediated by the TLE/Groucho corepressors. *Proc.Natl.Acad.Sci.U.S.A.* 95: 11590-11595.

97. Brannon, M., Brown, J. D., Bates, R., Kimelman, D., and Moon, R. T. (1999). XCtBP is a XTcf-3 co-repressor with roles throughout Xenopus development. *Development* 126: 3159-3170.

98. Nibu, Y., Zhang, H., and Levine, M. (4-3-1998). Interaction of short-range repressors with Drosophila CtBP in the embryo. *Science* 280: 101-104.

99. Smits, R., Kielman, M. F., Breukel, C., Zurcher, C., Neufeld, K., Jagmohan-Changur, S., Hofland, N., van Dijk, J., White, R., Edelmann, W., Kucherlapati, R., Khan, P. M., and Fodde, R. (5-15-1999). Apc1638T: a mouse model delineating critical domains of the adenomatous polyposis coli protein involved in tumorigenesis and development. *Genes Dev.* 13: 1309-1321.

100. Phippen, T. M., Sweigart, A. L., Moniwa, M., Krumm, A., Davie, J. R., and Parkhurst, S. M. (12-1-2000). Drosophila C-terminal binding protein functions as a context-dependent transcriptional co-factor and interferes with both mad and groucho transcriptional repression. *J.Biol.Chem.* 275: 37628-37637.

101. Waltzer, L. and Bienz, M. (1998). *Drosophila* CBP represses the transcription factor Tcf to antagonize Wingless signaling. *Nature* 395: 521-525.

102. Klingensmith, J. and Nusse, R. (1994). Signaling by wingless in Drosophila. *Dev.Biol.* 166: 396-414.

103. Van Genderen, C., Okamura, R. M., Farinas, I., Quo, R. G., Parslow, T. G., Bruhn, L., and Grosschedl, R. (1994). Development of several organs that require inductive epithelial- mesenchymal interactions is impaired in LEF-1-deficient mice. *Genes Dev.* 8: 2691-2703.

104. Zhou, P., Byrne, C., Jacobs, J., and Fuchs, E. (1995). Lymphoid enhancer factor 1 directs hair follicle patterning and epithelial cell fate. *Genes-Dev.* 9: 700-713.

105. Verbeek, S., Izon, D., Hofhuis, F., Robanus Maandag, E., te Riele, H., van de Wetering, M., Oosterwegel, M., Wilson, A., MacDonald, H. R., and Clevers, H. (1995). An HMG-box-containing T-cell factor required for thymocyte differentiation. *Nature* 374: 70-74.

106. Korinek, V., Barker, N., Moerer, P., van Donselaar, E., Huls, G., Peters, P. J., and Clevers, H. (1998). Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4. *Nat.Genet.* 19: 379-383.

107. Korinek, V., Barker, N., Morin, P. J., van Wichen, D., de Weger, R., Kinzler, K. W., Vogelstein, B., and Clevers, H. (1997). Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma [see comments]. *Science* 275: 1784-1787.

108. Merrill, B. J., Gat, U., DasGupta, R., and Fuchs, E. (7-1-2001). Tcf3 and Lef1 regulate lineage differentiation of multipotent stem cells in skin. *Genes Dev.* 15: 1688-1705.

109. Hamilton, F. S., Wheeler, G. N., and Hoppler, S. (2001). Difference in XTcf-3 dependency accounts for change in response to beta-catenin-mediated Wnt signaling in Xenopus blastula. *Development* 128: 2063-2073.

110. Roel, G., Hamilton, F. S., Gent, Y., Bain, A. A., Destree, O., and Hoppler, S. (11-19-2002). Lef-1 and Tcf-3 Transcription Factors Mediate Tissue-Specific Wnt Signaling during Xenopus Development. *Curr.Biol.* 12: 1941-1945.

111. Hovanes, K., Li, T. W., Munguia, J. E., Truong, T., Milovanovic, T., Lawrence, Marsh J., Holcombe, R. F., and Waterman, M. L. (2001). Beta-catenin-sensitive isoforms of lymphoid enhancer factor-1 are selectively expressed in colon cancer. *Nat.Genet.* 28: 53-57.

112. Atcha, F. A., Munguia, J. E., Li, T. W., Hovanes, K., and Waterman, M. L. (2-11-2003). A new beta -catenin dependent activation domain in T cell factor. *J.Biol.Chem.*

113. Hecht, A. and Stemmler, M. P. (2-7-2003). Identification of a promoter-specific transcriptional activation domain at the C terminus of the Wnt effector protein T-cell factor 4. *J.Biol.Chem.* 278: 3776-3785.

114. Miyoshi, Y., Nagase, H., Ando, H., Horii, A., Ichii, S., Nakatsuru, S., Aoki, T., Miki, Y., Mori, T., and Nakamura, Y. (1992). Somatic mutations of the APC gene in colorectal tumors: mutation cluster region in the APC gene. *Hum.Mol.Genet.* 1: 229-233.

115. Morin, P. J., Sparks, A. B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B., and Kinzler, K. W. (1997). Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. *Science* 275: 1787-1790.

116. Liu, W., Dong, X., Mai, M., Seelan, R. S., Taniguchi, K., Krishnadath, K. K., Halling, K. C., Cunningham, J. M., Boardman, L. A., Qian, C., Christensen, E., Schmidt, S. S., Roche, P. C., Smith, D. I., and Thibodeau, S. N. (2000). Mutations in AXIN2 cause colorectal cancer with defective mismatch repair by activating beta-catenin/TCF signaling. *Nat.Genet.* 26: 146-147.

117. Rubinfeld, B., Robbins, P., El Gamil, M., Albert, I., Porfiri, E., and Polakis, P. (1997). Stabilization of beta-catenin by genetic defects in melanoma cell lines. *Science* 275: 1790-1792.

118. Miyoshi, Y., Iwao, K., Nagasawa, Y., Aihara, T., Sasaki, Y., Imaoka, S., Murata, M., Shimano, T., and Nakamura, Y. (6-15-1998). Activation of the beta-catenin gene in primary hepatocellular carcinomas by somatic alterations involving exon 3. *Cancer Res.* 58: 2524-2527.

119. De La, Coste A., Romagnolo, B., Billuart, P., Renard, C. A., Buendia, M. A., Soubrane, O., Fabre, M., Chelly, J., Beldjord, C., Kahn, A., and Perret, C. (7-21-1998). Somatic mutations of the beta-catenin gene are frequent in mouse and human hepatocellular carcinomas. *Proc.Natl.Acad.Sci.U.S.A* 95: 8847-8851.

120. Palacios, J. and Gamallo, C. (4-1-1998). Mutations in the beta-catenin gene (CTNNB1) in endometrioid ovarian carcinomas. *Cancer Res.* 58: 1344-1347.

121. Chan, E. F., Gat, U., McNiff, J. M., and Fuchs, E. (1999). A common human skin tumour is caused by activating mutations in beta-catenin. *Nat.Genet.* 21: 410-413.

122. Van De, Wetering M., Sancho, E., Verweij, C., de Lau, W., Oving, I., Hurlstone, A., van der, Horn K., Batlle, E., Coudreuse, D., Haramis, A. P., Tjon-Pon-Fong, M., Moerer, P., van den, Born M., Soete, G., Pals, S., Eilers, M., Medema, R., and Clevers, H. (10-18-2002). The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. *Cell* 111: 241-250.

123. Batlle, E., Henderson, J. T., Beghtel, H., van den Born, M. M., Sancho, E., Huls, G., Meeldijk, J., Robertson, J., van De, Wetering M., Pawson, T., and Clevers, H. (10-18-2002). Beta-catenin and TCF mediate cell positioning in the intestinal epithelium by controlling the expression of EphB/ephrinB. *Cell* 111: 251-263.

124. Wilkinson, D. G. (2001). Multiple roles of EPH receptors and ephrins in neural development. *Nat.Rev.Neurosci.* 2: 155-164.

CHAPTER 2

ALL TCF HMG BOX TRANSCRIPTION FACTORS INTERACT WITH GROUCHO RELATED CO-REPRESSORS

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Abstract

Tcf/Lef family transcription factors are the downstream effectors of the Wingless/Wnt signal transduction pathway. Upon Wingless/Wnt signaling, β-catenin translocates to the nucleus, interacts with Tcf (1, 2, 3) and thus activates transcription of target genes (4, 5). Tcf factors also interact with members of the Groucho (Grg/TLE) family of transcriptional co-repressors (6). We have now tested all known mammalian Groucho family members for their ability to interact specifically with individual Tcf/Lef family members. Transcriptional activation by any Tcf could be repressed by Grg-1, Grg-2/TLE-2, Grg-3 and Grg-4 in a reporter assay. Specific interactions between Tcf and Grg proteins may be achieved *in vivo* by tissue or cell type limited expression. To address this, we determined the expression of all Tcf and Grg/TLE family members in a panel of cell lines. Within any cell line, several Tcfs and TLEs are co-expressed. Thus, redundancy in Tcf/Grg interactions appears to be the rule. The "long" Groucho family members containing 5 domains are repressors of Tcf mediated transactivation, whereas Grg-5 which only contains the first two domains acts as a de-repressor. As previously shown for *Drosophila* Groucho, we show that long Grg proteins interact with histone deacetylase-1. Although Grg-5 contains the GP homology domain that in long Grg proteins mediates HDAC binding, Grg-5 fails to bind this co-repressor, explaining how it can de-repress transcription.

Introduction

Recent studies have demonstrated that members of the Tcf/Lef family of HMG box transcription factors are important downstream effectors of the Wnt/Wingless signaling cascade in mammalian, *Xenopus*, *Drosophila* and *C.elegans* development (7). In the absence of a Wnt signal, β-catenin associates with Axin, APC and GSK-3β in the cytoplasm (8, 9, 10). In this complex β-catenin is phosphorylated by GSK-3β, resulting in ubiquitination and degradation of β-catenin by the proteasome pathway (11, 12). Wnt signaling results in inhibition of GSK-3β, leading to the accumulation of β-catenin in the cytoplasm and its translocation to the nucleus. Association of β-catenin with Tcf in the nucleus leads to the formation of a bipartite transcription factor activating target gene expression, such as cyclin D1 (5), Tcf-1 (13) and PPARδ (14).

Experiments in *Drosophila* and *Xenopus* indicated that Tcf molecules could also function as transcriptional repressors in the absence of a Wnt/Wg signal (15, 16, 17, 18). Several recent studies have proposed a molecular basis for this phenomenon. Tcf can associate with a number of different transcriptional co-repressors. Binding of Tcf with CBP (19) or CtBP (20) can lead to repression of the Wnt/Wingless response. We and others have shown that the co-repressor Groucho and its vertebrate homologs can bind to Tcf. The association of Tcf and Groucho results

in repression of Tcf targets both in the context of synthetic promoters and of endogenous genes (21, 22, 6).

The vertebrate Tcf family of transcription factors consists of four members Tcf-1, Lef-1, Tcf-3 and Tcf-4 (Figure 1A). Studies have indicated that Tcf-1 is largely expressed in cells of the T cell lineage (23). Inactivation of the Tcf-1 gene by homologous recombination results in mice with an incomplete block in T cell development (24). Lef is expressed in pre-B and T cells in adult mice, and mainly in the neural crest, mesencephalon, tooth germs and whisker follicles during embryogenesis. Lef-1 deficient mice die shortly after birth and lack teeth, mammary glands, whiskers and hair but show no defects in lymphoid cell populations (25). T cell development in mice lacking both Tcf-1 and Lef-1 is completely arrested and impaired at an earlier stage than in mice lacking only Tcf-1, revealing that Tcf-1 and Lef-1 are partially redundant in the regulation of T cell development (26). Tcf-3 is expressed in stomach epithelium, hair follicles and keratinocytes of the skin (27). Tcf-4 expression occurs much later in embryogenesis than Tcf-1, Lef-1 or Tcf-3 and is most highly expressed in the midbrain and in intestinal and mammary epithelium. Tcf-4 exhibits a highly restricted expression pattern in the epithelium of the developing gut (27, 28). Continued expression of Tcf-4 expression at this site is essential for the maintenance of the progenitor compartment of gut epithelium, as indicated in Tcf-4 deficient mice by the abnormal development of their small intestines (29). Constitutively active Tcf-4-β-catenin complexes are found in the nuclei of colon carcinoma cells with mutations in APC or β-catenin (30). Presumably this results in the uncontrolled activation of Tcf target genes, which by implication transforms colon epithelial cells and initiates polyp formation (31).

Drosophila Groucho is the founding member of a conserved family of transcriptional co-repressors. Other members of this family are found from *C.elegans* to vertebrates. The murine and human genome harbour four full-length homologs of Groucho as well as a gene that encodes a truncated Groucho protein. The human Groucho homologs are TLE-1, -2, -3, -4 (for Transducin-Like Enhancer of Split) and the truncated variant hAES (for Amino terminal Enhancer of Split). The mouse Groucho family consists of the Groucho-related genes, *Grg-1, -2, -3, and -4* and the *Grg-5* gene encoding a shorter variant (32). The Grg/TLE proteins are highly similar to Drosophila Groucho in their domain structure. They contain 5 protein domains: an amino terminal Q (Glutamine rich) domain, followed by a GP (glycin/proline rich) domain, a CcN domain (containing putative casein kinase II/cdc2 phosphorylation sites and nuclear localisation signal), a SP (serine/proline rich) domain and four WD40 repeats (protein interaction domain) (Figure 1B). Of these the Q and WD40 domain sequences are most highly conserved. *Grg-5* and *hAES* encode only the two amino terminal domains of these proteins. Groucho related proteins have been described to tetramerize through a leucine zipper-like structure in the amino terminal Q domain (33).

Groucho proteins function as co-repressors for specific subsets of DNA binding transcription factors, including the Hairy-related proteins, Runt domain proteins (34), Engrailed (35), Dorsal (36) Pax-5 (37), NK-3 (38), NK-4 (39), NF-κB (39) and HNF3β (40). For some of these transcription factors, the domain which interacts with Groucho is mapped to a short peptide motif. Hairy related bHLH transcription factors are involved in diverse developmental processes such as sex determination, segmentation, neurogenesis and myogenesis in the fly. The carboxyterminal WRPW motif of these bHLH transcription factors binds to the SP domain of Groucho, but also the WD40 domain may be involved (41, 35, 42). Runt domain proteins like *Drosophila* Runt and human AML associate with Groucho proteins through a related motif, VWPRY (34). Another conserved Groucho interaction motif is found in a number of transcription

factors such as Engrailed and Goosecoid that associate with Groucho through their eh1/GEH domain (35, 43). Engrailed has been shown to interact with the WD40 domain of Groucho (44). Dorsal, a Rel domain transcription factor involved in dorsal ventral patterning, has been described to associate with Groucho (36). Some of these transcription factors are genuine repressors, depending on Groucho for their activity, like Hairy (41), HES (45) and Blimp-1 (46). Others like Tcf (6), Runt (34) and Dorsal (36) are activators and convert to repressors upon association with Groucho-related proteins.

Recently, the Drosophila histone deacetylase Rpd3 was shown to interact with Groucho (47). Recruitment of Rpd3 to the promotor of target genes will result in modulation of the local chromatin structure. Histone deacetylases remove acetyl groups from lysin residues in the amino-terminal tails of core histones. By mechanisms which are not yet fully understood this appears to result in a more compact chromatin structure is being formed that is associated with a repressed transcriptional state (48).

The current study characterises the Tcf/Groucho interaction in more detail. We describe the (co-) expression of individual Tcf and Grg/TLE family members in a panel of cell lines. In addition, we have mapped the Tcf interaction domain of Grg proteins and analysed whether there is specificity in the interaction between individual Tcf and TLE family members. Deletion analysis of XGrg-4 showed that only the first two domains, Q and GP, are sufficient to repress Tcf mediated transcriptional activation. However, Grg-5 which contains the same highly conserved domains, functions as a de-repressor. To determine the basis of the opposing functions of the truncated XGrg-4-QGP and XGrg-5 we examined their relative ability to interact with the human homolog of RPD3 (HDAC-1).

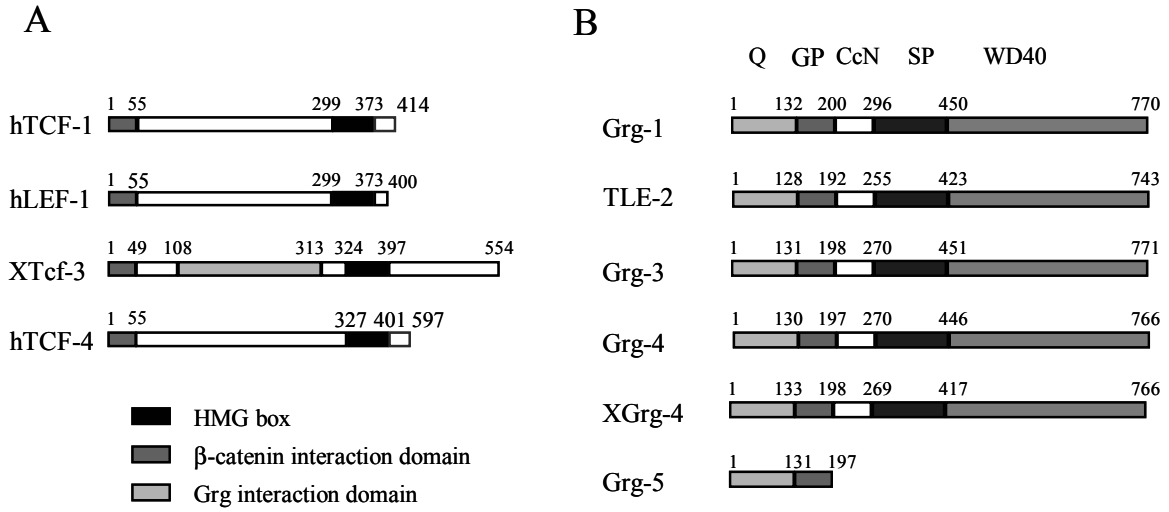


Figure 1. Domain structure of Tcf (A) and Grg (B) constructs.

Tcf proteins contain a centrally located DNA-binding HMG box and the N-terminal β-catenin interaction domain. As previously reported (6) the Grg interaction domain is located between these domains. The Grg proteins contain five distinct domains: Q, GP, CCN, SP and WD40 regions (49), while Grg-5 consists only of the Q and GP domain.

Materials and Methods

Transfections and Luciferase assays
2.5 x 106 IIAI.6 B cells were transfected by electroporation with various combinations of a luciferase reporter plasmid containing three optimal Tcf sites upstream of the minimal HSV-TK promoter (1 μg of pTKTOP) or its negative control vector containing mutated Tcf sites (pTKFOP) in combination with the following plasmids: an internal transfection control (50 ng of pRNL-TK, Promega) and one ore more of the following expression constructs: Tcf expression vectors (2 μg); Grg expression plasmids (0.5 or 5.0 μg); β-catenin expression plasmid (5.0 μg). For repression and de-repression experiments (see Figure 5), 250 μg XTcf-3, 100, 50, and 25 ng Armadillo and 5 ng of Grg-5 or XGrg-4-QGP (1-197) expression plasmids were used. Additional pCDNA3 plasmid was included where necessary to make the total amount of DNA equivalent. cDNAs encoding hTCF-1, hLEF-1, XTcf-3, hTCF-4, and Myc-tagged versions of Grg-1, TLE-2, Grg-3 were expressed in pCDNA3. The expression construct for Grg-4 in pKW2T was kindly provided to us by Dr. M. Busslinger (Research Institute of Molecular Pathology, Vienna, Austria). Luciferase activities were determined 24 h after transfection using the DUAL luciferase system according to the manufacturer's protocol (Promega). Luciferase activity was normalised relative to RNL-luciferase activity. For every experiment transfections were performed in duplicate and several independent experiments were performed.

Yeast Two Hybrid Assay
Two Hybrid experiments were carried out as described previously (2). Briefly, the Tcf (4-359) and Grg-5 (1-197) bait constructs were created by fusing the regions encoding the indicated amino acids to the GAL4 binding domain of pMD4. pVA3 encodes a murine P53-GAL4 binding domain hybrid in pGBT9 (Clontech). Multiple preys: mGrg-5 (1-197, 4-106, 82 –197, 4-62, 46-106, 82-106) were inserted in frame with the GAL4 activation domain in pGADGH (Clontech) or pGADRX (Stratagene). pTD1 encodes SV40 large T antigen in pGAD3F (Clontech). Baits and preys were transformed into the *Saccharomyces cerevisiae* strain HF7C (Clontech) and true interactions were scored by growth on selective nutrient agar plates. Equivalent results were obtained monitoring β-galactosidase activity (data not shown).

Cell lines
For the transfection experiments the mouse B cell line IIAI.6 was used. For the reverse transcription coupled PCR experiments the following human cell lines were used as a source of RNA: Jurkat and CEM (T cell lines), Reh and Raji (B cell lines), 293T (embryonic kidney cell line), HT29, LS174, SW480, SW620, DLD-1, RKO (colon carcinoma cell lines) and SKBR3 and T47D (mammary carcinoma cell lines). Cells were cultured in RPMI-1640 supplemented with 10% fetal calf serum and antibiotics.

Reverse transcriptase (RT)-PCR analysis

Total RNA for detection of *hTcf1, hLef-1, hTcf3, hTcf4, TLE-1, TLE-2, TLE-3* and *TLE-4* was isolated from the different cell lines using RNAzolB solution (Tel Test inc.) according to the manufacturer's instructions. Random-primed cDNA from total RNA was prepared using standard techniques. The following primers were used for the different PCR reactions: for *Tcf-1*, *Lef-1* and *Tcf-4* PCRs specific primers were used to amplify part of the mRNA corresponding to the NH_2-terminus upstream of the HMG box. For *hTcf-3* primers were developed to amplify the HMG box since this was the only sequence available at the time. *Tcf1* primers: 5'-ACCAGCGGCATGTACAAAGAG-3' (sense), 5'-TTCAGGTTGCGGTCGAAGGGC-3' (antisense). *Lef-1* primers: 5'-TTCTCCACCCATCCCGAGAAC-3' (sense), 5'-CTGAGGCTTCACGTGCATTTAG-3' (antisense). *Tcf-4* primers: 5'-CCATCACCGGCACACATTGTC-3'(sense), 5'-ACTATGGTGTGAGCCGACATC-3'. *Tcf-3* primers: 5'-GAAATCACCAGTCACCGTGAAA- 3'(sense), 5'-ACCAGGTTGGGTAGAGCTGCG-3' (antisense)

For *TLE-1*, *TLE-2*, *TLE-3* PCRs, specific primers were used to amplify a fragment of GP domain, CcN domain and part of the SP domain. For *hTLE-4* a part of the WD domain was amplified since that was the only sequence available for this gene. *hTLE-1* primers: 5'-GGCAGTGCCGGCCTTCTTGCG-3'(sense), 5'-AGGCTTGCCGAGACCTGGACG-3' (antisense). *hTLE-2* primers: 5'-AGTGCTACGGGGCTGCTTGCT-3'(sense), 5'-CGTTGAGAGTGCTGTGGGAGC-3' (antisense). *hTLE-3* primers: 5'-AGCAGCTCCGGGCTGCTGGCA-3'(sense), 5'-AGCCGAGGCCATTATACCTAT-3' (antisense). *hTLE-4* primers: 5'-TACACGGGTGGGAAGGGCGCG-3'(sense), 5'-GGCTTGGTGACATGCAAAACT-3' (antisense)

The primers for the *GAPDH* control PCR: 5'-AAGGTGAAGGTCGGAGTCAAC-3'(sense) 5'-TTACTCCTTGGAGGCCATGTG-3'(antisense)

Immunopreciptations

293T cells were co-transfected with pCDNA3-Flag-HDAC-1 (kindly provided to us by T. Kouzarides, Wellcome/CRC Institute Cambridge, UK) and pCDNA3-MYC-QGP-XGrg4 (amino acid 1-197) or pCDNA3-MYC-Grg5. Cells were harvested and whole cell lysate was prepared in Triton X-100 lysisbuffer (20 mM Tris ph 8.0, 140mM NaCl, 1% triton X-100 and 10% Glycerol) containing protease inhibitors (Protease inhibitor cocktail tablets; Roche Diagnostics) The extract was incubated with 1 µg anti-Myc monoclonal antibody and 5 µl protein A/G beads (Santa Cruz) or with 5 µl FLAG coupled beads (Sigma) over night at 4^oC in Triton X-100 lysis buffer. The beads were washed twice in Triton X-100 buffer and twice in Wash buffer (20 mM Tris pH 8.0, 150 mM NaCl and 0.5% NP-40) Supernatants were resolved by SDS-PAGE and transferred on nitrocellulose membrane (Immobilon-P, Millipore) The membrane was incubated with anti-FLAG (Sigma) or anti-Myc antibodies and immune reactive proteins were visualised by enhanced chemiluminescence. (Amersham Pharmacia Biotech)

Results and discussion

A 100 amino acid region in Grg-5 is essential for interaction with Tcf

The interaction between Tcf and Grg was previously mapped to the QGP domains of Groucho since Tcf interacts with Grg-5, consisting only of these two domains (50). Grg proteins can also tetramerize through their Q domain (33). We used the yeast two-hybrid assay to more precisely map the domain of Grg that interacts with Tcf versus the domain that is responsible for multimerization with other Grg molecules (Figure 2). Constructs expressing different parts of Grg-5 were fused to the Gal-4 activation domain and their interaction with Tcf-1 (4-359) and Grg-5 (1-197) fused to the gal-4 DNA binding domain was determined. The results shown in figure 2 imply that the Tcf interaction domain resides in the first 106 amino acids of Grg-5. All other deletion constructs of Grg-5 failed to interact with Tcf. Our data indicate that this N terminal region also contains the Grg interaction domain, in accordance with the domain previously described to mediate tetramerization (33). Further truncation of this region probably prevents proper folding of the tetramerization domain and thereby results in loss of the interaction with both Grg and Tcf.

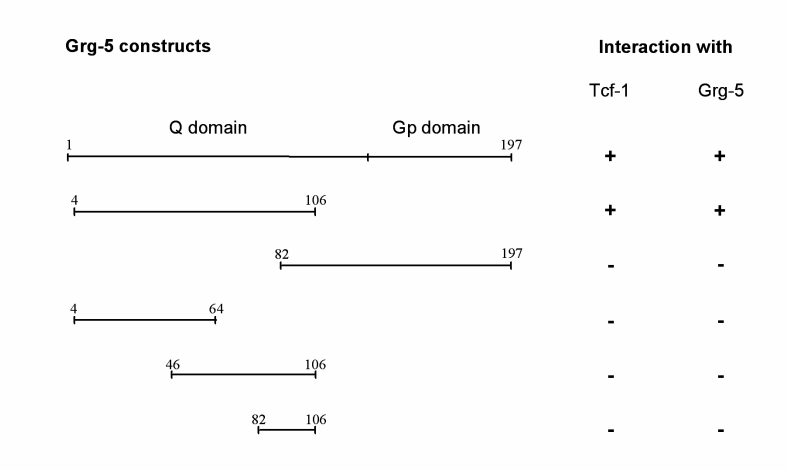


Figure 2. Mapping of the Grg-Tcf interaction domain and Grg multimerization domain.
The different bait Grg-5 constructs used in the yeast two hybrid assay are shown on the left. The ability of these constructs to interact with prey molecules Tcf-1 (4-359) or Grg-5 (1-197) and allow growth of yeast on selective plates is indicated. The minimal interaction domain of Grg-5 required for interaction with Tcf resides in the first 106 amino acids. Further truncation of this domain results in loss of interaction.

Repression of TCF mediated transcription by Groucho related genes

To further investigate the issue of specificity in the interaction between different Tcf family members and Grg proteins, we used the previously established β-catenin-Tcf reporter gene assay (3). In this assay co-transfection of Tcf and β-catenin results in transactivation of a luciferase reporter gene, which we previously demonstrated could be repressed by introduction of Groucho (6). Here the four different Tcf family members were co-transfected with β-catenin and increasing amounts of either *Grg-1*, *TLE-2*, *Grg3* or *Grg-4*. An expression construct of human *TLE-2* was included in stead of mGrg-2 for the latter had not been described at the time. Three human Tcf family members were used *hTcf-1*, *hLef-1*, *hTcf-4* (Figure 3A, B, and D respectively). For Tcf-3 we used the *Xenopus* ortholog (Figure 3C) because human *Tcf-3* proved difficult to express (data not shown). Identical transfections were carried out using the reporter with mutated Tcf sites. A *Renilla* luciferase vector was also transfected to correct for transfection efficiency. [Note that the B cell line used for these assays expresses moderate levels of both *Grg-1* and *Grg-4*. (6)] These experiments show that all different Grg proteins can repress transcriptional activation mediated by all Tcf family members. Grg-1 and Grg-4 seem to be more potent repressors than Grg3 and TLE-2. In this assay the Tcf proteins show differences in transactivation capacity: *hTcf-1* and *Lef-1* transactivate stronger than *XTcf-3* and *hTcf-4*, but for all Tcf family members repression is observed upon co-transfection with the different Groucho homologues.

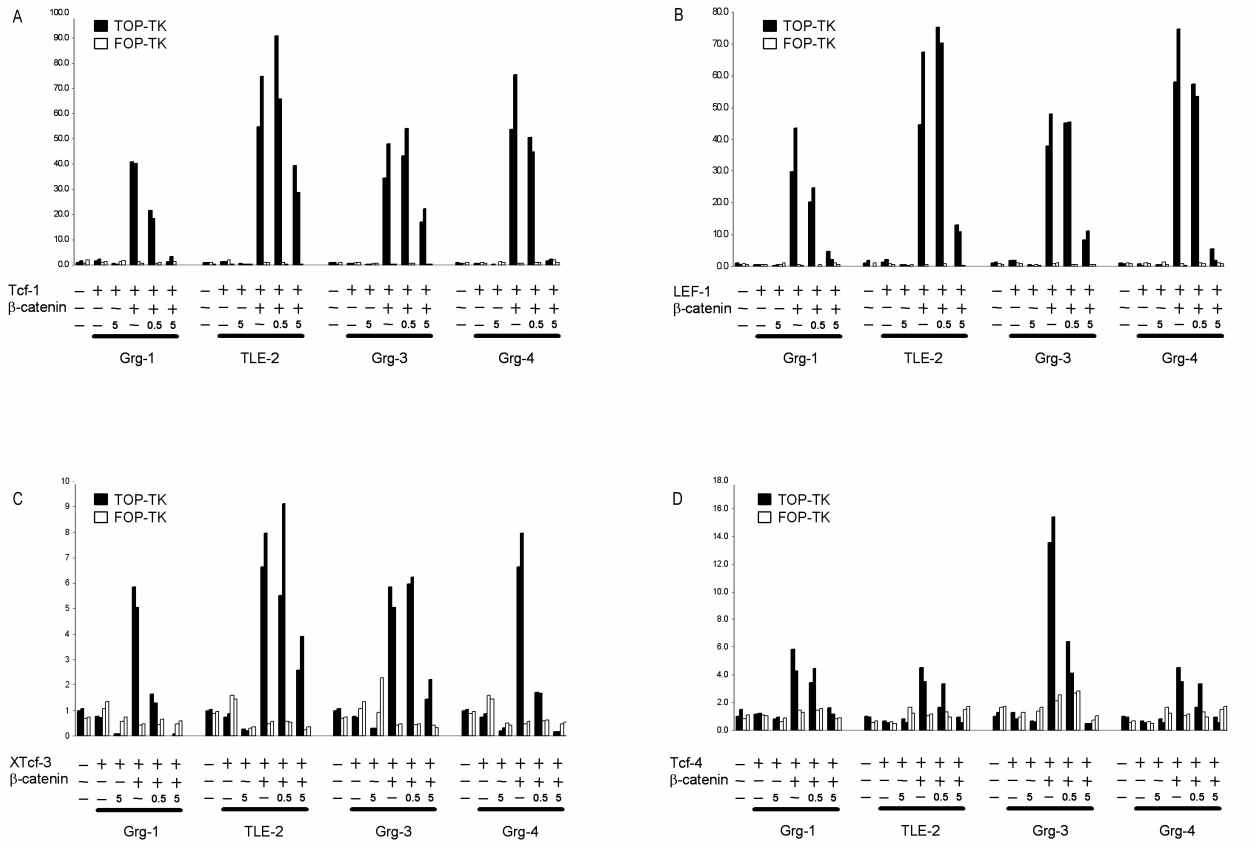


Figure 3. All 'long' Grg proteins can repress transactivation mediated by all Tcf family members.
Mouse B cell line IIAI.6 was co-transfected with the Tcf reporter construct (containing either wild-type Tcf binding sites, TOP-TK, or mutated Tcf binding sites, FOP-TK as a negative control) and a specific Tcf family member and β-catenin. This results in the transactivation and increase of luciferase activity. Upon addition of increasing amounts of any of the Grg family members, the luciferase activity decreases proportionally. Transfections were performed in duplicate, and results from one representative independent experiment are depicted in each case. luciferase values were corrected for the efficiency of transfection using the internal *Renilla* transfection control by determining the luciferase/Renilla ratio. This ratio is given on the Y-axis, and was arbitrarily set at 1 for the sample in which the TOP-TK reporter construct alone was transfected.

A. Repression of Tcf-1 mediated transcriptional activation by Grg-1, TLE-2, Grg-3 and Grg-4
B. Repression of Lef-1 mediated transcriptional activation by Grg-1, TLE-2, Grg-3 and Grg-4
C. Repression of Tcf-3 mediated transcriptional activation by Grg-1, TLE-2, Grg-3 and Grg-4
D. Repression of Tcf-4 mediated transcriptional activation by Grg-1, TLE-2, Grg-3 and Grg-4

Expression of the different Tcf and TLE family members in a panel of cell lines

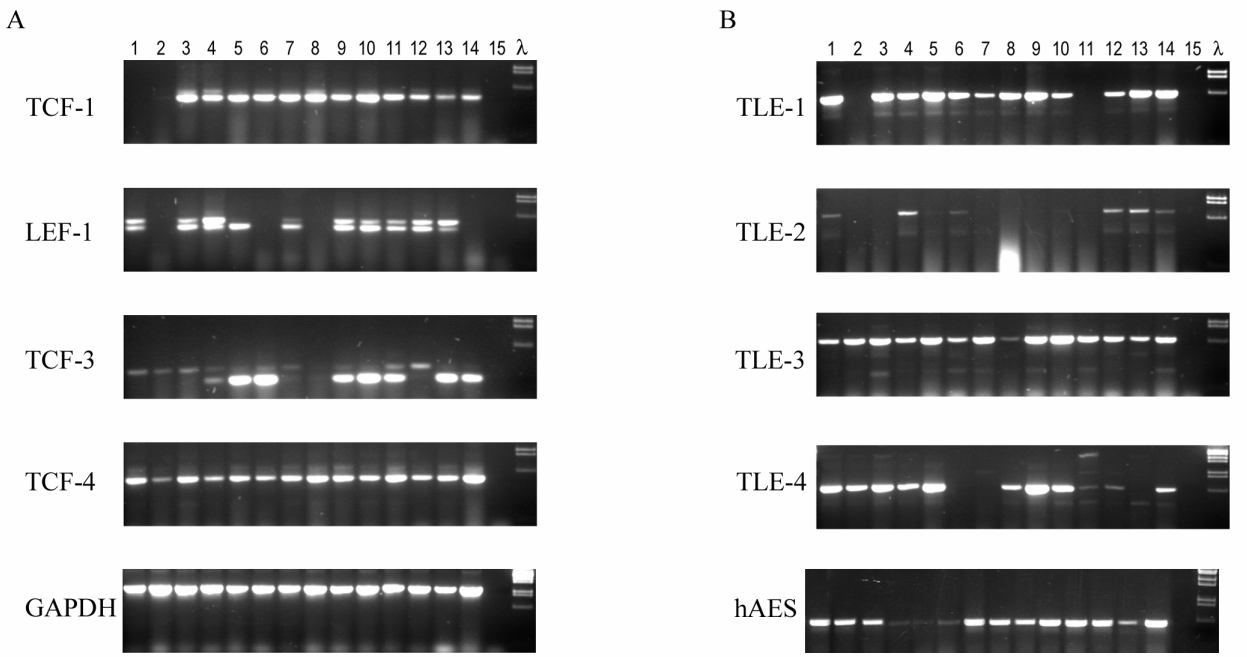
We examined whether there was any specificity in the interaction between Tcf and TLE proteins based on their expression pattern in a panel of cell lines. Two B cell lines, Reh and Raji and two T cell lines, Cem and Jurkat were tested, as well as a number of colon carcinoma cell lines (HCT116, LS174, HT29, DLD-1, SW480, SW620 and RKO) and two mammary carcinoma cell lines (T47D and SKBR3). In colon carcinoma cell lines the Wnt signaling cascade is constitutively activated. In all but one (RKO) of these colon carcinoma cell lines, mutations in either APC or β-catenin have been identified. Absence of TLE expression in the RKO cell line could potentially account for activation of the pathway in this cell line.

RNA was isolated from the above cell lines and first strand cDNA was prepared by reverse transcription (RT). This cDNA was used as a template in PCR to assay for the expression of the different Tcf and TLE family members (Figure 4). As a negative control, reverse transcriptase was omitted from the RT reaction. PCR for the different Tcf and TLE family members was carried out. A GAPDH PCR served as a internal control for the quality of the prepared cDNA. The PCR products were designed to be of different sizes to distinguish between the different family members. A product of each PCR was sequenced to confirm the identity of the amplified product and to exclude any cross reactivity between family members (data not shown).

Expression of *hTCF-1* was detected in all cell lines tested except in the B cell lines. In the cell lines where *hTCF-1* is expressed a very faint band was also observed running more slowly in the gel. This corresponds to a splice variant of *Tcf-1* containing an extra exon as described earlier (51). *hLEF-1* expression was found in Reh, CEM, Jurkat, 293T, LS174T, DLD-1, SW480, SW620, RKO and T47D. It was known previously to be expressed in pre-B and T cells and several other cell types (25), but apparently it is not expressed in all colon- or mammary carcinoma cell lines. The two different products present in most *hLEF-1* expressing cell lines correspond to the presence of two different splice variants (52) as was confirmed by sequencing (data not shown). *hTCF-3* expression was restricted to Jurkat, 293T, some colon carcinoma cell lines (HCT116, DLD-1, SW480 and SW620) and both mammary carcinoma cell lines T47D and SKBR3. For this family member a more restricted expression pattern was expected since *hTCF-3* is mainly expressed during early embryogenesis (27). *hTCF-4* is expressed ubiquitously in all cell lines tested.

The expression of the Groucho family members also showed a relatively broad pattern. Within this panel of cell lines, only the expression pattern of *hTLE-2* was restricted, being expressed in Reh, Jurkat, HCT-116, RKO, T47D and SKBR-3. For *hAES*, *hTLE-1*, *hTLE-3*and *hTLE-4* a broad expression pattern was observed. *hAES* is expressed in all cell lines tested but at a low level in Raji, 293T and T47D. *hTLE-1* is only absent in Raji and SW620 cell lines. *hTLE-4* is present in all cell lines of this panel with the exception of HCT116 and LS174T. *hTLE-3* is present in all cell lines in this panel. Corresponding with this broad expression of TLEs in cell lines, it has been shown that the TLE proteins are also broadly expressed in adult tissues (49).

These data show that a number of diverse cell lines all express multiple TCF and TLE family members. This indicates that the pattern of expression does not contribute to a possible specificity of interaction between Tcf and TLE molecules. It does imply, however, that two or more Groucho repressor proteins are normally available for a tight control over the TCF transcriptional activation pathway, since deregulation of this pathway can result in tumorigenesis, as has been shown for colon carcinoma (31) and melanoma (53).



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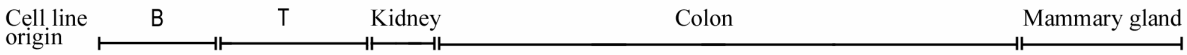

	Reh	Raji	CEM	Jurkat	293T	HCT116	LS174T	HT29	DLD-1	SW480	SW620	RKO	T47D	SKBR3
hTCF-1	-	-	+	+	+	+	+	+	+	+	+	+	+	+
hLEF	+	-	+	+	+	-	+	-	+	+	+	+	+	-
hTCF-3	-	-	-	+	+	+	-	-	+	+	+	-	+	+
hTCF-4	+	+	+	+	+	+	+	+	+	+	+	+	+	+
TLE-1	+	-	+	+	+	+	+	+	+	+	-	+	+	+
TLE-2	+	-	-	+	-	+	-	-	-	-	-	+	+	+
TLE-3	+	+	+	+	+	+	+	+	+	+	+	+	+	+
TLE-4	+	+	+	+	+	-	-	+	+	+	+	+	-	+
hAES	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Figure 4. Expression of *hTCF-1*, *hLEF-1*, *hTCF-3*, *hTCF-4* (A), *TLE-1*, *TLE-2*, *TLE-3*, *TLE-4* and *hAES* (B)
Expression was determined by RT PCR using cDNA generated from a panel of cell lines. Reh (lane 1), Raji (lane 2), CEM (lane 3), Jurkat (lane 4), 293T (lane 5), HCT116 (lane 6), LS174T (lane 7), HT29 (lane 8), DLD-1 (lane 9), SW480 (lane 10), SW620 (lane 11), RKO (lane 12), T47D (lane 13), SKBR3 (lane 14) and H_2O control for the PCR (lane 15). The lane marked with λ depicts the DNA marker (bacteriophage DNA digested with EcoRI and HindIII) Expression is tabulated for Tcfs and TLEs for the given cell lines. + visible RT-PCR product; -, no visible RT-PCR product

Domains of Grg required for repression of Tcf mediated transactivation and dependency on HDAC interaction

Deletion constructs of *Xenopus* Grg-4 (XGrg-4) were tested for their ability to repress Tcf mediated transcriptional activation (data not shown). The minimal construct of XGrg-4 required for repression consists of only the first two N-terminal domains. This truncation of XGrg-4, consisting of amino acids 1-197, encodes the Q domain, involved in tetramerization and interaction with Tcf, and the GP domain, essential for interaction with the histone deacetylase-1, HDAC (47). This construct is highly homologous to Grg-5, which is comprised of only the Q and GP domains, but functions as a de-repressor (6) (Figure 5A). In figure 5B, an alignment of the GP domains of mGrg-1, mGrg-3, XGrg-4 and mGrg-5 is depicted, showing the high homology between these proteins. The amino acids unique for Grg-5 are indicated in bold.
Cells were transfected with the Tcf reporter construct (TK-TOP), XTcf-3 and different amounts of Armadillo (the *Drosophila* homologue of β-catenin). XGrg-4-QGP consistently repressed transcription while Grg-5 enhanced the Armadillo mediated transactivation (Figure 5C.)

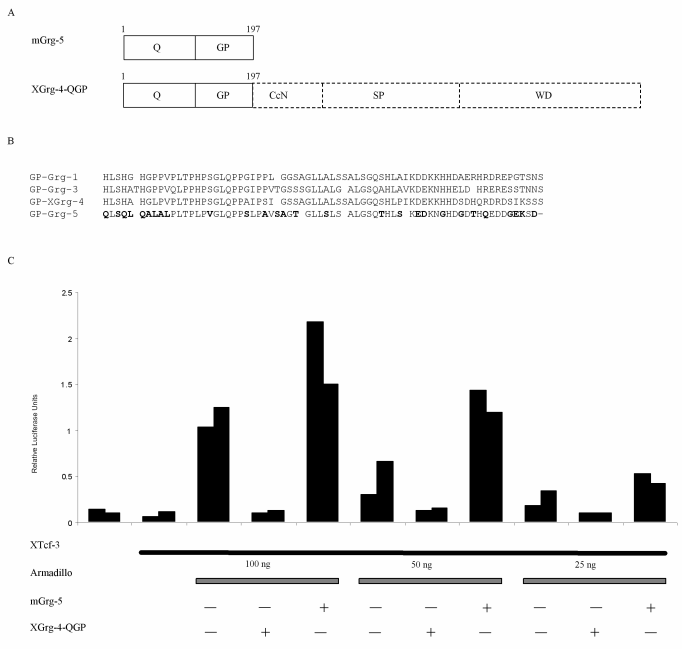

Figure 5. Comparison of Grg-5 with XGrg-4-QGP

A. The structure of the truncated XGrg-4 QGP molecule compared to that of Grg-5.

B. Alignment of the GP domains of long Grg-homologues with mGrg-5. The amino acid residues that are unique for mGrg-5 when compared to mGrg-1, mGrg-3 and XGrg-4 are depicted in bold.

C. XGrg-4-QGP functions as a repressor, but mGrg-5 functions as a de-repressor for Tcf mediated transcriptional activation. Mouse B cell line IIAI.6 was co-transfected with the Tcf reporter construct (TOP-TK), XTcf-3 and Armadillo (the *Drosophila* β-catenin homologue). This results in transactivation and increase in luciferase activity. This transactivation is de-repressed when Grg-5 is co-transfected, whereas the transactivation is repressed upon co-transfection with a truncated version of XGrg-4 that only contains the Q and GP domains. transfections were performed in duplicate and luciferase values were corrected for the efficiency of transfection using the internal *Renilla* transfection control pRNL-TK by determining the luciferase/*Renilla* ratio. This ratio is given on the y-axis

The GP domains of proteins of the Groucho family are highly conserved, and this domain is involved in interaction with HDAC (47). The subtle differences between the GP domain of the long Groucho homologs and the GP domain of the shorter variants like Grg-5 are possibly responsible for these antagonistic functions (Figure 5B). To address this, the interaction of HDAC with XGrg-4-QGP and mGrg-5 was studied in immunoprecipitation experiments (Figure 6). A Flag-HDAC-1 construct was co-transfected with the Myc-tagged Grg constructs of mGrg-5 and the truncated XGrg-4-QGP. Cell lysates were immunoprecipitated with anti-Myc antibody and protein A/G beads. The presence of HDAC was assayed on an immunoblot using the Flag antibody. Figure 6A shows that precipitation of Myc tagged Grg-5 and XGrg-4-QGP results in co-precipitation of HDAC with XGrg-4-QGP (lane 3) but not with Grg-5 (lane 4), although HDAC expression in the lysate of the cells co-transfected with Grg-5 and HDAC-1 is apparent (lane 5). Lanes 6 and 7 show that the two Myc tagged Grg constructs were immunoprecipitated with similar affinity. Similar results are obtained when Flag tagged HDAC is immunoprecipitated and the presence of mGrg-5 and XGrg-4-QGP is assayed (Figure 6B). Precipitation of Flag tagged HDAC co-precipitates XGrg-4-QGP but not Grg-5 (Figure 6B, lanes 3 and 4, respectively). Association of HDAC with full length Grg-3 was also observed (data not shown).

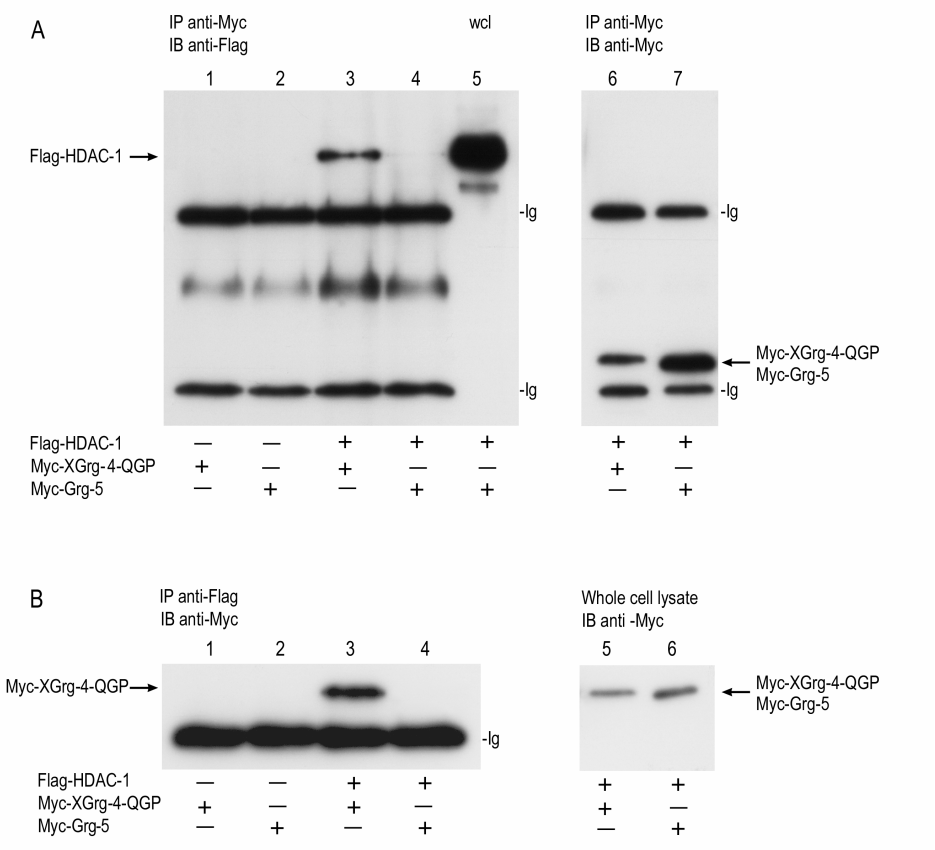

Figure 6. HDAC-1 associates with XGrg-4-Q-GP, but not with mGrg-5.
293T cells were transfected with either Myc-tagged XGrg-4-QGP or Myc-tagged mGrg-5 alone (lanes 1 and 2), or with a Flag-tagged HDAC-1 (lanes 3 and 4). Cell lysates were prepared 24 hours after transfection.

A. Extracts from transfected cells were immunoprecipitated (IP) with an anti-Myc antibody against Myc-tagged XGrg-4-QGP and Myc-tagged mGrg-5, and the presence of Flag-HDAC-1 in the immunoprecipitate was assayed on immunoblot (IB) with ant-Flag antibody. In lanes 1 and 2 only Myc-tagged XGrg-4-QGP or Myc-tagged mGrg-5 were transfected. In lanes 3 and 4 Myc-XGrg-4-QGP or Myc-Grg-5 were co-transfected with Flag-tagged HDAC-1. Lane 5 shows the presence of HDAC-1 in the co-transfection of Myc-Grg-5 and Flag-HDAC.
B. Extracts from transfected cells were immunoprecipitated (IP) with an anti-Flag antibody against Flag-HDAC-1. The presence of Myc-XGrg-4-QGP and Myc-Grg-5 was assayed ion immunoblot (IB) with anti-Myc antibody. In lanes 1 and 2 only Myc-XGrg-4-QGP or Myc-Grg-5 were transfected. In lanes 3 and 4, Myc-XGrg-4-QGP or Myc-Grg-5 were co-transfected with Flag-HDAC-1. Lanes 5 and 6 show an immunoblot with anti-Myc antibody, indicating the expression of Myc-XGrg-4-QGP or Myc-Grg-5 when co-transfected with Flag-HDAC-1.
Bands corresponding to HDAC, Grg-5, XGrg-4-QGP are indicated with arrows. Bands labelled "Ig" indicate the heavy and light chains of the antibodies used for the immune precipitation.

These data show that the long, repressive vertebrate Groucho homologs readily associate with the histone deacetylase HDAC-1 as was described previously in the fly (47). The de-repressor Grg-5, however, does not bind HDAC, explaining why Grg-5 does not function as a repressor.

Grg-5 may function to fine-tune the repression mediated by Groucho family members. In one possible model, Grg-5 would associate with long repressive Grg proteins and thereby decreases the amount of HDAC that is tethered to the template, reducing the level of repression. Alternatively, Grg-5 could bind directly to a subclass of transcription factors interacting with the first two domains of Groucho and prevent long Grg family members associating with such factors, abrogating repression entirely (Figure 7). *In vivo*, the precise activation status of a given transcription factor interacting with Groucho proteins would be partially determined by the mutual antagonism between long and short forms, the balance of which could be influenced by protein expression levels, sub cellular distribution or post-translational modifications of Groucho proteins.

We find no specificity in the interactions between the different members of the Tcf and Groucho families. Every cell line tested expresses several TLE and TCF family members. Further more, reporter assays reveal that any long Grg can repress any given Tcf, suggesting that all partners are capable of interacting *in vivo*. The abundance of Groucho molecules and the redundancy with which they interact with TCF proteins probably highlights the requirement to silence target gene expression adequately, in order to prevent unrestrained cell growth and/or aberrant differentiation, hall marks of cancer cells. It still remains a possibility that some specificity is achieved by other means not identified in this study. For example, TLE phosphorylation may influence their repressive activity, since CDC2 and Casein Kinase II consensus sites are found in the Grg/TLE sequences and it has previously been shown that the TLE phosphorylation pattern changes during differentiation (54).

The mechanism of Groucho mediated repression remained unclear until recently, when it was shown that Groucho functionally interacts with the histone de-acetylase Rpd3 (47). Recruitment of Rpd3 to a target promotor would result in modification of the local chromatin structure and, consequently, the formation of a more compact chromatin structure, associated with a repressed transcriptional state. This is likely the main mechanism through which Groucho related proteins exert their function, although other data indicate an additional mechanism of repression may exist. When different Grg constructs are tethered to a Gal4 DNA binding domain,

domains capable of mediating repression are also found outside the GP domain (41). Moreover, when Groucho mediated repression is assayed in the presence of the HDAC inhibitor Trichostatin A, some residual repression activity remains (47). When the histone deacetylase Rpd3 in *Drosophila* is mutated, only mild defects are observed (55). All these studies suggest that HDAC is an important, but not the sole, mediator of transcriptional repression. Further study of the mechanism of Groucho mediated repression will address these subjects in the future.

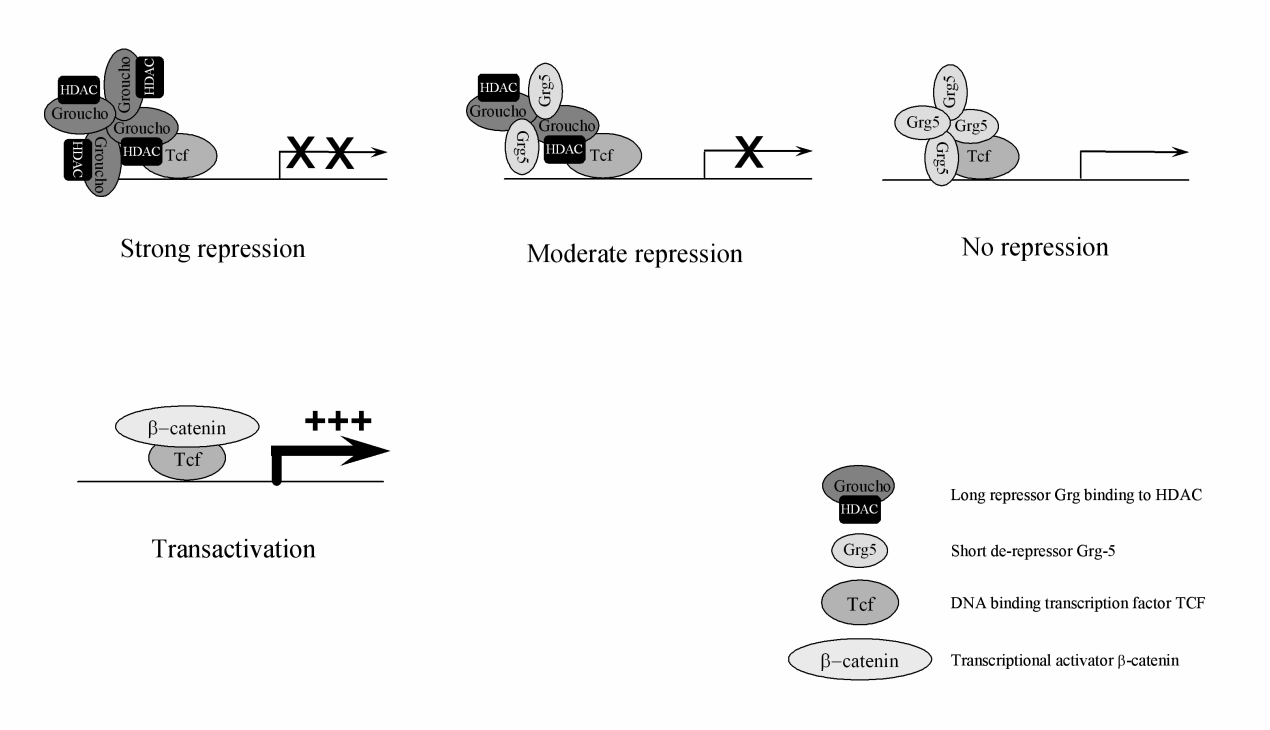

Figure 7. Model for Tcf , "long" Grg and Grg-5 functions.
Expression of Tcf target genes is activated when Tcf is associated with β-catenin and repressed when Tcf binds Groucho. Here we show that the function of Grg-5 provides another level of regulation. When in a complex, both the long repressor Grg and Grg-5 are bound to Tcf, a less competent repressor is formed, since less HDAC activity is tethered to the promotor. When Grg-5 replaces the longer repressor proteins entirely from the complex with Tcf, repression is absent because HDAC activity is lacking.

Reference list

1. Behrens, J., von Kries, J. P., Kuhl, M., Bruhn, L., Wedlich, D., Grosschedl, R., and Birchmeier, W. (1996). Functional interaction of beta-catenin with the transcription factor LEF-1. *Nature* 382: 638-642.

2. Molenaar, M., van de Wetering, M., Oosterwegel, M., Peterson Maduro, J., Godsave, S., Korinek, V., Roose, J., Destree, O., and Clevers, H. (1996). XTcf-3 transcription factor mediates beta-catenin-induced axis formation in Xenopus embryos. *Cell* 86: 391-399.

3. Van de Wetering, M., Cavallo, R., Dooijes, D., van Beest, M., van Es, J., Loureiro, J., Ypma, A., Hursh, D., Jones, T., Bejsovec, A., Peifer, M., Mortin, M., and Clevers, H. (1997). Armadillo coactivates transcription driven by the product of the Drosophila segment polarity gene dTCF. *Cell* 88: 789-799.

4. He, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., da Costa, L. T., Morin, P. J., Vogelstein, B., and Kinzler, K. W. (1998). Identification of c-MYC as a target of the APC pathway [see comments]. *Science* 281: 1509-1512.

5. Tetsu, O. and McCormick, F. (4-1-1999). Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature* 398: 422-426.

6. Roose, J., Molenaar, M., Peterson, J., Hurenkamp, J., Brantjes, H., Moerer, P., van de Wetering, M., Destree, O., and Clevers, H. (1998). The *Xenopus* Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors. *Nature* 395: 608-612.

7. Cadigan, K. M. and Nusse, R. (1997). Wnt signaling: a common theme in animal development. *Genes Dev.* 11: 3286-3305.

8. Behrens, J., Jerchow, B. A., Wurtele, M., Grimm, J., Asbrand, C., Wirtz, R., Kuhl, M., Wedlich, D., and Birchmeier, W. (1998). Functional interaction of an axin homolog, conductin, with beta- catenin, APC, and GSK3beta. *Science* 280: 596-599.

9. Nakamura, T., Hamada, F., Ishidate, T., Anai, K., Kawahara, K., Toyoshima, and Akiyama, T. (1998). Axin, an inhibitor of the Wnt signaling pathway, interacts with beta-catenin, GSK-3beta and APC and reduces the beta-catenin level. *Genes Cells* 3: 395-403.

10. Rubinfeld, B., Albert, I., Porfiri, E., Fiol, C., Munemitsu, S., and Polakis, P. (1996). Binding of GSK3beta to the APC-beta-catenin complex and regulation of complex assembly [see comments]. *Science* 272: 1023-1026.

11. Aberle, H., Bauer, A., Stappert, J., Kispert, A., and Kemler, R. (1997). beta-catenin is a target for the ubiquitin-proteasome pathway. *EMBO J.* 16: 3797-3804.

12. Kitagawa, M., Hatakeyama, S., Shirane, M., Matsumoto, M., Ishida, N., Hattori, K., Nakamichi, I., Kikuchi, A., and Nakayama, K. (5-4-1999). An F-box protein, FWD1, mediates ubiquitin-dependent proteolysis of beta-catenin. *EMBO J.* 18: 2401-2410.

13. Roose, J., Huls, G., van Beest, M., Moerer, P., van der, Horn K., Goldschmeding, R., Logtenberg, T., and Clevers, H. (9-17-1999). Synergy between tumor suppressor APC and the beta-catenin-Tcf4 target Tcf1. *Science* 285: 1923-1926.

14. He, T. C., Chan, T. A., Vogelstein, B., and Kinzler, K. W. (10-29-1999). PPARdelta is an APC-regulated target of nonsteroidal anti-inflammatory drugs. *Cell* 99: 335-345.

15. Bienz, M. (1998). TCF: transcriptional activator or repressor? *Curr.Opin.Cell Biol.* 10: 366-372.

16. Brannon, M., Gomperts, M., Sumoy, L., Moon, R. T., and Kimelman, D. (1997). A beta-catenin/XTcf-3 complex binds to the siamois promoter to regulate dorsal axis specification in Xenopus. *Genes Dev.* 11: 2359-2370.

17. Fan, M. J., Gruning, W., Walz, G., and Sokol, S. Y. (1998). Wnt signaling and transcriptional control of Siamois in Xenopus embryos. *Proc.Natl.Acad.Sci.U.S.A.* 95: 5626-5631.

18. Riese, J., Yu, X., Munnerlyn, A., Eresh, S., Hsu, S. C., Grosschedl, R., and Bienz, M. (1997). LEF-1, a nuclear factor coordinating signaling inputs from wingless and decapentaplegic. *Cell* 88: 777-787.

19. Waltzer, L. and Bienz, M. (1998). *Drosophila* CBP represses the transcription factor Tcf to antagonize Wingless signaling. *Nature* 395: 521-525.

20. Brannon, M., Brown, J. D., Bates, R., Kimelman, D., and Moon, R. T. (1999). XCtBP is a XTcf-3 co-repressor with roles throughout Xenopus development. *Development* 126: 3159-3170.

21. Cavallo, R., Cox, R., Moline, M., Roose, J., Polevoy, G., Clevers, H., Peifer, M., and Bejsovec, A. (1998). *Drosophila* Tcf and Groucho interact to repress Wingless signaling activiy. *Nature* 395: 604-608.

22. Levanon, D., Goldstein, R. E., Bernstein, Y., Tang, H., Goldenberg, D., Stifani, S., Paroush, Z., and Groner, Y. (9-29-1998). Transcriptional repression by AML1 and LEF-1 is mediated by the TLE/Groucho corepressors. *Proc.Natl.Acad.Sci.U.S.A.* 95: 11590-11595.

23. Oosterwegel, M., van de Wetering, M., Timmerman, J., Kruisbeek, A., Destree, O., Meijlink, F., and Clevers, H. (1993). Differential expression of the HMG box factors TCF-1 and LEF-1 during murine embryogenesis. *Development.* 118: 439-448.

24. Verbeek, S., Izon, D., Hofhuis, F., Robanus Maandag, E., te Riele, H., van de Wetering, M., Oosterwegel, M., Wilson, A., MacDonald, H. R., and Clevers, H. (1995). An HMG-box-containing T-cell factor required for thymocyte differentiation. *Nature* 374: 70-74.

25. Van Genderen, C., Okamura, R. M., Farinas, I., Quo, R. G., Parslow, T. G., Bruhn, L., and Grosschedl, R. (1994). Development of several organs that require inductive epithelial- mesenchymal interactions is impaired in LEF-1-deficient mice. *Genes Dev.* 8: 2691-2703.

26. Okamura, R. M., Sigvardsson, M., Galceran, J., Verbeek, S., Clevers, H., and Grosschedl, R. (1998). Redundant regulation of T cell differentiation and TCRalpha gene expression by the transcription factors LEF-1 and TCF-1. *Immunity.* 8: 11-20.

27. Korinek, V., Barker, N., Willert, K., Molenaar, M., Roose, J., Wagenaar, G., Markman, M., Lamers, W., Destree, O., and Clevers, H. (1998). Two members of the Tcf family implicated in Wnt/β-catenin signaling during embryogenesis in the mouse. *Mol.Cell.Biol.* 18: 1248-1256.

28. Barker, N., Huls, G., Korinek, V., and Clevers, H. (1999). Restricted high level expression of Tcf-4 protein in intestinal and mammary gland epithelium. *Am.J.of Pathology* 154:

29. Korinek, V., Barker, N., Moerer, P., van Donselaar, E., Huls, G., Peters, P. J., and Clevers, H. (1998). Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4. *Nat.Genet.* 19: 379-383.

30. Korinek, V., Barker, N., Morin, P. J., van Wichen, D., de Weger, R., Kinzler, K. W., Vogelstein, B., and Clevers, H. (1997). Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma [see comments]. *Science* 275: 1784-1787.

31. Morin, P. J., Sparks, A. B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B., and Kinzler, K. W. (1997). Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. *Science* 275: 1787-1790.

32. Fisher, A. L. and Caudy, M. (1998). Groucho proteins: transcriptional corepressors for specific subsets of DNA-binding transcription factors in vertebrates and invertebrates. *Genes Dev.* 12: 1931-1940.

33. Chen, G., Nguyen, P. H., and Courey, A. J. (1998). A role for Groucho tetramerization in transcriptional repression. *Mol.Cell Biol.* 18: 7259-7268.

34. Aronson, B. D., Fisher, A. L., Blechman, K., Caudy, M., and Gergen, J. P. (1997). Groucho-dependent and -independent repression activities of Runt domain proteins. *Mol.Cell Biol.* 17: 5581-5587.

35. Jimenez, G., Paroush, Z., and Ish Horowicz, D. (1997). Groucho acts as a corepressor for a subset of negative regulators, including Hairy and Engrailed. *Genes Dev.* 11: 3072-3082.

36. Dubnicoff, T., Valentine, S. A., Chen, G., Shi, T., Lengyel, J. A., Paroush, Z., and Courey, A. J. (1997). Conversion of dorsal from an activator to a repressor by the global corepressor Groucho. *Genes Dev.* 11: 2952-2957.

37. Eberhard, D., Jimenez, G., Heavey, B., and Busslinger, M. (5-15-2000). Transcriptional repression by Pax5 (BSAP) through interaction with corepressors of the Groucho family. *EMBO J.* 19: 2292-2303.

38. Choi, C. Y., Kim, Y. H., Kwon, H. J., and Kim, Y. (11-19-1999). The homeodomain protein NK-3 recruits Groucho and a histone deacetylase complex to repress transcription. *J.Biol.Chem.* 274: 33194-33197.

39. Choi, C. Y., Lee, Y. M., Kim, Y. H., Park, T., Jeon, B. H., Schulz, R. A., and Kim, Y. (10-29-1999). The homeodomain transcription factor NK-4 acts as either a transcriptional activator or repressor and interacts with the p300 coactivator and the Groucho corepressor. *J.Biol.Chem.* 274: 31543-31552.

40. Wang, J. C., Waltner-Law, M., Yamada, K., Osawa, H., Stifani, S., and Granner, D. K. (6-16-2000). Transducin-like enhancer of split proteins, the human homologs of Drosophila groucho, interact with hepatic nuclear factor 3beta. *J.Biol.Chem.* 275: 18418-18423.

41. Fisher, A. L., Ohsako, S., and Caudy, M. (1996). The WRPW motif of the hairy-related basic helix-loop-helix repressor proteins acts as a 4-amino-acid transcription repression and protein-protein interaction domain. *Mol.Cell Biol.* 16: 2670-2677.

42. Paroush, Z., Finley, R. L., Jr., Kidd, T., Wainwright, S. M., Ingham, P. W., Brent, R., and Ish Horowicz, D. (1994). Groucho is required for Drosophila neurogenesis, segmentation, and sex determination and interacts directly with hairy-related bHLH proteins. *Cell* 79: 805-815.

43. Jimenez, G., Verrijzer, C. P., and Ish, Horowicz D. (1999). A conserved motif in goosecoid mediates groucho-dependent repression in Drosophila embryos. *Mol.Cell Biol.* 19: 2080-2087.

44. Tolkunova, E. N., Fujioka, M., Kobayashi, M., Deka, D., and Jaynes, J. B. (1998). Two distinct types of repression domain in engrailed: one interacts with the groucho corepressor and is preferentially active on integrated target genes. *Mol.Cell Biol.* 18: 2804-2814.

45. Grbavec, D. and Stifani, S. (1996). Molecular interaction between TLE1 and the carboxyl-terminal domain of HES-1 containing the WRPW motif. *Biochem.Biophys.Res.Commun.* 223: 701-705.

46. Ren, B., Chee, K. J., Kim, T. H., and Maniatis, T. (1-1-1999). PRDI-BF1/Blimp-1 repression is mediated by corepressors of the Groucho family of proteins. *Genes Dev.* 13: 125-137.

47. Chen, G., Fernandez, J., Mische, S., and Courey, A. J. (9-1-1999). A functional interaction between the histone deacetylase Rpd3 and the corepressor groucho in Drosophila development. *Genes Dev.* 13: 2218-2230.

48. Chen, G. and Courey, A. J. (5-16-2000). Groucho/TLE family proteins and transcriptional repression. *Gene* 249: 1-16.

49. Stifani, S., Blaumueller, C. M., Redhead, N. J., Hill, R. E., and Artavanis-Tsakonas, S. (1992). Human homologs of a Drosophila Enhancer of split gene product define a novel family of nuclear proteins [published erratum appears in Nat Genet 1992 Dec;2(4):343]. *Nat.Genet.* 2: 119-127.

50. Mallo, M., Franco del Amo, F., and Gridley, T. (1993). Cloning and developmental expression of Grg, a mouse gene related to the groucho transcript of the Drosophila Enhancer of split complex. *Mech.Dev.* 42: 67-76.

51. Van de Wetering, M., Castrop, J., Korinek, V., and Clevers, H. (1996). Extensive alternative splicing and dual promoter usage generate Tcf-1 protein isoforms with differential transcription control properties. *Mol.Cell Biol.* 16: 745-752.

52. Carlsson, P., Waterman, M. L., and Jones, K. A. (1993). The hLEF/TCF-1 alpha HMG protein contains a context-dependent transcriptional activation domain that induces the TCR alpha enhancer in T cells. *Genes Dev.* 7: 2418-2430.

53. Rubinfeld, B., Robbins, P., El Gamil, M., Albert, I., Porfiri, E., and Polakis, P. (1997). Stabilization of beta-catenin by genetic defects in melanoma cell lines. *Science* 275: 1790-1792.

54. Liu, Y., Dehni, G., Purcell, K. J., Sokolow, J., Carcangiu, M. L., Artavanis Tsakonas, S., and Stifani, S. (1996). Epithelial expression and chromosomal location of human TLE genes: implications for notch signaling and neoplasia. *Genomics* 31: 58-64.

55. Mannervik, M. and Levine, M. (6-8-1999). The Rpd3 histone deacetylase is required for segmentation of the Drosophila embryo. *Proc.Natl.Acad.Sci.U.S.A* 96: 6797-6801.

CHAPTER 3

GENERATION OF MONOCLONAL ANTIBODIES RAISED AGAINST THE MEMBERS OF THE GROUCHO PROTEIN FAMILY

Helen Brantjes, Jeroen Kuipers, Mascha van Noort and Hans Clevers

Abstract

Groucho proteins comprise a family of very conserved transcriptional co-repressors, which are found in a broad range of species, ranging from mammals to *Xenopus* and *Drosophila*. In mouse there are five family members denoted Groucho-related genes: *Grg-1*, *Grg-2*, *Grg-3*, *Grg-4* and *Grg-5*. Groucho repressors do not bind DNA directly, but affect transcription by binding transcription factors. A large variety of transcription factors including Hairy related genes (1), Runt domain proteins (2) and TCF/LEF factors (3;4) are known to use Groucho proteins to mediate repression. Recently we demonstrated that the five Grg family members can all functionally interact with each of the four different TCF proteins (5). In an attempt to determine if there is any specificity in the interaction between TCF and Grg proteins on the basis of their expression patterns in embryonic mouse tissues, we raised monoclonal antibodies specific to Grg-1, Grg-2, Grg-3, Grg-4 and Grg-5.

Introduction

The family of Groucho proteins is a conserved family of transcriptional co-repressors. Members of this protein family are present in many species, including human, mouse, frog, zebra fish, chicken and worm. *Drosophila* Groucho, the founding member of these proteins, comprises five different domains (Figure 1): an amino terminal Q (Glutamine rich) domain, followed by a GP (glycin/proline rich) domain, a CCN domain (containing putative casein kinase II/cdc2 phosphorylation sites and nuclear localisation signal), a SP (serine/proline rich) domain and four WD40 repeats (protein interaction domain) (6). In *Drosophila*, Groucho is the single family member present, whereas four different five-domain-containing Groucho homologs are present in both human (TLE-1, -2, -3, -4, for transducin-like enhancer of split) (7) and mouse (Grg-1, -2, -3, -4, for Groucho related genes). In addition, a family member has been identified in humans, AES1 (amino terminal enhancer of split) and in mice, Grg-5 (8), that resembles a truncated variant consisting of only the first two N-terminal domains.

As co-repressors, Groucho proteins do not bind DNA directly, but are recruited to the promotors by interacting with a variety of DNA binding transcription factors to modulate their transcriptional activity. One class of Groucho interacting proteins are DNA binding factors that function solely transcriptional repressors where Groucho provides the repressive activity, e.g. Hairy related proteins (1;9), Engrailed (10;11), Brinker (12) and NK-3 and -4 (13;14) Alternatively, transcriptional activators, such as Dorsal (15), TCF/LEF factors (3;4) and PAX-5 (16) can be modified to become transcriptional repressors upon association with Groucho proteins.

Groucho proteins function as transcriptional co-repressors by binding to histone deacetylases (HDACs) (17,18). HDACs de-acetylate the N-terminal lysine tails of histones, restoring a positive charge which facilitates DNA wrapping around histone structures, thus condensing the chromatin structure of the DNA and making it less accessible for transcription factors. In general, decreased acetylation levels of chromatin are associated with repression of gene expression (19).

Previously, we described that all five mouse Groucho proteins can interact with each of the four different Tcf family members (5). Transcriptional activation by the individual TCF/LEF proteins can be repressed by each Grg protein. We raised monoclonal antibodies against the different mouse Grg proteins to determine if there is any specificity between the Tcf-Grg interactions based on expression patterns in the mouse embryo. In this report we describe the generation of mouse monoclonal antibodies specific for Grg-1, Grg-2, Grg-3, Grg-4 and Grg-5 and their functionality in several assays.

Materials and Methods

Cell lines and reagents
COS and 293T cell lines were routinely cultured in RPMI (Life Technologies), supplemented with 10% fetal calf serum, and antibiotics.

Production and purification of the His-tagged fusion proteins.
His-tagged proteins were constructed by cloning different PCR fragments into a pET 21 plasmid: mGrg-1 a PCR fragment corresponding to 765-1061 bp (tcttctcca…gcagcagct), mGrg-2 822-1123 bp (cctgtgacc…agcagctct), mGrg-3 601-993 bp (atgacggag…aagagtgac), mGrg-4 351-742 bp (atggcagaa…agagtgat) and mGrg-5 431-684 bp (acggctcct…aagtcggat). The bacterial strain BL21 was transformed with these recombinant plasmids and cultured in LB ampicillin medium (100 µg/ml) at 37^{o}C till an OD of 0.6. Isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM to induce production of the different histidine fusion proteins, the bacteria were cultured for an additional 3 hours. Bacteria were harvested by centrifugation for 15 minutes at 4000X *g* at 4^{o}C and the pellet was resuspended in 8 ml ice cold binding buffer (5 mM imidazole, 500 mM NaCl, 160 mM Tris HCl, pH 7.9). The suspension was sonicated for 10 minutes and subsequently centrifuged at 10.000 rpm for 45 minutes at 4 ^{o}C. The supernatant was passed over a Ni^{2+} column and the bound Grg-His fusion proteins were eluted in fractions of 1 ml of elution buffer (20 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH7.9)

Generation of mouse monoclonal antibodies
Six week old BALB/c mice were immunized by intraperitoneal injection of 100 µg of fusion protein in Freund's complete adjuvant (Difco) with a second injection in Freund's incomplete adjuvant (Difco) 14 days later. Three to five additional injections were performed with 100 µg fusion protein in PBS at weekly intervals. A mouse with titer 1/1000 was sacrificed, the spleen isolated and 1x 10^{8} splenocytes were fused with a ratio of 2:1 with SP2/0 mouse myeloma cells

using polyethylene glycol. The fused cell population was resuspended in hypoxanthine aminotropterin thymidine selection medium (Life) and plated into twenty 96-well flat bottom culture plates. Supernatants were screened 10-14 days after the hybridoma fusion. Positive hybridomas were repeatedly sub cloned to generate clonal hybridomas secreting monoclonal Grg-1, mGrg-2, mGrg-3, mGrg-4 and mGrg-5 antibodies.

Hybridoma screening assay

To screen the hybridoma supernatants, approximately $10x10^6$ COS cells were transiently transfected with 10 µg pCDNA3 vector containing Myc-mGrg-1, Myc-mGrg-2, Myc-mGrg-3, Flag-mGrg-4 or Myc-mGrg-5 constructs using DEAE Dextran, according to standard procedures. Cells were subsequently plated into 96-well flat bottom culture plates at a concentration of 10^4 per well. Cells were cultured for 48 hours, washed once with PBS, fixed with methanol and stored at -20°C. For screening the plates were washed with PBS and incubated with the hybridoma supernatants for 60 minutes. As a positive control staining with anti-Myc or anti-Flag antibody was used. Detection was carried out with peroxidase labelled rabbit-α-mouse horseradish peroxidase 1:100 (DAKO) and 0.02% aminoethylcarbonate / 0.1% hydrogen peroxide in 0.1 M sodium acetate, pH 4.8 as a colour substrate. Individual wells were examined for staining using an inverted microscope.

Western blotting

For detection of transfected Grg protein, approximately 0.3 x 10^6 293T cells were transfected with an expression vector (pCDNA3) for a full length Grg protein using fugene (Roche) according to manufacturer's instructions. 24 hours after transfection the cells were lysed in 200 µl of 2X SDS-PAGE sample buffer (15 mM 1 M Tris pH6.8, 5% SDS, 40% Glycerol, 0.005% BPB, 8% β-mercapto-ethanol), vortexed vigorously, denatured by heating at 98°C for 5 min and sonicated. 10 µl of protein extract was resolved on a 10% (w/v) denaturing polyacrylamide gel, and transferred to PVDF membrane (Immobilon-P, Millipore). The protein was detected by probing the blot for one hour in the hybridoma supernatant (cleared by centrifugation 15 minutes at 4000 *g*) followed by a horse-radish-peroxidase-conjugated rabbit anti-mouse IgG polyclonal antibody (Pierce), and finally visualized by enhanced chemiluminescence (Amersham).

Immunohistochemistry

Mouse embryos were harvested between time points E10.5 and E16.5 days, and fixed in NOTOX (Yvsolab) at 4°C. They were subsequently embedded in paraffin and sectioned at 5µm thickness. After de-paraffination, endogenous peroxidase activity was blocked by incubating the sections in 1.5% peroxide in methanol for 20 minutes. The sections were then boiled in 0.01 M citrate, pH 6.0 or in Tris/EDTA (40 mM/1mM, pH 8.0) buffer pH 8, for 20 minutes and cooled slowly. Before staining, the sections were blocked with 1% BSA in phosphate buffered saline (PBS) for 15 minutes. The sections were washed in PBS and incubated with hybridoma culture supernatant for 60 minutes. The sections were washed in PBS and the primary antibody was detected with Poly-HRP-goat-α-mouse IgG (Immunovision) and DAB solution.

Results and discussion

Mice were immunized with recombinant Grg proteins to generate monoclonal antibodies against the different Grg family members. The proteins fragments used for immunizations were carefully designed from unique regions to prevent cross reactivity between the antibodies against these highly related proteins (Figure 1). The chosen fragment of each cDNA was cloned into the pET21 vector and large amounts of protein were produced and purified using the HIS tag of the constructs. The Grg-2, -3, 4-, and -5 constructs readily produced several mg of protein, whereas several different constructs had to be tested before sufficient Grg-1 recombinant protein was obtained.

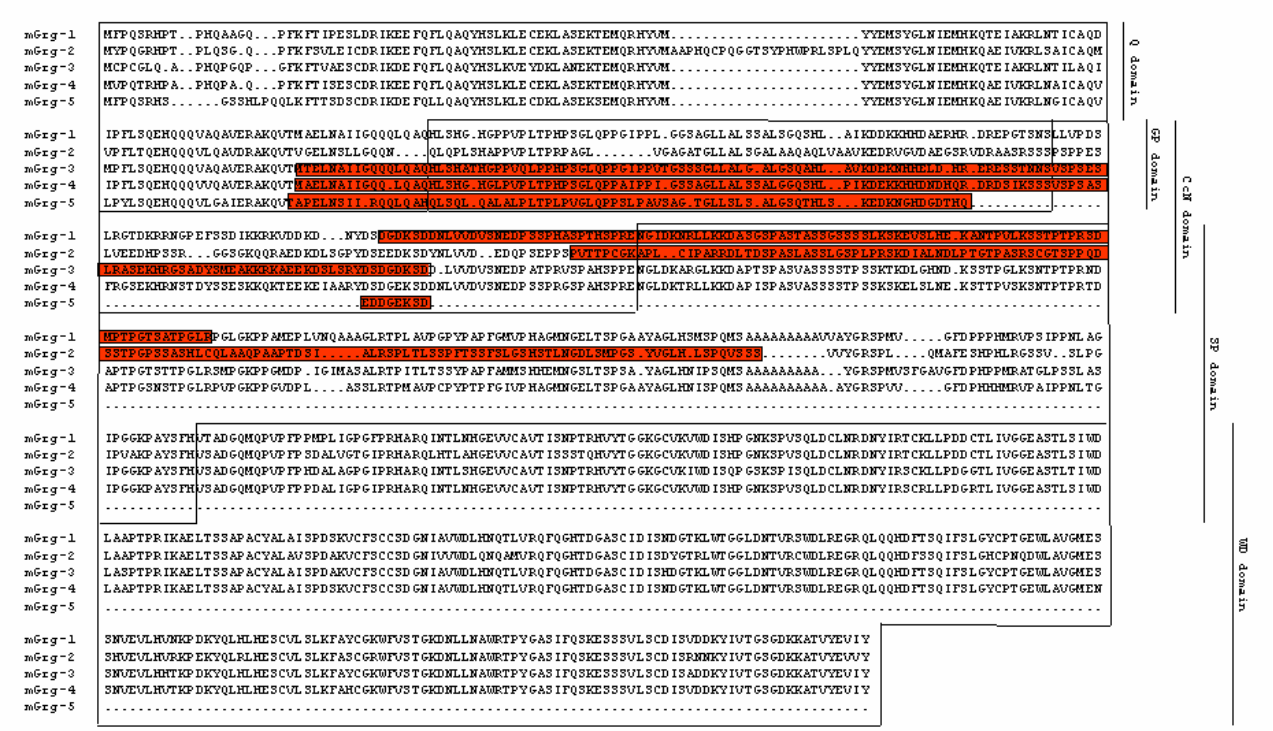

Figure 1. Protein alignment of the mouse Grg protein family members Grg-1, Grg-2, Grg-3, Grg-4 and Grg-5. In mouse there are five Groucho family members. The "long" variants Grg-1, -2, -3, and -4 contain of five distinct domains: Q, GP, CCN, SP and WD40 regions, whereas the short variant Grg-5 only consists of the two N-terminal domains, the Q and GP domains. The regions of the individual Grg proteins used to generate antibodies are indicated in the grey box.

The first fusion experiment of myeloma cell line SP2/0 with splenocytes from a mouse immunized with Grg-1 protein resulted in one clonal hybridoma cell line producing antibodies reactive with both Grg-1 and Grg-4 (data not shown). Another construct was designed, this time resulting in excess of 1700 hybridomas. Five of these were positive on COS cells transfected with mGrg-1. These were sub cloned and only two (42.2 and 44.1) were still positive for Grg-1 and did not cross react with Grg-4.

Fusion of the splenocytes of the Grg-2 immunized mice with the SP2/0 cell line yielded in excess of 1500 hybridomas. Fifteen of these supernatants reacted positively on mGrg-2 transfected COS cells. After sub cloning, two hybridomas (9E4 and 3D4) continued to specifically recognize Grg-2 in transfected COS cells.

The fusion experiment yielding monoclonal lines producing Grg-3 antibody, resulted in excess of 1500 hybridomas. Twenty-nine of these were positive in the first screen on mGrg-3 transfected COS cells. These were sub cloned, resulting in the generation of three monoclonal hybridoma lines, BG11, AA8 and CC3.

The fusion experiment for Grg-4 antibody producing lines resulted in excess of 1500 hybridomas. Eight were positive on mGrg4 transfected COS cells and after sub cloning 6 lines remained which produced anti-Grg-4 antibodies (GE11B4, NB886, F11B4, GR11C7F6, FB8C11D9, DD9CF05).

The fusion between the SP2/0 cell line and splenocytes from mice immunized with mGrg-5 protein yielded 1800 hybridomas, of which 2 were positive. These were sub cloned, resulting in two monoclonal lines (MG3 and KF12).

Characterization of the specificity of the monoclonal antibodies directed against the Grg proteins

The supernatants were tested on COS cells transfected with Myc-Grg-1, Myc-Grg-2, Myc-Grg-2, Myc-Grg-3, Flag-Grg-4 and Myc-Grg-5 to determine the specificity of the monoclonal antibodies produced by these hybridomas. The antibodies were shown to be specific for the Grg family member they were raised against. (Figure 2.)

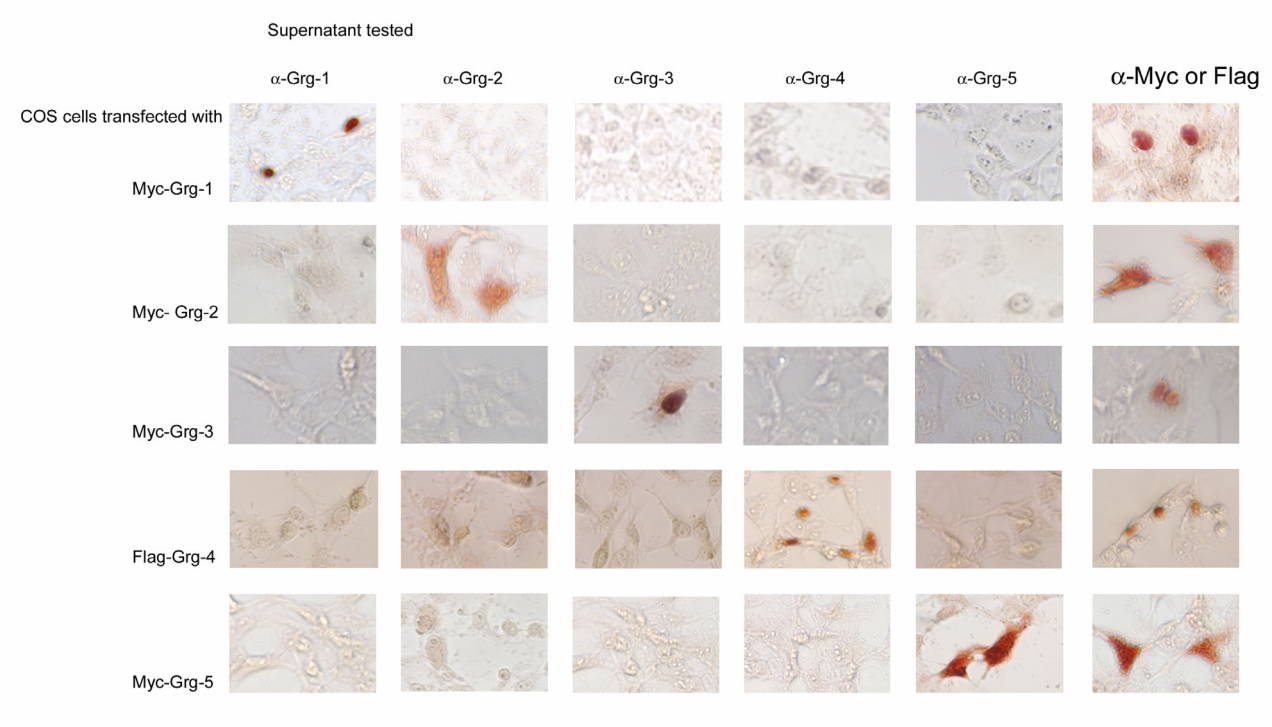

Figure 2. Characterization of the monoclonal antibodies specificity on transfected COS cells.
COS cells were transfected with Flag or Myc- tagged expression vectors of the different Grg family members, Myc-Grg-1 (row 1), Myc-Grg-2 (row 2), Myc-Grg-3 (row 3), Flag-Grg-4 (row 4) and Myg-Grg-5 (row 5). Supernatant with the monoclonal antibodies raised against the different Grg proteins were used to stain these COS cells, anti-Grg-1 in column 1, anti-Grg-2 in column 2, anti-Grg-3 in column 3, anti-Grg-4 in column 4 and anti-Grg-5 in column 5. The transfected COS cells were stained with the Myc or Flag antibody to control for expression of the Grg proteins (column 6).

The hybridomas were subsequently tested for reactivity and specificity by Western blot. 293T cells were transfected with tagged versions of the cDNAs for the different Grg proteins. A blot with lysates of Grg-1, Grg-2, Grg-3 and Grg-4 was incubated with the selected supernatants. Cross reactivity with the much smaller Grg-5 protein was determined on a separate blot. All supernatants for the Grg proteins were found to be reactive in Western blot and exhibited no cross reactivity (Figure 3.) The antibodies were also tested for detection of endogenous protein in mouse tissue extracts, but without success. Possibly the expression levels of endogenous proteins were too low to be detected in this way.

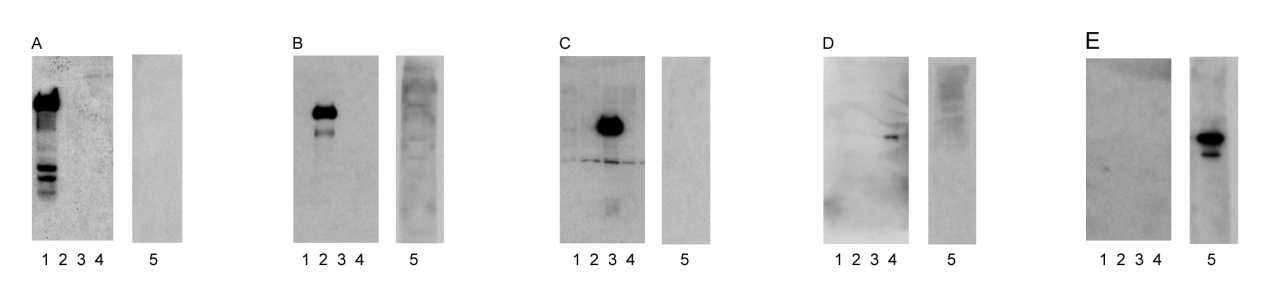

Figure 3. The antibodies against the mouse Grg proteins are specific for the different family members on Western blot.

Cell extracts from 293T cells transfected with Myc-Grg-1 (lane 1), Grg-2 (lane 2), Myc-Grg-3 (lane 3), Flag Grg-4 (lane 4) and Myc Grg-5 (lane 5) were analyzed by Western Blot with the supernatants containing the Grg monoclonal antibodies α-Grg1 (A), α-Grg-2 (B), α-Grg3 (C), α-Grg-4 (D), α-Grg-5 (E)

Immunohistochemistry with the monoclonal antibodies directed against the Grg family members

The monoclonal antibodies were initially designed to determine the expression pattern of the different Grg proteins in mouse tissues. The supernatants, and later the purified versions, were therefore tested on immunohistochemistry. Only the antibody against Grg-3 worked in this assay (Figure 4). This protein demonstrated ubiquitous nuclear expression. Several variations of the procedure were tested for the other antibodies, including the use of different fixatives (formalin or NOTOX or frozen sections) different antigen retrieval methods, different secondary antibodies. However, none of the other antibodies showed any specific staining using these methods.

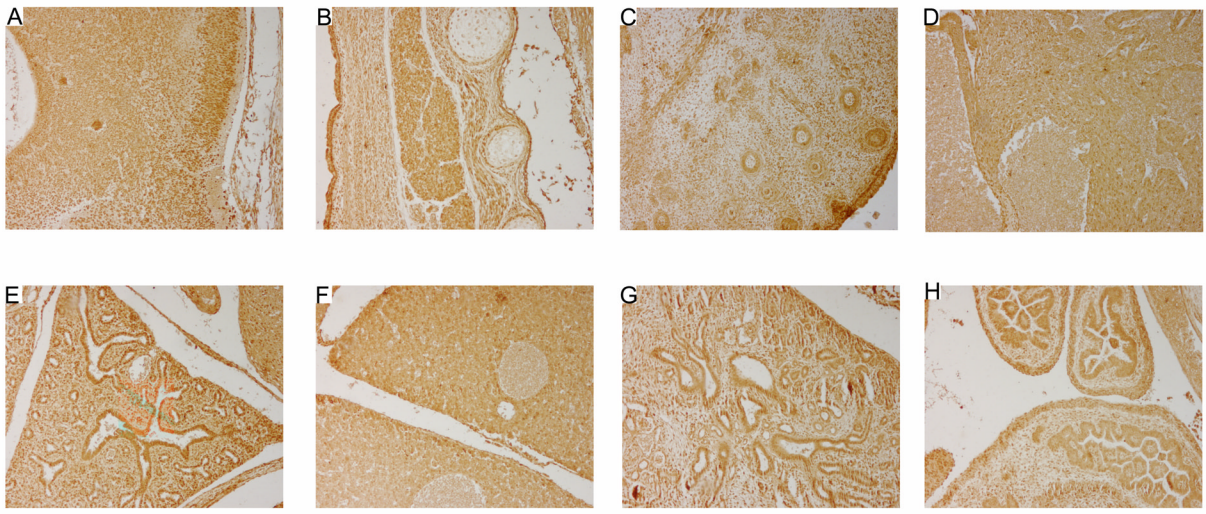

Figure 4. Grg-3 expression in the mouse embryo

Day 16 mouse embryo tissue sections were stained with the Grg-3 antibody, showing an ubiquitous expression of mGrg-3 in the different tissues, brain (A), notochord and cartilage (B), whiskers (C), heart (D), lung (E), liver (F), kidney (G) and colon (H).

The expression of the different Grg family members was determined by Northern Blot on mouse tissues, to get additional information on their expression pattern. RNA was isolated from several mouse organs: brain, thyroid gland, thymus, heart, lung, spleen, liver, kidney, stomach, small intestine, colon and testis. Northern blot experiments were carried out using probes for the different mGrg RNA's (Fig. 5). Grg proteins, like their human TLE counterparts (20), were quite ubiquitously expressed. However the expression pattern of Grg-2, was much more restricted, being found only in heart tissue.

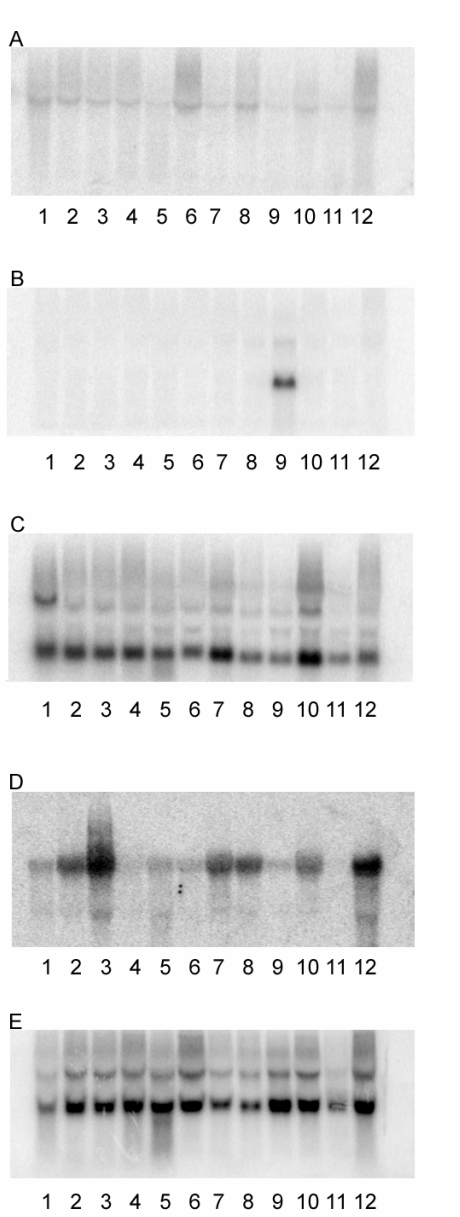

Figure 5. Expression of the different Grg family members in mouse tissues by Northern blot.
RNA was isolated from a selection of adult mouse tissues: testis (lane 1), colon (lane 2), small intestine (lane 3), stomach (lane 4), kidney (lane 5), liver (lane 6), spleen (lane 7), lung (lane 8), heart (lane 9), thymus (lane 10), thyroid gland (lane 11) and brain (lane 12). Probes specific for the different Groucho family members were used: Grg-1 (A), Grg-2 (B), Grg-3 (C), Grg-4 (D) and Grg-5 (E).

Here we present the generation of specific monoclonal antibodies against the five different members of the Grg protein family. These proteins are fully active when used to stain methanol fixed transfected cells and in western blot procedures on transfected proteins. Only the mGrg-3 showed activity in immunohistochemistry, showing that the protein is ubiquitously expressed at a high level. Regrettably, the other Grg antibodies are not appropriate for immunohistochemistry, so the expression patterns of the different Grg proteins in mouse tissues could not be revealed. To address this issue alternative techniques can be applied such as *in situ* hybridization. The specific expression of Grg-2 as revealed by Northern blotting might indicate a unique role for Grg-2 in heart tissue and could be interesting to be studied in more detail.

However, the antibodies described here will be useful in biochemical experiments. They could be used to confirm the interaction between Groucho and novel binding partners or they could be instrumental to study the mechanism whereby Grg proteins function. Grg proteins occur in large protein complexes with transcription factors, HDACs, possibly containing proteins of the Sir-3 repressor complex (21). The exact composition of this complex could be determined into more detail using the Grg antibodies in large scale protein purification experiments. Moreover, it has been described that not all repression conferred by Groucho can be explained by HDAC activity, since the HDAC inhibitor TSA can only partially block Groucho mediated repression (17) and there are also domains outside the HDAC interaction domain that contain repression activity (9). By purifying this so called repressosome, additional novel components involved in Groucho mediated repression could be identified.

Reference list

1. Paroush, Z., Finley, R. L., Jr., Kidd, T., Wainwright, S. M., Ingham, P. W., Brent, R., Ish Horowicz, D. (1994) Groucho is required for Drosophila neurogenesis, segmentation, and sex determination and interacts directly with hairy-related bHLH proteins. *Cell* 79, 805-815

2. Aronson, B. D., Fisher, A. L., Blechman, K., Caudy, M., Gergen, J. P. (1997) Groucho-dependent and -independent repression activities of Runt domain proteins. *Mol.Cell Biol.* 17, 5581-5587

3. Roose, J., Molenaar, M., Peterson, J., Hurenkamp, J., Brantjes, H., Moerer, P., van de Wetering, M., Destree, O., Clevers, H. (1998) The *Xenopus* Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors. *Nature* 395, 608-612

4. Levanon, D., Goldstein, R. E., Bernstein, Y., Tang, H., Goldenberg, D., Stifani, S., Paroush, Z., Groner, Y. (1998) Transcriptional repression by AML1 and LEF-1 is mediated by the TLE/Groucho corepressors. *Proc.Natl.Acad.Sci.U.S.A.* 95, 11590-11595

5. Brantjes, H., Roose, J., van De, W. M., Clevers, H. (2001) All Tcf HMG box transcription factors interact with Groucho-related co- repressors. *Nucleic Acids Res.* 29, 1410-1419

6. Fisher, A. L., Caudy, M. (1998) Groucho proteins: transcriptional corepressors for specific subsets of DNA-binding transcription factors in vertebrates and invertebrates. *Genes Dev.* 12, 1931-1940

7. Dajani, R., Fraser, E., Roe, S. M., Young, N., Good, V., Dale, T. C., Pearl, L. H. (2001) Crystal structure of glycogen synthase kinase 3beta. structural basis for phosphate-primed substrate specificity and autoinhibition. *Cell* 105, 721-732

8. Miyasaka, H., Choudhury, B. K., Hou, E. W., Li, S. S. (1993) Molecular cloning and expression of mouse and human cDNA encoding AES and ESG proteins with strong similarity to Drosophila enhancer of split groucho protein. *Eur.J.Biochem.* 216, 343-352

9. Fisher, A. L., Ohsako, S., Caudy, M. (1996) The WRPW motif of the hairy-related basic helix-loop-helix repressor proteins acts as a 4-amino-acid transcription repression and protein-protein interaction domain. *Mol.Cell Biol.* 16, 2670-2677

10. Jimenez, G., Paroush, Z., Ish Horowicz, D. (1997) Groucho acts as a corepressor for a subset of negative regulators, including Hairy and Engrailed. *Genes Dev.* 11, 3072-3082

11. Tolkunova, E. N., Fujioka, M., Kobayashi, M., Deka, D., Jaynes, J. B. (1998) Two distinct types of repression domain in engrailed: one interacts with the groucho corepressor and is preferentially active on integrated target genes. *Mol.Cell Biol.* 18, 2804-2814

12. Zhang, H., Levine, M., Ashe, H. L. (2001) Brinker is a sequence-specific transcriptional repressor in the Drosophila embryo. *Genes Dev.* 15, 261-266

13. Choi, C. Y., Kim, Y. H., Kwon, H. J., Kim, Y. (1999) The homeodomain protein NK-3 recruits Groucho and a histone deacetylase complex to repress transcription. *J.Biol.Chem.* 274, 33194-33197

14. Choi, C. Y., Lee, Y. M., Kim, Y. H., Park, T., Jeon, B. H., Schulz, R. A., Kim, Y. (1999) The homeodomain transcription factor NK-4 acts as either a transcriptional activator or repressor and interacts with the p300 coactivator and the Groucho corepressor. *J.Biol.Chem.* 274, 31543-31552

15. Dubnicoff, T., Valentine, S. A., Chen, G., Shi, T., Lengyel, J. A., Paroush, Z., Courey, A. J. (1997) Conversion of dorsal from an activator to a repressor by the global corepressor Groucho. *Genes Dev.* 11, 2952-2957

16. Eberhard, D., Jimenez, G., Heavey, B., Busslinger, M. (2000) Transcriptional repression by Pax5 (BSAP) through interaction with corepressors of the Groucho family. *EMBO J.* 19, 2292-2303

17. Chen, G., Fernandez, J., Mische, S., Courey, A. J. (1999) A functional interaction between the histone deacetylase Rpd3 and the corepressor groucho in Drosophila development. *Genes Dev.* 13, 2218-2230

18. Courey, A. J., Jia, S. (2001) Transcriptional repression: the long and the short of it. *Genes Dev.* 15, 2786-2796

19. De Ruijter, A. J., Van Gennip, A. H., Caron, H. N., Kemp, S., Van Kuilenburg, A. B. (2002) Histone deacetylases: characterisation of the classical HDAC family. *Biochem.J.* Pt,

20. Stifani, S., Blaumueller, C. M., Redhead, N. J., Hill, R. E., Artavanis-Tsakonas, S. (1992) Human homologs of a Drosophila Enhancer of split gene product define a novel family of nuclear proteins [published erratum appears in Nat Genet 1992 Dec;2(4):343]. *Nat.Genet.* 2, 119-127

21. Yochum, G. S., Ayer, D. E. (2001) Pf1, a novel PHD zinc finger protein that links the TLE corepressor to the mSin3A-histone deacetylase complex. *Mol.Cell Biol.* 21, 4110-4118

CHAPTER 4

PYGOPUS AND BCL-9,

TWO NOVEL COMPONENTS OF

THE WNT SIGNALING CASCADE

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Abstract

Two novel components of the Wingless signaling cascade in drosophila were recently identified in *Drosophila*, Pygopus and Legless (1, 2, 3, 4). Here we study the function of their mammalian homologs, hPYGOPUS-1, hPYGOPUS-2 and BCL-9.1 and BCL-9.2. We show that PYGOPUS-2 and BCL-9.2 show a ubiquitous expression pattern in a panel of cell lines, whereas PYGOPUS-1 and BCL-9.1 expression is more restricted. PYGOPUS and BCL-9 together enhance WNT signaling in a TCF reporter assay, only when the cascade is activated by WNT-1, not by any of the other stimuli tested, including co-transfection of β-catenin or dominant negative AXIN. Cellular localization studies suggest a role of BCL-9 in nuclear transport of β-catenin.

Introduction

WNT signaling controls multiple cell fate decisions in animal development (as reviewed in (5)), like embryonic segmentation, axis specification and the regulation of stem cell number and differentiation in epithelia, such as those of the skin and colon (6, 7). Initially the outline of this signaling route was identified in Drosophila genetic screens, but this pathway is conserved throughout the animal kingdom, from flies, worms, frogs, mice to man.

Activation of the WNT signaling pathway results in activation of downstream target genes, resulting in cell fate specification. Genetic and biochemical approaches have outlined the canonical WNT signaling pathway as we now know it (reviewed in (8)). At the heart of this cascade is the regulated stability of free, cytoplasmic β-catenin. In the absence of a WNT signal, β-catenin is targeted for degradation by the action of a multi-protein complex of glycogen synthase kinase 3β (GSK-3β), the scaffold protein Axin and the adenomatous polyposis coli (APC) tumour suppressor. This complex phosphorylates the N terminus of β-catenin and primes it for ubiquitination and subsequent degradation by the proteasome. When a WNT ligand binds to its receptors Frizzled and LRP, Dishevelled (DVL) inhibits the complex of Axin, APC and GSK-3 from phosphorylating β-catenin, resulting in the accumulation and nuclear translocation of β-catenin. In the nucleus, β-catenin binds the DNA binding factor TCF and this bi-partite transcription factor will activate target genes.

Deregulation of the WNT signaling cascade is associated with carcinogenesis. 80 % of the sporadic and hereditary colorectal tumours show inactivation of APC (9). Mutated APC is no longer able to down regulate β-catenin, causing free β-catenin to accumulate, translocate to the nucleus and aberrantly activate target genes. Among the 20 % colon carcinomas that have wild-type APC, about 5% have mutations in β-catenin (10). Typically, these mutations affect the GSK3 phosphorylation sites rendering β-catenin insensitive to degradation. These β-catenin mutations

are also found in cancers of many other tissues, including melanomas, gastric cancer and hair follicle tumours (11, 12).

It is well established that the formation of the β-catenin-TCF complex is the prerequisite for the activation of target genes. Some questions however still remain to be answered. For example, we do not know how the localization of β-catenin is controlled, how translocation to the nucleus is regulated when the cell receives a WNT signal and how β-catenin activity is dampened when WNT signaling decreases. The mechanism of β-catenin nuclear import is largely unknown. It was shown to be independent of the importins, the classical NLS receptors, and possibly mediated by direct interaction with the nuclear pore complex (13, 14, 15). This was all shown in *in vitro* permeabilized cells. Possibly, in live cells other mechanisms are involved. There is evidence that β-catenin constantly shuttles between the nucleus and the cytoplasm. Its localization would be determined by competition between constant export mediated by APC (16, 17) and nuclear retention by binding to TCF. To address these and other yet unidentified aspects of the WNT signaling cascade, new protein interaction screens and genetic screens have been carried out.

Pygopus (1, 2, 3, 4) and Legless (4) have recently been identified as two novel components of the Wingless (Wg) signaling pathway in *Drosophila* mutagenesis screens for dominant suppressors of Wg overactivation. Inactivation of these proteins rescues the phenotype that is caused by ectopic Wg signaling activation, either by ectopic expression of Wingless (1, 4, 3) or activated Armadillo (2). Both *Pygopus* and *Legless* mutant flies fail to transduce Wg signaling, as was shown by the loss of expression of Wingless target genes, like Distalless in the wing imaginal disk (2, 3, 4), Engrailed in the ventral epidermis (1), and labial in midgut development (1). This demonstrates that Pygopus and Legless are essential for Wingless signal transduction *in vivo*. No effects were observed on the expression levels of several EGF and Hedgehog target genes in Pygopus or Legless mutant flies (2, 4), indicating that these protein are dedicated to the WNT signaling cascade, nor were any changes in localization or levels of Armadillo protein expression observed in the Pygopus mutant flies, signifying that Pygopus is not implicated in stabilization or localization of Armadillo (2, 1). Genetic epistasis experiments show that these proteins function downstream of Axin (1, 3) and Shaggy/GSK3 (4) and even downstream of a constitutively active form of Armadillo (4).

The Legless protein consists of 1464 amino acids and reportedly contains three homology domains, HD1, HD2 and HD3, of which the HD1 domain mediates binding to Pygopus and the HD2 domain is the interaction domain for β-catenin (4). *Drosophila* Pygopus is comprised of 815 amino acids and contains two conserved domains, an N-terminal homology domain (NHD) and a PHD domain at the c-terminus, which mediates interaction with Legless (4). PHD domains are frequently found in proteins involved in chromatin remodelling (18, 19), which would hint at a possible function of Pygopus in β-catenin-Tcf mediated target gene activation, although also the PHD domains have also been described to function as a ubiquitine ligase or as a more general protein scaffold (20, 21). Both Legless (4) and Pygopus (3, 1) proteins show a nuclear localization upon over-expression in mammalian cells. The mammalian homolog of Legless BCL-9, PYGOPUS, β-catenin and TCF are found in one multi-protein complex in *in vitro* translation experiments.

A model was proposed where Legless functions to bring Pygopus to Tcf-β-catenin complex and affect target gene expression. The role of these proteins in the mammalian system has hardly been addressed. Pygopus and Legless have two mammalian family members each. The role of these proteins in WNT signaling will be studied more closely in this chapter.

Materials and methods

Cell lines and reagents
For transfection experiments the human embryonic kidney cell line, 293T, was used. For the Northern blot the following human cell lines were used as a source of RNA: Jurkat (T cell line), XGL and RPMI (B cell lymphoma lines), 293T, HT29, LS174, SW480, HCT116, DLD-1, RKO and CaCO2 (colon carcinoma cell lines). HEK-TR cell line (Invitrogen) was used for the RNAi experiments. Cells were cultured in RPMI (Life technologies), supplemented with 5% fetal calf serum and antibiotics.

Northern blot
Northern blot was performed by standard procedures. RNA from cell lines was isolated using the Chomczynski and Sacchi procedure (22). For each sample 10 µg RNA was loaded on a 1.2% agarose gel containing 2% formaldehyde. RNA was transferred to Zeta-Probe membranes (Biorad). Loading was checked by ethidium bromide staining. Hybridization was performed using ExpressHyb Hybridization Solution (Clontech). Probes were labelled using the RadPrime DNA labelling system (Invitrogen)

Transfections and Luciferase assays
0.3 x 10^6 293T cells were transfected using fugene (Roche) according to manufacturer's instructions. Cells were transfected with various combinations of a luciferase reporter plasmid containing three optimal Tcf sites upstream of the minimal HSV-TK promoter (100 ng of pTK-TOP) or its negative control vector containing mutated Tcf sites (pTK-FOP), an internal transfection control (50 ng of pRNL-TK, Promega) and one or more of the following expression constructs: hBCL-9.1; hPYGOPUS-2 expression vectors (50 ng -500 ng); WNT-1 expression plasmids (5 ng). The total amount of plasmid DNA transfected was made equivalent with pCDNA3. cDNAs encoding hBcl-9.1 and hPygopus2 (a kind gift from K. Basler) and their deletion mutants were expressed as Flag or Myc tagged versions in pCDNA3. Luciferase activities were determined 24 h after transfection using the DUAL luciferase system according to the manufacturer's protocol (Promega). Luciferase activity was normalised relative to RNL-luciferase activity. For every experiment transfections were performed in duplicate and several independent experiments were performed.

Immunoprecipitation and Western blotting
For immunoprecipitation experiments, 293T cells were co-transfected with Flag-hBCL-9.1-pCDNA3, and Myc-hPygopus2-pCDNA3 or β-catenin-pCi. Cells were harvested and lysed on ice for 30 minutes in IP lysis buffer (50 mM Tris pH 7.8, 150 mM NaCl, 5mM MgCl2, 0.1% Non-Idet-P40, 1mM dithiothreitol) containing protease inhibitors (Protease inhibitor cocktail tablets, Roche Diagnostics). After clearance the lysate was incubated overnight at 4^{o}C with 5 µl Flag coupled beads (Sigma), or with 5 µl Myc coupled beads (Sigma), or with 4 µl anti-β-catenin antibody (Transduction Labs) and 5 µl protein A/G beads (Santa Cruz). Immunoprecipitates were washed once with lysis buffer and three times in wash buffer (20 mM Tris pH 7.8, 100 mM NaCl, 1 mM EDTA and 0.1% Non-Idet-P40). Material bound to the beads was eluted in SDS-loading

buffer and resolved by SDS-PAGE gels, transferred on PVDF membrane (Biorad) The membrane was incubated with anti-Flag (Sigma), anti-Myc or anti-β-catenin antibodies and immune reactive proteins were visualised by enhanced chemiluminescence. (Amersham Pharmacia Biotech)

Tandem affinity (TAP) procedure

The TAP procedure was used to purify proteins associated with hPYGOPUS-2. For this use, a fusion protein was constructed between PYGOPUS-2 and the TAP tag at the c-terminus of the protein (the vector containing the TAP tag was kindly provided by Dr. J. van Es). 293T cells were transfected with this vector and harvested as described above. The vector only encoding the TAP tag was used as a negative control. The lysate was adjusted to IgG binding conditions (180 mM NaCl, 10 mM Tris pH 8, 0.2% NP-40 and 0.5 mM DTT). This was incubated over night at 0C with IgG beads (Amersham Bioscience) for 1st extraction of protein A binding proteins. IgG beads were washed twice with IgG binding buffer and once with TEV cleavage buffer (10 mM Tris pH 8, 150 mM NaCl, 0.3% NP-40, 0.5 mM EDTA, 0.5 mM DTT). The beads were resuspended in 1 ml TEV cleavage buffer and 150 U of TEV protease was added and incubated at 16 0C, mixing regularly for 2 hrs. Supernatant was collected and adjusted to calmodulin binding conditions (45 mM Tris pH 8, 150 mM NaCl, 0.7 mM MgAc, 0.7 mM Imidazole, 2.5 mM CaCl2, 0.2% NP-40, 10 mM β-mercapto-ethanol). 50 µl calmodulin affinity resin (Stratagene) was added and this was incubated for at least 2 hrs at 4^{o}C. The protein complexes with beads were washed three times with calmodulin binding buffer, and separated on SDS-polyacrylamide gels. Proteins were detected by silver stain, specific bands were cut from the gel and analyzed by mass spectrometry. (J. Krijgsveld, Utrecht)

RNAi studies

The doxycyclin regulated vector pTER, as recently described by Van de Wetering et al (23) as used to knock down Pygopus2 gene expression. The oligonucleotides used were as follows:
For oligo 5, 5'-
GATCCCTCCACCTGCTTCTACTGCTTTCAAGAGAAGCAGTAGAAGCAGGTGGATTTTT
GGAAA-3' and 5'-
AGCTTTTCCAAAAATCCACCTGCTTCTACTGCTTCTCTTGAAAGCAGTAGAAGCAGGT
GGAGG-3; for oligo 6, 5'-
GATCCCCGATGACCAGGATGCCATTTTCAAGAGAAATGGCATCCTGGTCATCGTTTTT
GGAAA-3' and 5'-
AGCTTTTCCAAAAACGATGACCAGGATGCCATTTCTCTTGAAAATTGGCATCCTGGTC
ATCGGG; for oligo7, 5'-
GATCCCGCTTCTGCCGTCTGGGCCTTTCAAGAGATGGCCCAGACGGCAGAAGCTTTTT
GGAAA-3' and 5'-
AGCTTTTCCAAAAAGCTTCTGCCGTCTGGGCCTTCTCTTGAATGGCCCAGACGGCAGA
AGCGG-3'

The HEK-TR line from Invitrogen was used to generate pTER stable cell lines. This cell line expresses the Tet-repressor, that can be switched off upon treatment of the cells with doxycyclin. HEK-TR cells were transfected with the pTER-Pygopus2 oligo's and zeocin resistant clones were selected for their ability to down regulate PYGOPUS-2 by Northern blotting.

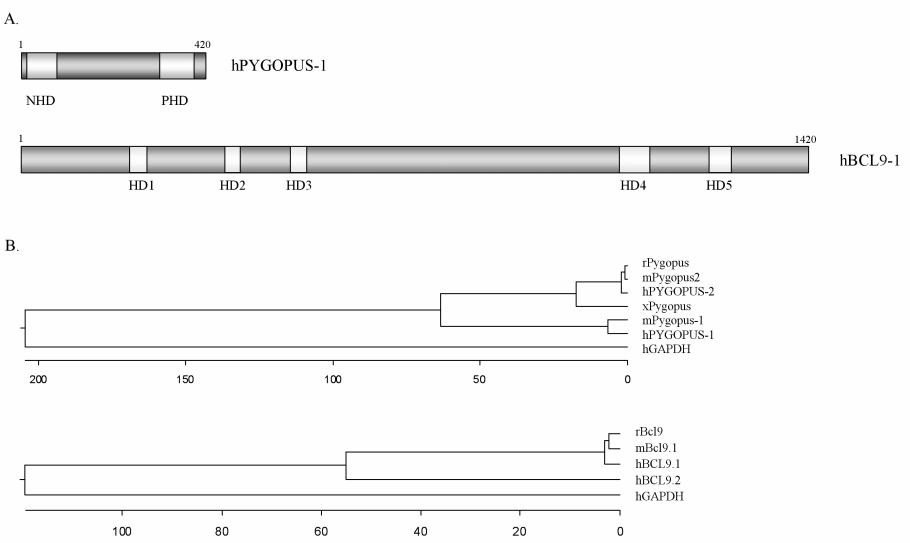
Immunofluorescence
0.3 x 10^6 293T cells were grown on a glass cover slip and transfected using fugene (Roche) with various combinations of the following expression constructs: Flag-BCL-9, GFP-5'-BCL-9, VSV-PYGOPUS, CFP-Pygopus, Myc-β-catenin. After 24 hours cells were fixed by adding 1/10 volume 37% formaldehyde directly to the culture medium. After 5 minutes at room temperature, cells were washed in PBS and directly embedded in Vectashield (Vector Laboratories Inc.) or used for antibody staining. For primary antibody the mouse anti-Flag (Sigma) or anti-Myc (8E9, monoclonal cell line) monoclonal antibody were used, these were detected either with anti-mouse IgG-FITC or anti-mouse IgG-FITC (Southern Biotechnology Associated).

Results

In mammals two proteins were identified that share high homology with *Drosophila* Pygopus, hPYGOPUS-1 and hPYGOPUS-2. These proteins are smaller than *Drosophila* Pygopus, but they also have the conserved NHD and PHD domains (Figure 1A). BCL-9, the putative mammalian homologue of *Drosophila* Legless, is hardly conserved on overall amino acid sequence, but clearly the conserved homology domains HD1, HD2 and HD3 can be identified (Figure 1A).

BCL-9 has initially been identified as the gene involved in the t(1;14) (q21;q32) translocation in the B cell lymphoma line CEMO (24). Chromosome 1q21-25 is one of the hotspots of chromosomal abnormalities including translocations and duplications in Non-Hodgkin's lymphoma and multiple myeloma. Rearrangements at this site are associated with tumour progression (reviewed in (25)). Nevertheless considerable promiscuity exists in the translocation partners with which 1q regions rearrange, BCL-9 is found in some cases, but also LHX4 (26), FCγRIIB (27), MUC1 (28) and IRTA1 and IRTA2 (29) are often found. This suggests that the deregulation of BCL-9 expression is probably not causing B cell lymphoma.

The two protein families of BCL-9 and PYGOPUS have been conserved throughout vertebrate evolution, given that homologous proteins are found in mouse, rat and frog (Figure 1B). The conserved domains are almost identical between the family members of different species (figure 1C), both for the PYGOPUS NHD and PHD domains and the HD domains of BCL-9. Moreover two previously unidentified conserved domains were recognized in the C-terminus of the BCL-9 proteins, HD4 and HD5 (Figure 1C).



C.

Bcl9 Homology domains

HD1 (28 aa)

hBcl9.1 (177-204)	VYVFSTEMANKAAEAVLKGQVETIVSFH
hBcl9.2 (240-267)	VYVFTTHLANTAAEAVLQGRADSILAYH
mBcl9.1 (265-292)	VYVFSTEMANKAAEAVLKGQVETIVSFH
rBcl9.1 (410-437)	VYVFSTEMANKAADAVLKGQVETIVSFH

HD2 (23 aa)

hBcl9.1 (349-371)	DGLSQEQLEHRERSLQTLRDIQR
hBcl9.2 (397-419)	EGLSKEQLEHRERSLQTLRDIER
mBcl9.1 (437-459)	DGLSQEQLEHRERSLQTLRDIQR
rBcl9.1 (582-604)	DGLSQEQLEHRERSLQTLRDIQR

HD3 (24 aa)

hBcl9.1 (467-490)	TPEQIAWLKLQQEFYEEKRRKQEQ
hBcl9.2 (515-538)	TPEQVAWRKLQEEYYEEKRRKEEQ
mBcl9.1 (554-577)	TPEQIAWLKLQQEFYEEKRRKQEQ
rBcl9.1 (799-822)	TPEQIAWLKLQQEFYEEKRRKQEQ

HD4 (52 aa)

hBcl9.1 (997-1048)	PEPTLSQNPLSIMMSRMSKFAMPSSTPLYHDAIKTVASSDDDSPPARSPNLP
hBcl9.2 (1075-1126)	PFPTPSQNPLSLMMTQMSKYAMPSSTPLYHNIAKTIATSDDELLPDRPLLPP
mBcl9.1 (1084-1135)	PEPTLSQNPLSIMMSRMSKFAMPSSTPLYHDAIKTVASSDDDSPPARSPNLP
rBcl9.1 (1219-1270)	PEPTLSQNPLSIMMSRMSKFAMPSSTPLYHDAIKTVASSDDDSPPARSPNLP

HD5 (32 aa)

hBcl9.1 (1223-1254)	EVIRPGATGIPEFDLSRIIPSEKPSQTLQYFP
hBcl9.2 (1312-1343)	EVIRPTPTGIPEFDLSRIIPSEKPSSTLQYFP
mBcl9.1 (1255-1286)	EVIRPGATGIPEFDLSRIIPSEKPSQTLQYFP
rBcl9.1 sequence not known yet	

Pygopus conserved domains

NHD domain (46 bp)

hPygopus1 (30-76)	VQLGSPDKKKRKANTQGPSFPPLSEYAPPPNPNSDHLVAANPFDDN
hPygopus2 (35-81)	LQMKSPEKKRRKSNTQGPAYSHLTEFAPPPTPMVDHLVASNPFEDD
mPygopus1 (30-76)	IQLGSPDKKKRKANTQGSSFPPLSEYAPPPNPNSDHLVAANPFDDS
mPygopus2 (35-81)	LQMKSPEKKRRKSNTQGPAYSHLTEFAPPPTPMVDHLVASNPFEDD
rPygopus2 (35-81)	LQMKSPEKKRRKSNTQGPAYSHLTEFAPPPTPMVDHLVASNPFEDD
XPygopus (22-68)	MQMKSPEKKRRKSNTQGPAYSHLSEFAPPPTPMVDHLVASNPFEDD

PHD domain (64 aa)

hPygopus1 (339-403)	PVYPCGICTNEVNDDQDIALCEASCQKWFHRICTGMTESAYGLLTAEASAVWGCDTCMADKDVQI
hPygopus2 (326-390)	LVYPCGACRSEVNDDQDIALCEASCQKWFHRECTGMTESAYGLLTTEASAVWACDLCLKTKEIQS
mPygopus1 (337-401)	PVYPCGICTNEVNDDQDIALCEASCQKWFHRICTGMTESAYGLLTAEASAVWGCDTCMADKDVQI
mPygopus2 (325-389)	LVYPCGACRSEVNDDQDIALCEASCQKWFHRECTGMTESAYGLLTTEASAVWACDLCLKTKEIQS
rPygopus2 (325-389)	LVYPCGACRSEVNDDQDIALCEASCQKWFHRECTGMTESAYGLLTTEASAVWACDLCLKTKEIQS
XPygopus (309-373)	LIYPCGACEREVNDDQDIALCEASCQKWFHRECTGMTESAYGLLTREVSAVWACDYCLKTKDIQS

Figure 1. Domain structure of BCL-9 and PYGOPUS proteins.

1A. Schematic representation of the BCL-9 protein with the conserved homology domains (HD) 1-5, and PYGOPUS with the N-terminal homology domain (NHD) and plant homology domain (PHD)

1B. BCL-9 and PYGOPUS are conserved proteins throughout the animal kingdom, homologues have been identified in human (h), rat (r), mouse (m) and frog (x).

1C. Alignment of the amino acid sequences of the conserved domains of BCL-9 and PYGOPUS proteins.

The expression patterns of PYGOPUS and BCL-9 were determined in a panel of human cell lines by Northern blot (Figure 2). Several colon carcinoma cell lines were tested (SW480, DLD-1, HT29, LS174, HCT116, RKO and CACO2), an embryonic kidney cell line (239T), a T cell line (Jurkat) and two B-cell lymphoma cell lines (XGL and RPMI). In colon carcinoma cell lines, the WNT signaling cascade is constitutively activated. In all but one of these (RKO) mutations in APC of β-catenin have been identified. Aberrant expression of PYGOPUS or BCL-9 might account for the activation of the pathway in the RKO cell line. The B cell lymphoma cell lines were used to determine if these would show over-expression of the BCL-9, as has been described for the cell line CEMO (24).

Expression of PYGOPUS-1 was detected only in the cell lines HT29, 293T, Jurkat and XGL, whereas PYGOPUS-2 showed a more broad expression pattern, being present in all cell lines tested. BCL-9.1 also showed expression, though weak in all cell lines tested, except for HT29, 293T, Jurkat and XGL where it is highly expressed. BCL-9.2 is highly expressed in all cell lines from this panel. The levels of PYGOPUS and BCL-9 in RKO cell line is not different from the other cell lines, indicating that aberrant expression levels of PYGOPUS and BCL-9 are not implicated in the elevated WNT signaling activity in this cell line. The expression of BCL-9.2 in the RPMI cell line and BCL-9.1 and BCL-9.2 in the cell line RPMI, might be slightly increased, but is not strongly over expressed as described for the CEMO cell line (24), indicating that these two cell lines probably have no activated BCL-9 as a result from a translocation t(1;14) (q21;q32).

These data show that at least one member of the PYGOPUS and one BCL-9 family is expressed in every cell line.

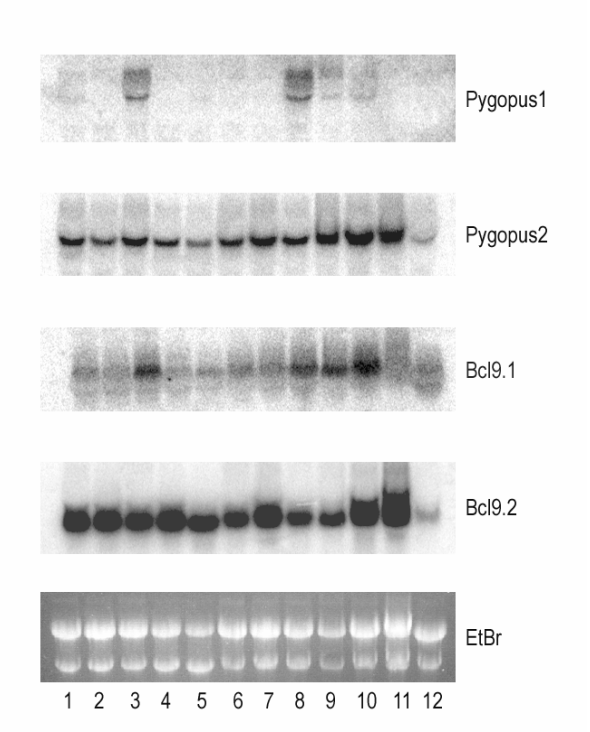


Figure 2A. The expression patterns of PYGOPUS-1, PYGOPUS-2, BCL-9.1 and BCL-9.2 in several human cell lines. Expression patterns were determined by Northern blot in a panel of human cell lines. SW480 (lane1), DLD-1(lane 2), HT29 (lane 3), LS147 (lane 4), HTC116 (lane 5), RKO (lane 6), CACO2 (lane7), 293T (lane 8), Jurkat (lane 9), XGL (lane 10), RPMI (lane 11).

Cell line	SW480	DLD1	HT29	LS174	HTC116	RKO	CACO2	293T	Jurkat	XGL	RPMI
PYGOPUS1	+/-	-	+	-	-	-	-	+	+/-	+/-	+/-
PYGOPUS2	+	+	+	+	+	+	+	+	+	++	++
BCL9.1	+/-	+/-	+	+/-	+/-	+/-	+/-	+	+	++	+/-
BCL9.2	++	++	++	++	++	+	++	+	+	+++	++++

Table 1. The expression of PYGOPUS-1, PYGOPUS-2, BCL-9.1 and BCL-9.2, is tabulated for the different cell lines. - , no visible signal, +/- weak signal, + signal, ++++ strong signal

To determine the role of PYGOPUS and BCL-9 in the mammalian WNT signaling cascade, the effect of over-expression of these proteins was determined in a β-catenin-TCF reporter gene assay (30). In this assay, transfection of Wnt-1 in 293T cells activates the WNT signaling pathway, resulting in transactivation of a luciferase reporter gene containing a promoter with optimal TCF sites (TOP-TK). As a negative control a luciferase reporter is used with mutated TCF sites (FOP-TK), which is not sensitive to activation of the WNT signaling cascade. Co-transfection of different amounts and combinations of expression constructs for *PYGOPUS-2* and *BCL-9.1* were used to determine the effect of these proteins on Wnt-1 mediated activation (Figure 3A.).

Transfection of PYGPOPUS alone does not affect WNT signaling. Upon transfection of BCL-9, only a marginal, 2 fold increase in TCF signaling is observed. Co-transfection of both PYGOPUS and BCL-9 results in an enhancement of WNT signaling of about 5-10 times. This was shown to be concentration dependent and specific for TOP-TK, since no effect was observed on FOP-TK.

The WNT signaling pathway in these 293T cells was activated in several ways, to determine where in the cascade PYGOPUS and BCL-9 exert their function. Activation of the cascade was carefully optimized to about 5-10 fold, ensuring that it still is possible to enhance the transactivation, since WNT signaling in 293T cells can be activated at least 50 fold with mutant β-catenin (data not shown). In figure 3B, WNT signaling was stimulated by addition of LiCl or by transfection of either wild-type or mutant β-catenin, lacking the S33 phosphorylation site rendering the protein insensitive to degradation. Activation of the cascade by these stimuli could not be enhanced by the combination of PYGOPUS and BCL-9, in contrast to the published (4) enhancement of stabilized β-catenin signaling by PYGOPUS alone. No enhancement by PYGOPUS and BCL-9 was detected when the cascade was activated by a dominant negative version of β-TRCP or a dominant negative version AXIN, or by DISHEVELLED (figure 3C), nor was any effect observed of BCL-9 and PYGOPUS single or co-transfection in colon carcinoma cell lines (data not shown). WNT signaling in these cell lines is constitutively activated as a consequence of β-catenin or APC mutation, but apparently PYGOPUS and BCL-9 can not enhance this any further.

These data show that BCL-9 and PYGOPUS can enhance β-catenin-TCF mediated signaling, but only when activation of the cascade is initiated by Wnt-1. Data from *Drosophila* show that Pygopus and Legless are genetically downstream of Axin, GSK-3β and stabilized Armadillo. This apparent discrepancy may reflect over-expression of BCL-9 and PYGOPUS in the TCF reporter assays as described here versus the inactivation of either protein in the *Drosophila* system.

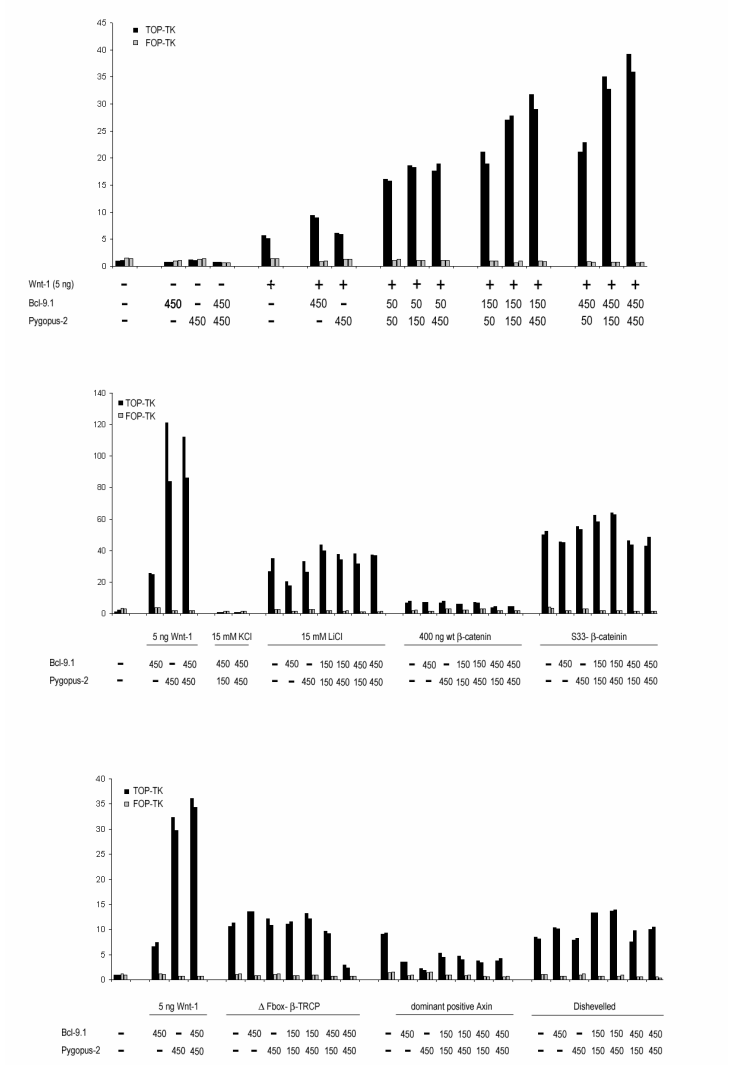


Figure 3. BCL-9 and PYGOPUS together enhance TCF mediated WNT signaling

239T cells were transfected with the TCF reporter construct (containing either wild type TCF binding sites, TOP-TK, or mutated TCF binding sites, FOP-TK, as a negative control), Renilla luciferase as a transfection efficiency control, WNT-1 and several combinations of BCL-9 and PYGOPUS expression constructs.

3A. Only co-transfection of PYGOPUS with BCL-9 enhances TCF signaling when the cascade is activated by Wnt-1 (5 ng)

3B. PYGOPUS and BCL-9 do not enhance TCF mediated transactivation when the cascade is activated with 15 mM LiCl or upon co-transfection with wild type β-catenin (400 ng) or mutated S33-β-CATENIN (25 ng)

3C. PYGOPUS and BCL-9 do not enhance TCF mediated transactivation when the cascade is activated with a dominant negative version of β-TRCP, dominant positive AXIN, or DISHEVELLED

To determine if the interactions for the *Drosophila* proteins also are found for human PYGOPUS and BCL-9, immunoprecipitation experiments were carried out. 293T cells transiently transfected with expression constructs for Flag-BCL9.1 and Myc-PYGOPUS-2. Cell lysates were immunoprecipitated with Myc-antibody coupled directly to sepharose beads and the presence of BCL-9 was assayed in Western blot using a the Flag antibody (Figure 4A). To exclude aspecific binding of Flag-BCL-9 to the beads, a negative control was included with lysates of cells only transfected with Flag-BCL-9 that were also immunoprecipitated with Myc-coupled beads. The Flag-BCL9 protein is readily expressed, when the whole cell lysate (WCL) is analyzed on Western blot. Flag-BCL-9 is also immunoprecipitated efficiently with Flag antibody. Only when Myc-Pygopus is co-transfected Flag-BCL-9 is present in the Myc immunoprecipitate.

Similar results are obtained in the reverse experiment, when Flag-BCL-9 is immunoprecipitated and the presence of Myc-PYGOPUS is assayed (Figure 5B). The molecular weight of Myc-PYGOPUS is about 50 kDa. A biotinilated anti-Myc antibody was used in the Western blot to prevent the rabbit-anti-mouse-HRP secondary antibody crossreacting with the immunoglobulin heavy chain from the antibody used in the immunoprecipitation. This was detected with streptavidin-coupled HRP, that does not cross react with the immunoglobulin. The Myc-PYGOPUS is expressed (WCL) and immunoprecipitated efficiently (IP a-Myc), and only upon presence of Flag-BCL-9 is Myc-PYGOPUS present in the immunoprecipitate using the Flag antibody (Figure 4B).

The interaction between BCL-9 and β-catenin was addressed by immunoprecipitation. Cells were transfected with Flag-BCL-9 with or without β-catenin, and lysates were precipitated with Flag coupled beads. The precipitate was analyzed by Western blot (figure 4C). β-catenin is efficiently expressed (WCL), and immunoprecipitated (IP anti-β-catenin) and only when Flag-BCL-9 is present, β-catenin is present in the Flag-precipitation. Similar results were obtained when the reversed experiment was carried out. Lysates from cells with β-catenin with or without Flag-BCL-9 were used for immunoprecipitation with β-catenin antibody.

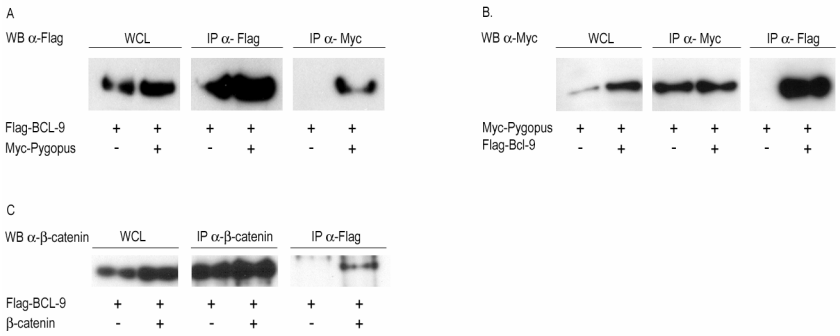


Figure 4. BCL-9 interacts with PYGOPUS and β-catenin.

Flag-BCL-9 and Myc-PYGOPUS (4A and 4B) or Flag-BCL-9 with Myc-β-catenin (4C) are co-transfected in 293T cells, 24 hours after transfection cells are harvested and lysed. Protein complexes were immunoprecipitated using anti-Flag or anti-Myc coupled beads and analyzed by Western blot (WB) of total expressed protein (WCL) and immunoprecipitated (IP) protein.

4A. Flag-BCL-9 and Myc-PYGOPUS co-immunoprecipitation with Myc antibody.

4B. Myc-PYGOPUS and Flag-BCL-9 co-immunoprecipitation with Flag antibody.

4C. Flag-BCL-9 and β-catenin, co-immunoprecipitation with Flag antibody.

Studies from *Pygopus* mutants in *Drosophila* (4, 2) show that both the PHD domain and the NHD domains of *Drosophila* Pygopus are essential for its function. The PHD domain is used for interaction with BCL-9 but the function of the NHD is unknown. We used the Tandem Affinity Purification (TAP) procedure to identify novel PYGOPUS interacting proteins (31, 32). This procedure is a tool to allow rapid purification of protein complexes under native conditions. The TAP-tag consists of two immunoglobulin-binding domains of protein A from Staphylococcus aureus, a cleavage site for the tobacco etch virus (TEV) protease, and the calmodulin binding peptide. Protein purification is achieved via two step affinity chromatography, carried out under native conditions. The TAP tag is fused to the protein of interest and expressed, the fusion protein and associated proteins are recovered from cell extracts by affinity selection on an IgG matrix. After washing, the TEV protease is added to release bound material. This leaves a bulk of non-specifically associated proteins on the beads, resulting in much less background compared with conventional precipitation methods. The eluate is incubated with calmodulin coated beads for a second affinity purification step and after washing the bound material can be eluted with EGTA.

The TAP tag was fused to the c-terminus of PYGOPUS-2. 293T cells were either stably or transiently transfected with this expression construct, cells were lysed in TX100-lysis buffer and TAP-PYGOPUS was purified from the extract using the described procedure. After binding to the calmodulin column the protein complexes were not eluted with EGTA, since this did not result in a very efficient recovery. Protein complexes on the calmodulin beads were directly eluted in SDS loading buffer and resolved on SDS-polyacrylamide gel and silver stained (Figure 5). No obvious difference was observed between the pattern of bands of associated proteins in the extracts from stably transfected or transiently transfected protein.

From several experiments bands of associated proteins were cut from silver stained gel and analyzed by mass spectrometry. Proteins identified using this procedure were the uncleaved Pygopus-TAP protein, often HSP70-1,-5,-8 were identified, but also BCL-9 was recovered from the precipitate (Figure 6). The presence of heat shock proteins is probably caused by the over-expression of TAP-PYGOPUS. Several different buffers were used for the preparation of the extract, also TAP-PYGOPUS was stably transfected into LS174 cell line and purified from these cell extracts, but no associated proteins other than heat shock proteins were identified, BCL-9 was not found in these purifications. Upon co-transfection of Wnt-1 with TAP-PYGOPUS, BCL-9 is still found associated with PYGOPUS, but no novel associated proteins were identified, as far as detectable on silver stain gel.

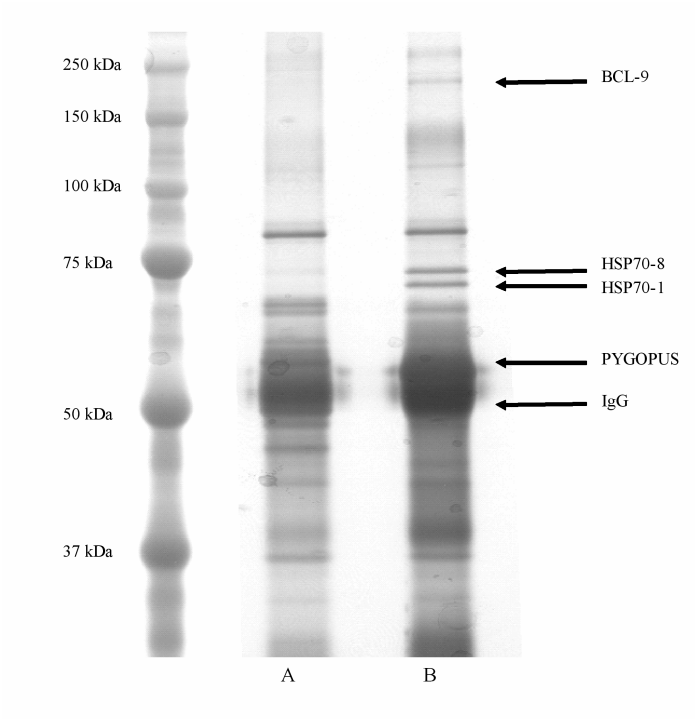


Figure 5. Purification of PYGOPUS associated proteins using the TAP procedure.
293T cells were transiently transfected with an expression vector for the TAP-PYGOPUS fusion protein (B) or the TAP expression vector alone as negative control (A). Protein complexes were isolated, purified and separated on denaturing protein gel and stained using silver stain. Specific bands of interacting proteins were cut from gel and analyzed by mass spectrometry.

To determine if PYGOPUS really is essential for WNT signaling, we used RNAi to inducibly knock down the protein in mammalian cells. For this we used RNAi oligo's expressed by the pTER system as recently described by Van de Wetering et al (23). The pTER vector contains Tet repressor sites between the Histone H1 promoter and the RNAi sequences. In cell lines expressing the Tet repressor protein, it will bind to its target sites inserted in the H1 promoter, resulting in a block of transcription. The cell lines used here express the tetracycline repressor under the control of doxycyclin: in absence of doxycycline, binding of the Tet repressor blocks the H1 promoter, and addition of doxycycline inhibits the binding of the Tet repressor to the H1 promoter, releasing transcription of the RNAi sequences.

The human embryo carcinoma cell line that expresses the Tet repressor under the control of doxycycline (HEK-TR) was used for the RNAi experiments to knock down PYGOPUS. This cell line was used because PYGOPUS and BCL-9 enhance TCF signaling in the related 293T cell line and not in the colon carcinoma cell lines with a constitutively activated WNT signaling pathway.

Seven RNAi oligo's for PYGOPUS-2 were developed and cloned into the pTER vector. The effectivity of these oligo's was first tested on transiently transfected Flag-PYGOPUS-2 in 293T cells. Cells were transiently transfected with 100 ng Flag-PYGOPUS and 1 µg of the RNAi oligo. After one, two and three days cells were harvested, lysed in TX100 lysis buffer and the

amount of Flag-PYGPOUS was determined by western blot, after correction for total amount of protein by Bradford.

Only three oligo's worked in this assay (Figure 6). One day after transfection no effect of the oligo's is visible, but after 2, and most obvious after 3 days the amount of PYGOPUS protein is strongly down regulated.

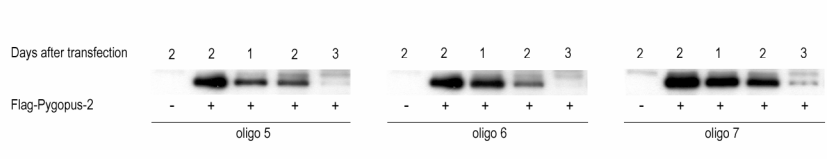


Figure 6. RNAi vectors for PYGOPUS-2 efficiently knock down transiently transfected protein.
293T cells were transfected with 100 ng Flag-tagged PYGOPUS-2 and 1 µg of either of the PYGOPUS-2 RNAi vector in pTER, 5, 6 or 7. Cells were harvested after 2 and 3 days of transfection. Flag-PYGOPUS-2 levels were determined on denaturing polyacrylamide, after correction for total protein amount with Bradford.

The RNAi oligo's 5, 6 and 7 were used to make stable transfectants in the HEK-TR cell line. Clones were selected that down regulate the PYGOPUS mRNA after induction with doxycyclin. Three clones were selected and induced with doxycycline for three days (Figure 7). Down-regulation of the PYGOPUS-2 mRNA is clearly observed in the lines with oligo 6 and 7, the line with oligo 5 only gives a moderate reduction of mRNA levels. Induction does not show any affect on the HEK-TR cell line.

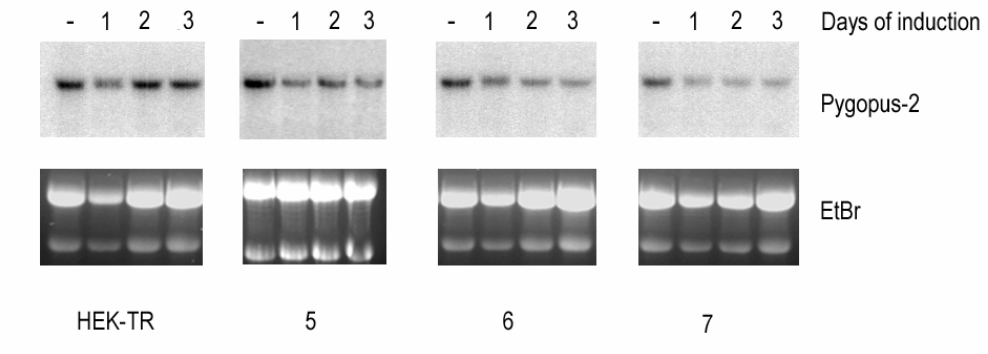


Figure 7. Stably integrated RNAi vectors for PYGOPUS-2 knock down the endogenous mRNA of PYGOPUS-2 in the HEK TR cell line
HEK-TR cells were stably transfected with three different RNAi oligo's in pTER, 5, 6 and 7. Cells were seeded and induced with doxycycline for a time course of three days. Each day cells were harvested, RNA was isolated and analyzed by Northern Blot. Ethidium bromide staining served as a loading control.

To determine if the down-regulation of PYGOPUS-2 in the HEK TR cell line has an affect on WNT signaling, the TOP/FOP reporter assay was used. The PYGOPUS RNAi cell lines were seeded and doxycyclin was added, after 1,2 and 3 days these were transfected with the TCF reporter (TK-TOP) or its negative control (TK-FOP) with or without WNT-1. As a negative control, cells were transfected similarly, but not induced with doxycycline. Also the HEK-TR cell line was included to exclude any non-specific effects (Figure 8). One day after transfection cells were lysed and luciferase activity was measured. Transfection of Wnt-1 in the PYGOPUS RNAi cell lines does result in an increase of β-catenin-TCF mediated transcriptional activation. Addition of doxycycline and subsequent down-regulation of the PYGOPUS mRNA however, has no effect on this WNT-1 mediated transcriptional activation. This could be caused by the fact that the PYGOPUS-2 mRNA is not completely down regulated after 3 days of induction (Figure 8). Moreover, PYGOPUS-1 still present in these cell lines, could be sufficient to maintain TCF signaling. Currently RNAi oligo's for PYGOPUS-1 are transfected in these cell lines in an attempt to down regulate both PYGOPUS messages and to be able to observe an effect on WNT signaling.

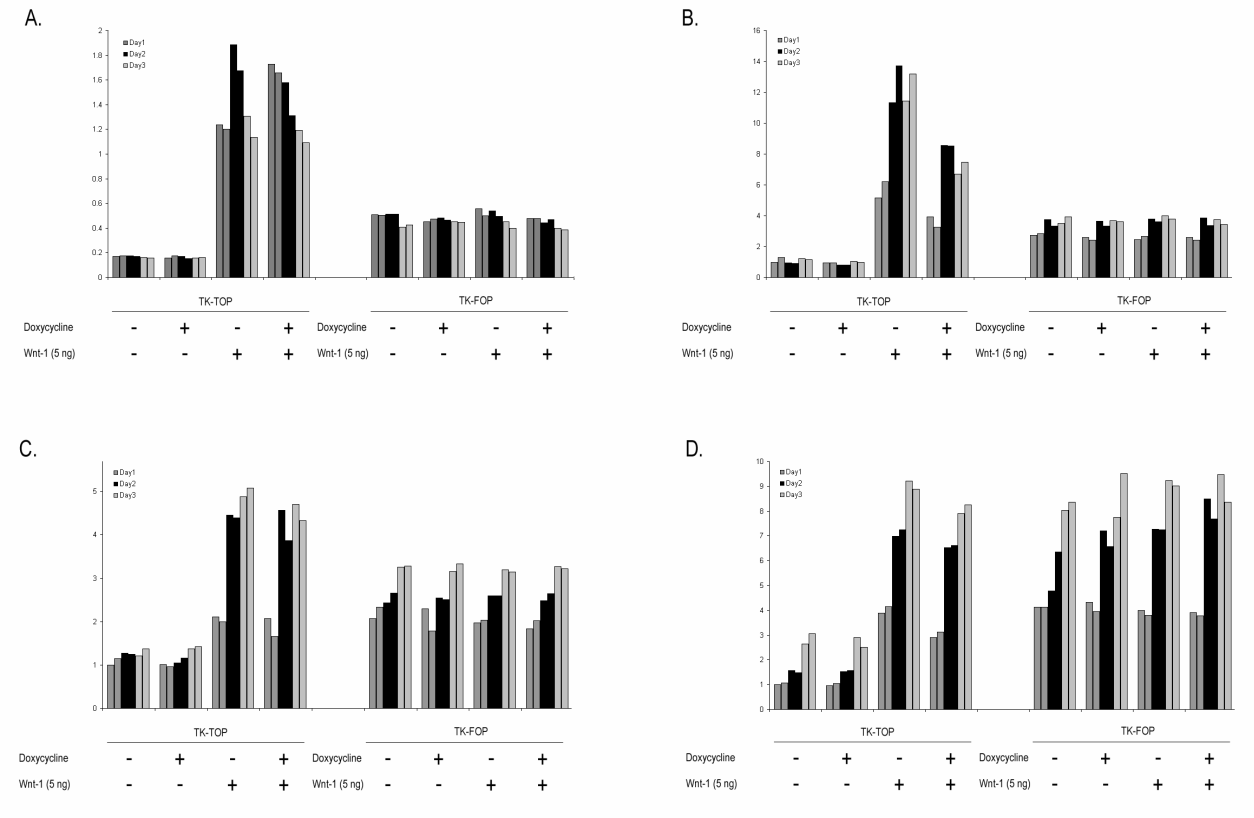


Figure 8. Effect of knock down of PYGOPUS-2 on TCF mediated signaling

HEK-TR cells were stably transfected with the pTER vectors encoding RNAi oligo's for PYGOPUS-2. Cells were seeded and induced with doxycycline. After 1, 2 or 3 days of induction these were transfected with the Tcf reporter (TK-TOP) or its negative control (TK-FOP) with or without Wnt-1. One day after transfection cell were harvested and luciferase levels were determined. Three different cell lines were tested, each expressing a different RNAi oligo for Pygopus-2, panel A. cell line 5; panel B. cell line 6 and panel C. cell line 7. The parental cell line was used as a negative control, panel D. HEK-TR.

The cellular localization of PYGOPUS and BCL-9 was studied using immunofluorescence on fixed cells transfected with expression constructs of BCL-9, PYGOPUS or β-catenin. Initially, Flag-BCL-9 and VSV-tagged PYGOPUS were detected using the Flag or VSV antibody and visualized with a secondary antibody labelled with FITC or TRITC. Later also fusion proteins were constructed with green fluorescent protein (GFP) or cyan fluorescent protein (CFP)

Localization of GFP-BCL-9 was hard to visualize, since this protein appeared to accumulate in strongly fluorescent dots. To circumvent this problem we used a fusion between GFP and a N terminal part of BCL-9 comprising of amino acid 1 to 582, still containing the HD1, HD2 and HD3 domains (GFP-5' BCL-9). This truncated version of BCL-9 however, does not enhance WNT-1 mediated TCF signaling as determined by the TOP/FOP reporter assay (Figure 12). Apparently for full function of BCL-9 also the HD4 and HD5 domains are indispensable. This GFP-5'-BCL-9 showed a similar distribution to Full length Flag-BCL-9. We are currently repeating the localization experiments of co-transfection of Flag-BCL-9 with CFP-β-catenin.

Flag-5'-BCL-9 localization showed three types of localization in 293T cells, in about 30% of the cells BCL-9 is exclusively cytoplasmic, in 50% of the cells the protein is homogeneously distributed between cytoplasm and nucleus, while in 20% of the cells show BCL-9 localization in the nucleus (Figure 9A). This is in contrast with the previously described nuclear localization of BCL-9 in the wing blade primordium (4). When the β-catenin interaction domain HD2 is deleted from this BCL-9 construct, the localization of the protein shifts to a total nuclear localization (Figure 9B). This effect is not caused by lack of the HD4 and HD5 domains, since deletion of HD1 or HD3 of GFP-5' BCL-9 results in a distribution similar to wild type GFP-5'-BCL-9 (as summarized in Table 3). The nuclear localization of the deletion mutant lacking the HD2 domain, therefore must be caused by the deletion of its β-catenin interaction domain. Apparently, BCL-9 localization is affected by β-catenin or conversely, β-catenin localization may be affected by BCL-9, or both.

PYGOPUS is localized in the nucleus, as observed when VSV-PYGOPUS is transfected in 293T cells and detected with anti-VSV and a secondary antibody labelled with TRITC (Figure 9C.), similar to the localization of CFP-PYGOPUS (data not shown). Transfection of Myc-β-catenin and subsequent detection with anti-Myc and TRITC shows that the protein is evenly distributed between the nucleus and the cytoplasm. (Figure 9D.)

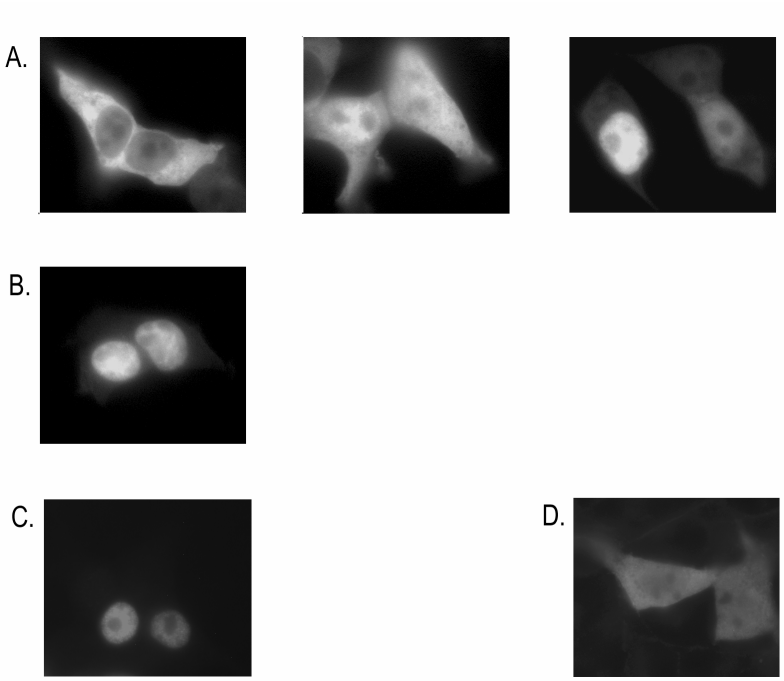


Figure 9. Localization of BCL-9, PYGOPUS and β-catenin in transiently transfected 393T cells.
9A. Distribution of GFP-5'-BCL-9 in different cells. About 20% of the cells show an exclusive cytoplasmic localization, in 50% the protein is homogeneous distributed between the cytoplasm and the nucleus, and 30% of the cells show an exclusive nuclear BCL-9 localization.
9B. Deletion of the β-catenin interaction domain HD2 form BCL-9 results in exclusive nuclear localization of the protein
9C. VSV-PYGOPUS localization is determined by staining with anti-VSV antibody and a TRITC labelled second antibody. The protein is exclusively present in the nucleus.
9D. Myc-β-catenin is distributed evenly between cytoplasm and nucleus. the protein was detected with anti-Myc antibody and a TRITC labelled second antibody.

When CFP-PYGOPUS and GFP-5'-BCL are co-transfected, a complete co-localization is observed, both proteins now reside in the nucleus (Figure 10.)

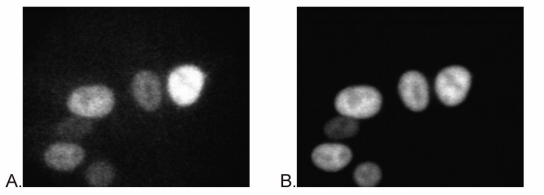


Figure 10. Co-localization in the nucleus of GFP-5'-BCL-9 and CFP-PYGOPUS
293T cells were co-transfected with GFP-5'-BCL-9 and CFP-PYGOPUS and harvested after 24 hours. Localization of both proteins was determined by immunofluorescent mircoscopy

The nuclear localization of BCL-9 lacking the β-catenin interaction domain, ΔHD2-BCL-9, prompts the question if the localization of BCL-9 and β-catenin are inter-dependent. To address this, Myc-β-catenin and GFP-5'-BCL-9 were co-transfected and the localization of both proteins was determined using direct fluorescence for BCL-9 and anti-Myc combined with a secondary TRITC labelled antibody for Myc-β-catenin (Figure 11). A clear co-localization is observed for both proteins, either in the nucleus or homogeneously distributed throughout the cell. The number of cells with nuclear BCL-9 staining is increased when co-transfected with β-catenin from 30% to 65%.

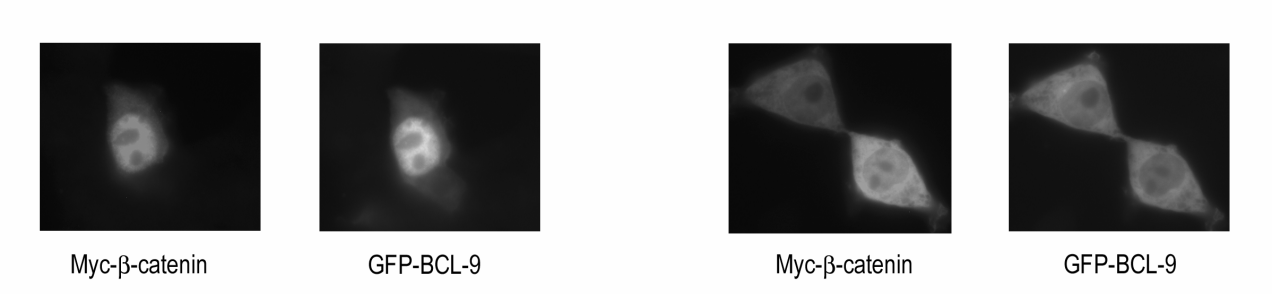


Figure 11. Co-transfection of GFP-5'-BCL-9 and Myc-β-catenin
293T cell were transfected with GFP-5'-BCL-9 and Myc-β-catenin, fixed after 24 hours and stained with anti-Myc antibody. This resulted in total co-localization of both proteins, either nuclear (65 %) or homogeneous distributed (35%).

This could indicate that BCL-9 can induce β-catenin translocation to the nucleus, suggesting a role for BCL-9 in β-catenin nuclear in- or export. One hypothesis could be that the HD2 domain of BCL-9 is a nuclear export signal, which is masked upon β-catenin binding, resulting in nuclear accumulation of BCL-9. In this model, BCL-9 would shuttle between cytoplasm and nucleus, and upon β-catenin binding BCL-9 would still translocate to the nucleus co-transporting β-catenin. Some similarities exist between the HD2 sequence and a classical NES. In this model localization of BCL-9 would then be affected by Leptomycin B (LMB), an inhibitor of the CRM1 mediated nuclear export machinery (33). However, when cells were transfected with GFP-5'-BCL-9 and treated with LMB, only a marginal increase in nuclear localization of BCL-9 was observed of maximum 1.5 - 2 fold. Moreover, when the HD2 domain is fused to GFP, it is distributed homogeneously over nucleus and cytoplasm and this does not change upon treatment with LMB. This indicates that the classical CRM1 nuclear export pathway does not play a major role in the localization of BCL-9. Nuclear export mechanisms exist that are insensitive to LMB, so the hypothesis that HD2 domain functions as a nuclear export signal can not be excluded.

Another experiment was designed to address the relation between β-catenin and BCL-9 localization. A colon carcinoma cell line (SW480) is used in which β-catenin can be inducibly knocked down, as recently described in Van de Wetering et al. (23). In this cell line, induction with doxycycline results in transcription of RNAi oligo's for β-catenin and subsequent knock down of β-catenin expression. These cells were transfected with GFP-5' BCL-9 and treated with doxycyclin. After three days, the localization of BCL-9 was determined. As a negative control, the same procedure was carried out with the SW480-TR cell line, lacking the RNAi oligo's (Table 2).

When the RNAi for β-catenin is induced, the number of cells with nuclear BCL-9 staining decreases from 30% to 13%. This effect is not observed in the cell line that does not contain the RNAi oligo's.

	Induction of β-catenin RNAi	% cells with nuclear BCL-9	% cells with homogenous BCL-9
SW480-TR	-	19	81
	+	17	83
SW480-TR with RNAi oligo's for β-catenin	-	30	70
	+	13	97

Table 2. Effect of β-catenin knock down on BCL-9 localization
Two cell lines, SW480-TR and SW480-TR with RNAi oligo's for β-catenin, were transfected with GFP-5'-BCL-9 and treated with doxycycline. BCL-9 localization was determined three days after transfection. (This experiment is one representative of four different experiments that were performed)

Deletion mutants for HD1, HD2 and HD3 were used to study the role of these domains in the function of BCL-9. These deletion mutants were constructed from full length BCL-9 as well as from the GFP-5'-BCL-9. Again, expression in 293T cells of the full length versions of ΔHD1, ΔHD2 and ΔHD3 show strong accumulation in highly fluorescent dots, which is impossible to visualize. Truncated variants of ΔHD2 and ΔHD3 give a clear fluorescence, hence these were used for the following experiments. The localization of these proteins upon transfection in 293T cells was determined, with and without co-transfection of PYGOPUS or β-catenin (Table 3). Moreover, the long versions of these full length deletion mutants were also tested in the TCF reporter assay to determine the effect on the signaling capacities of these BCL-9 mutants (Figure 12).

The localization pattern of BCL-9 lacking its PYGOPUS interaction domain (ΔHD1) is similar to wild type BCL-9, indicating that the nuclear localization we observe with 20% of the cells is not caused by interaction with PYGOPUS. As expected the localization of ΔHD1-BCL-9 is insensitive to co-transfection with PYGOPUS and still sensitive to co-transfection with β-catenin, an increase from 17% to 69% is observed in the number of cells with nuclear BCL-9.

The cellular distribution of the deletion mutant lacking the β-catenin interaction domain, ΔHD2, is somewhat more puzzling. Its localization shifts almost entirely to the nucleus as we have observed previously. Also upon co-transfection with PYGOPUS the protein localizes to the nucleus, as expected. Co-transfection with β-catenin results in a localization pattern similar to the wild type BCL-9, not to an increase in nuclear localization since this mutant lacks its β-catenin interaction domain.

The HD3 domain contains a putative nuclear localization signal (NLS) (Figure 1). Deletion of this domain was predicted to affect the localization of BCL-9 and have an effect on its signaling capacities. Unexpectedly, the ΔHD3 mutant stills shows nuclear localization in 16 % of the cells, apparently the HD3 domain does not contain a functional NLS Also this protein is sensitive to co-transfection with PYGOPUS, resulting in a nuclear localization, from 16 % to 96 % of the cells now showing nuclear localization. Also co-transfection of β-catenin results in a shift towards more nuclear localization, but with an attenuated effect, from 16 % to 58 %.

		% cells nuclear BCL-9 staining	% cells homogenous or cytoplasmic staining BCL-9
BCL-9	-	23	78
	+ PYGOPUS	97	3
	+ β-catenin	65	35
ΔHD1-BCL-9	-	17	83
	+ PYGOPUS	4	96
	+ β-catenin	69	31
ΔHD2-BCL-9	-	84	16
	+ PYGOPUS	94	6
	+ β-catenin	66	34
Δ-HD3-BCL-9	-	16	84
	+ PYGOPUS	96	4
	+ β-catenin	58	42

Table 3. Localization of deletion mutants of GFP-5'-BCL-9

293T cell were transfected with 500 ng of either of the GFP-tagged deletion constructs of BCL-9, ΔHD1, ΔHD2, and ΔHD3 in combination with pCDNA3 (empty vector), with Myc-PYGOPUS-2, or with β-catenin. 24 hours after transfection cells were fixed and the localization of BCL-9 was determined.

When these deletion mutants are tested in a TCF reporter assay, it is clear that only the wild type BCL-9 protein and the ΔHD3 mutant are still capable of enhancing the Wnt-1 induced signaling (Figure 12). As expected, since both interaction with PYGOPUS and β-catenin are essential for proper function of BCL-9 as has been shown in literature.

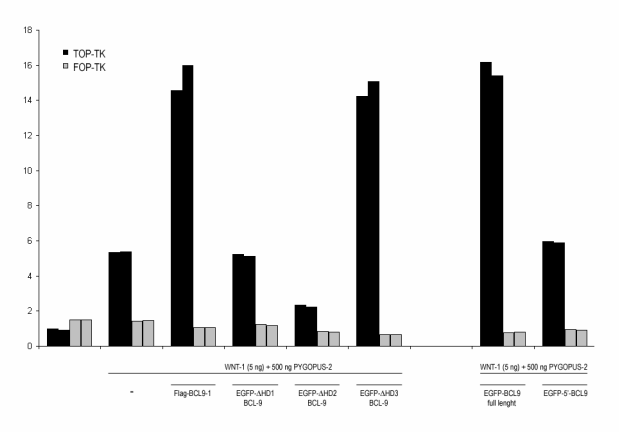


Figure 12. Effect of ΔHD1-, ΔHD2- and ΔHD3-BCL-9 with PYGOPUS on Wnt-1 activation
293T cells were transfected with the Tcf reporter (TK-TOP) or its negative control (TK-FOP) with 5 ng WNT-1 and 500 ng PYGOPUS-2 and 150 ng of wild type Flag-BCL-9, ΔHD1, ΔHD2, ΔHD3-BCL-9-EGFP, full length EGFP-BCL-9 or EGFP-5'-BCL-9.

Discussion

We have shown that PYGOPUS and BCL-9 are broadly expressed in the mammalian system. They play a role in transduction of a WNT signal, as PYGOPUS and BCL-9 together enhance TCF mediated transactivation when the cascade is activated with WNT-1. The interactions reported for the *Drosophila* system are present in mammalian cells. PYGOPUS and BCL-9 bind to each other and BCL-9 also interacts with β-catenin. Over-expression of β-catenin and PYGOPUS re-locates BCL-9 from the cytoplasm to the nucleus, and down-regulation of β-catenin releases BCL-9 from the nucleus. The data obtained so far clearly point in the direction of BCL-9 affecting the localization of β-catenin, but not the transcriptional activity of β-catenin-TCF as has been proposed in literature (4, 2, 1, 3). The role of PYGOPUS is still unclear. It could function as an anchor for BCL-9 to enable β-catenin release to TCF, but also a direct role on transcriptional activation via BCL-9 and β-catenin interaction as proposed cannot be excluded.

Many questions on BCL-9 and PYGOPUS function remain unanswered. Using the TCF reporter assay and over-expression of the proteins, it cannot clearly be addressed where in the cascade these proteins exert their function. Identifying novel binding partners of these proteins might shed more light on this matter. Currently, we are involved in high throughput yeast two

hybrid screens, with both PYGOPUS and BCL-9 proteins. In the lab, mass spectrometry analysis on protein complexes from large scale purification of protein complexes using immunoprecipitation with a Flag antibody has proven to be quite successful. This method as well as the TAP affinity purification as described above can also be used to identify BCL-9 interaction partners.

A way to study the mechanism of this nuclear translocation more closely would be to use photo bleaching techniques together with the fluorescent labelled proteins. This way the effect of several proteins on nuclear import rate of β-catenin can be measured, like co-transfection with WNT, PYGOPUS or BCL-9.

To determine the role of these proteins *in vivo*, the generation of monoclonal antibodies will be very important. These can be used in several experimental procedures. For example, they can be used to identify binding partners of BCL-9 and PYGOPUS in studies without over-expression systems, or they can be used to determine the expression patterns and localization of these proteins in tissues where WNT signaling is activated, for example in crypts of the small intestine, versus the expression in tissues where there is no WNT signaling, like in the villi of the small intestine. This could provide evidence if the localization and possibly transport of BCL-9 and β-catenin also play a role *in vivo*.

Much information in PYGOPUS and BCL-9 function in the mammalian system will be gathered by determining the effect on WNT signal transduction in the absence of these proteins. Generating inducible knock-out mice for these proteins is elaborate work since both proteins have two mammalian homologs. RNAi technology to knock down PYGOPUS and BCL-9 expression is another way to study the effect of BCL-9 and PYGOPUS depletion on WNT signaling. So far, only inducible cell lines were generated in which the PYGOPUS-2 mRNA did not decrease very strongly. Possibly, efficient down-regulation of proteins by RNAi requires the effect of several RNAi oligo's. In the above described cell lines additional different oligo's for PYGOPUS-2 can be transfected to mediate a stronger down-regulation of the PYGOPUS-2 mRNA. Subsequent introduction of RNAi oligo's for PYGOPUS-1 in the same cell line would result in a cell line in which all Pygopus mRNA can be efficiently down modulated. The effect of depletion of PYGOPUS on WNT signal transduction could then be addressed in these cell lines. The same approach is currently used to make cell lines in which both BCL-9.1 and BCL-9.2 can be efficiently knocked down. This way also the effect of down modulation of BCL-9 on WNT signal transduction will be determined.

Future research will unravel the mechanism by which BCL-9 and PYGOPUS function in WNT signaling. This may very well be an essential role, in accordance with the data from *Drosophila*. If PYGOPUS and BCL-9 function are absolutely required and specific for WNT signal transduction, this would make these proteins and possibly their interaction partners suitable targets for drug development. More than > 85 % of the sporadic and hereditary colorectal tumours show aberrant activation of the WNT signaling cascade. If disruption of the β-catenin-BCL-9 interaction would inhibit WNT signal transduction, for example by blocking the nuclear translocation of BCL-9, then small molecular compounds might be designed to inhibit the interaction between BCL-9 and β-catenin. However, these would need to be very specific in blocking only this interaction, since β-catenin binding to E-cadherin should not be affected. Also PYGOPUS might be a target for small molecule inhibitors, especially if future research shows that this protein functions exclusively in WNT signaling. Possibly, elucidation of the mechanism by which PYGOPUS and BCL-9 function and the identification of their binding partners might provide insights to design novel drug targets.

Reference list

1. Belenkaya, T. Y., Han, C., Standley, H. J., Lin, X., Houston, D. W., Heasman, J., and Lin, X. (2002). pygopus Encodes a nuclear protein essential for wingless/Wnt signaling. *Development* 129: 4089-4101.

2. Thompson, B., Townsley, F., Rosin-Arbesfeld, R., Musisi, H., and Bienz, M. (2002). A new nuclear component of the Wnt signaling pathway. *Nat.Cell Biol.* 4: 367-373.

3. Parker, D. S., Jemison, J., and Cadigan, K. M. (2002). Pygopus, a nuclear PHD-finger protein required for Wingless signaling in Drosophila. *Development* 129: 2565-2576.

4. Kramps, T., Peter, O., Brunner, E., Nellen, D., Froesch, B., Chatterjee, S., Murone, M., Zullig, S., and Basler, K. (4-5-2002). Wnt/wingless signaling requires BCL9/legless-mediated recruitment of pygopus to the nuclear beta-catenin-TCF complex. *Cell* 109: 47-60.

5. Cadigan, K. M. and Nusse, R. (1997). Wnt signaling: a common theme in animal development. *Genes Dev.* 11: 3286-3305.

6. Alonso, L. and Fuchs, E. (5-15-2003). Stem cells in the skin: waste not, Wnt not. *Genes Dev.* 17: 1189-1200.

7. Van de, Wetering M., Sancho, E., Verweij, C., de Lau, W., Oving, I., Hurlstone, A., van der, Horn K., Batlle, E., Coudreuse, D., Haramis, A. P., Tjon-Pon-Fong, M., Moerer, P., van den, Born M., Soete, G., Pals, S., Eilers, M., Medema, R., and Clevers, H. (10-18-2002). The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. *Cell* 111: 241-250.

8. Willert, K. and Nusse, R. (1998). Beta-catenin: a key mediator of Wnt signaling. *Curr.Opin.Genet.Dev.* 8: 95-102.

9. Kinzler, K. W. and Vogelstein, B. (1996). Lessons from hereditary colorectal cancer. *Cell* 87: 159-170.

10. Morin, P. J., Sparks, A. B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B., and Kinzler, K. W. (1997). Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. *Science* 275: 1787-1790.

11. Rubinfeld, B., Robbins, P., El Gamil, M., Albert, I., Porfiri, E., and Polakis, P. (1997). Stabilization of beta-catenin by genetic defects in melanoma cell lines. *Science* 275: 1790-1792.

12. Polakis, P. (8-1-2000). Wnt signaling and cancer. *Genes Dev* 14: 1837-1851.

13. Fagotto, F., Gluck, U., and Gumbiner, B. M. (1998). Nuclear localization signal-independent and importin/karyopherin- independent nuclear import of beta-catenin. *Curr.Biol.* 8: 181-190.

14. Yokoya, F., Imamoto, N., Tachibana, T., and Yoneda, Y. (1999). beta-catenin can be transported into the nucleus in a Ran-unassisted manner. *Mol.Biol.Cell* 10: 1119-1131.

15. Wiechens, N. and Fagotto, F. (1-9-2001). CRM1- and Ran-independent nuclear export of beta-catenin. *Curr Biol.* 11: 18-27.

16. Henderson, B. R. (2000). Nuclear-cytoplasmic shuttling of APC regulates beta-catenin subcellular localization and turnover. *Nat.Cell Biol.* 2: 653-660.

17. Rosin-Arbesfeld, R., Townsley, F., and Bienz, M. (8-31-2000). The APC tumour suppressor has a nuclear export function. *Nature* 406: 1009-1012.

18. Aasland, R., Gibson, T. J., and Stewart, A. F. (1995). The PHD finger: implications for chromatin-mediated transcriptional regulation. *Trends Biochem.Sci.* 20: 56-59.

19. Dooijes, D., van de Wetering, M., Knippels, L., and Clevers, H. (1993). The Schizosaccharomyces pombe mating-type gene mat-Mc encodes a sequence-specific DNA-binding high mobility group box protein. *J.Biol.Chem.* 268: 24813-24817.

20. Lu, Z., Xu, S., Joazeiro, C., Cobb, M. H., and Hunter, T. (2002). The PHD domain of MEKK1 acts as an E3 ubiquitin ligase and mediates ubiquitination and degradation of ERK1/2. *Mol.Cell* 9: 945-956.

21. Borden, K. L. (2-4-2000). RING domains: master builders of molecular scaffolds? *J.Mol.Biol.* 295: 1103-1112.

22. Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium. *Anal-Biochem.* 162: 156-159.

23. Van De, Wetering M., Oving, I., Muncan, V., Pon Fong, M. T., Brantjes, H., Van Leenen, D., Holstege, F. C., Brummelkamp, T. R., Agami, R., and Clevers, H. (2003). Specific inhibition of gene expression using a stably integrated, inducible small-interfering-RNA vector. *EMBO Rep.* 4: 609-615.

24. Willis, T. G., Zalcberg, I. R., Coignet, L. J., Wlodarska, I., Stul, M., Jadayel, D. M., Bastard, C., Treleaven, J. G., Catovsky, D., Silva, M. L., and Dyer, M. J. (3-15-1998). Molecular cloning of translocation t(1;14)(q21;q32) defines a novel gene (BCL9) at chromosome 1q21. *Blood* 91: 1873-1881.

25. Macintyre, E., Willerford, D., and Morris, S. W. (2000). Non-Hodgkin's Lymphoma: Molecular Features of B Cell Lymphoma. *Hematology.(Am.Soc.Hematol.Educ.Program.)* 180-204.

26. Kawamata, N., Sakajiri, S., Sugimoto, K. J., Isobe, Y., Kobayashi, H., and Oshimi, K. (7-25-2002). A novel chromosomal translocation t(1;14)(q25;q32) in pre-B acute lymphoblastic leukemia involves the LIM homeodomain protein gene, Lhx4. *Oncogene* 21: 4983-4991.

27. Callanan, M. B., Le Baccon, P., Mossuz, P., Duley, S., Bastard, C., Hamoudi, R., Dyer, M. J., Klobeck, G., Rimokh, R., Sotto, J. J., and Leroux, D. (1-4-2000). The IgG Fc receptor, FcgammaRIIB, is a target for deregulation by chromosomal translocation in malignant lymphoma. *Proc.Natl.Acad.Sci.U.S.A* 97: 309-314.

28. Dyomin, V. G., Palanisamy, N., Lloyd, K. O., Dyomina, K., Jhanwar, S. C., Houldsworth, J., and Chaganti, R. S. (4-15-2000). MUC1 is activated in a B-cell lymphoma by the t(1;14)(q21;q32) translocation and is rearranged and amplified in B-cell lymphoma subsets. *Blood* 95: 2666-2671.

29. Hatzivassiliou, G., Miller, I., Takizawa, J., Palanisamy, N., Rao, P. H., Iida, S., Tagawa, S., Taniwaki, M., Russo, J., Neri, A., Cattoretti, G., Clynes, R., Mendelsohn, C., Chaganti, R. S., and Dalla-Favera, R. (2001). IRTA1 and IRTA2, novel immunoglobulin superfamily receptors expressed in B cells and involved in chromosome 1q21 abnormalities in B cell malignancy. *Immunity.* 14: 277-289.

30. Van de Wetering, M., Cavallo, R., Dooijes, D., van Beest, M., van Es, J., Loureiro, J., Ypma, A., Hursh, D., Jones, T., Bejsovec, A., Peifer, M., Mortin, M., and Clevers, H. (1997). Armadillo coactivates transcription driven by the product of the Drosophila segment polarity gene dTCF. *Cell* 88: 789-799.

31. Rigaut, G., Shevchenko, A., Rutz, B., Wilm, M., Mann, M., and Seraphin, B. (1999). A generic protein purification method for protein complex characterization and proteome exploration. *Nat.Biotechnol.* 17: 1030-1032.

32. Westermarck, J., Weiss, C., Saffrich, R., Kast, J., Musti, A. M., Wessely, M., Ansorge, W., Seraphin, B., Wilm, M., Valdez, B. C., and Bohmann, D. (2-1-2002). The DEXD/H-box RNA helicase RHII/Gu is a co-factor for c-Jun-activated transcription. *EMBO J.* 21: 451-460.

33. Fukuda, M., Asano, S., Nakamura, T., Adachi, M., Yoshida, M., Yanagida, M., and Nishida, E. (11-20-1997). CRM1 is responsible for intracellular transport mediated by the nuclear export signal. *Nature* 390: 308-311.

CHAPTER 5

GENERAL DISCUSSION

Regulation of TCF transcriptional activity, repression, activation and fine-tuning

The WNT signaling pathway plays a decisive role during embryonic patterning and cell fate determination during development (as reviewed in (1)). This signaling cascade is highly conserved throughout evolution. Indeed, orthologs of proteins that are involved in WNT signaling are found in many species ranging from fruit flies to man. The key effector of the canonical WNT pathway is the multi-functional and proto-oncogene β-catenin (2).

If the WNT pathway is not activated, free β-catenin is actively targeted for degradation in the cytoplasm by the so-called destruction complex. This multiprotein destruction complex contains the serine/threonine kinase GSK-3β, casein kinase CK1, the scaffold protein Axin and the tumour suppressor protein APC (reviewed in (3)). The destruction complex phosphorylates β-catenin, thereby priming it for ubiquitination and subsequent degradation by the proteasome. As a consequence, levels of free β-catenin in the cell remain low. Meanwhile in the nucleus, TCF transcription factors are associated with transcriptional repressors including members of the Groucho (Grg) family (4, 5, 6). This interaction leads to the active repression of TCF target genes. Upon activation of the WNT pathway, a cascade of events is relayed that destabilizes the destruction complex, allowing unphosphorylated β-catenin to accumulate and to translocate to the nucleus. Once in the nucleus, β-catenin associates with a member of the Tcf family. This interaction converts TCF from a transcriptional repressor into transcriptional activator (7, 8). This bipartite protein complex activates transcription of WNT target genes such as *c-Myc* (9), *EphB2* (10) and *c-Myb* (10).

Repression

In absence of a WNT signal, TCF confer target gene expression by its association with transcriptional co-repressors: CtBP (11) and/or Groucho (4, 5, 6). In *Drosophila*, CtBP is characterized to mediate short range repression, whereas Groucho mediates long range repression (12, 13, 14). Furthermore, CtBP only interacts with some TCF splice variants (Tcf-3 and Tcf-4E) (11). This suggests multiple mechanism of regulation through repression. *Drosophila* Groucho is the founding member of family of transcriptional co-repressors. The mouse genome contains four Groucho-related-genes (Grg 1-4) and a truncated variant (Grg-5). Grg proteins contain 5 protein domains. The amino-terminal tetramerization domain, the Q (glutamine rich) domain, followed by a GP (glycine/proline rich) domain, a CCN (containing putative casein kinaseII/cdc2 phosphorylation sites and a nuclear localization signal), a SP (serine/proline rich) domain and four WD repeats (protein interaction domains). Grg-5 only consists of the two most N-terminal domains of the Grg proteins, the Q and GP domain. Grgs bind to histone de-acetylases (HDAC) which confer repression by de-acetylation of the N-terminal tails of core histones, hereby condensing local chromatin structure (15).

In Chapter 2 we described the results of the study on the specificity of the interaction between the TCFs and individual members of Grg family. This was carried out in a functional

assay combined with careful documentation of the expression pattern. In addition, the role of Grg-5 was studied in more detail.

Using the TCF reporter assay we showed that transcriptional activation of each of the TCF family members can be repressed by each of the 'long' Grg proteins, Grg-1-4 (16). This indicates a functional interaction between all TCF and Grg family members. However, it shows no specificity in the interaction between the members of these two protein families. The TCFs and the human Groucho homologs (TLE's) show a broad expression pattern in cell lines. Several Grgs and TCFs are co-expressed in every cell line, as well as in different mouse tissues (Chapter 2 and 3). This shows that there is no unique combination of individual TCF and Grg family members based on their expression in cell lines. The abundance of the Grg proteins and the redundancy with which they interact with Tcf proteins, probably highlights the requirement to silence target gene expression adequately. This is essential in order to prevent unrestrained cell growth and/or aberrant differentiation. Indeed, deregulation of the Wnt pathway may cause cancer (reviewed in (2)).

The functional interaction between TCF and Grg-5 was studied in TCF reporter assays (Chapter 2). Grg-5 acts as a de-repressor of TCF transcriptional activity. Upon transfection of TCF and β-catenin, activity of a reporter is enhanced by adding Grg-5. Grg-5 can still interact with the "long" versions of the Grg family members through the Q domain (17). The de-repressive function of this protein was clarified by immune precipitation experiments studying interactions with HDAC. Grg-5 has lost the capacity to bind to the histone de-acetylase 1 (HDAC-1), whereas a truncated version of Grg-4, containing only the Q and GP domain, does still interact with HDAC-1. From this data the following model emerges, where Grg-5 is a natural inhibitor of Grg mediated repression. When Grg-5 associates with other Grg family members, the amount of HDAC tethered to the template is decreased, reducing the level of repression.

Another level of regulation of repression of target genes is conferred by the expression of different TCF splice variants. Several naturally occurring splice variants of the different TCF family members have been described, one of these lack the N-terminal part of the protein which encodes the β-catenin interaction domain (18), so called ΔN variants. These are no longer able to bind to β-catenin, but can interact with Groucho proteins. The ΔN-TCF proteins block transcriptional activation in colon carcinoma cell lines (19, 10). Since Grg proteins are abundantly expressed, these ΔN-Tcf variants do not only block activation by competing with endogenous TCF-β-catenin complexes, but probably even function as active transcriptional repressors.

Several questions regarding Grg mediated repression of TCF target genes however, still remain unanswered. The mechanism of Grg mediated repression is not entirely clear yet. Although it is well known that Grg proteins interact with histone deacetylase proteins (HDAC), Grg mediated repression is only partially dependent on interaction with HDAC. Inhibition of HDAC function by Trichostatin A (TSA) e.g. results only in a partial relieve of repression (15) Moreover, an additional repression domain, although less potent, has been identified in the WD40 repeat domains of the Grgs (20). These data suggest that Grgs may also utilize histone deacetylase-independent mechanisms for transcriptional repression. Possibly the generated and extensively characterized monoclonal antibodies described in Chapter 3 can be used to purify protein complexes and to identify other proteins associated with Grg repressors, elucidating the HDAC independent repression mechanism.

In addition, it is not clear how the transition is achieved from TCF as a repressor, in complex with Grg proteins, to an activator upon association with β-catenin. The β-catenin and Grg interaction domains do not overlap in the Tcf protein sequence. So far, it has not been addressed if

these two proteins can simultaneously bind to TCF. Grg proteins are large proteins and they function as tetramers. Association of a large Grg complex with TCF might make it structurally impossible for β-catenin to bind this complex at the same time. Moreover, it is thus far unknown if accumulation of β-catenin is enough to convert Tcf from a transcriptional repressor into a transcriptional activator. Perhaps an additional modification (e.g. phosphorylation) of Grgs is required.

Since Grg proteins are transcriptional co-repressors for many DNA binding factors, it still has to be clarified how the correct distribution is achieved amongst the many interaction partners. Several reports indicate that there might be non-redundant functions for the different Grg protein family members (21, 22). Clearly, not all regulation mechanisms of Grg function have been uncovered yet and await further analysis. This will undoubtedly be helped by new technologies, including genomics and proteomics.

Activation

Upon activation of the Wnt signaling cascade, β-catenin accumulates and translocates into the nucleus. The mechanism of nuclear import is still unknown. It is clear that the nuclear import of β-catenin is independent of transport factors like importins or Ran GTP (23). It has been suggested that β-catenin directly interacts with the nuclear pore as a possible mechanism to enter the nucleus (24, 25).

In the nucleus, TCF and β-catenin bind and from a bi-partite transcription factor. The C-terminus of β-catenin contains a transactivation domain, while Tcf is able to bind to DNA. This interaction results in the activation of TCF target gene expression. Interactions with additional proteins activate β-catenin/Tcf activity. These proteins include the TBP, CREB and Brg. More specifically, β-catenin can bind to the TATA binding protein TBP, either directly (26) or via Pontin 52 (27), recruiting proteins of the general transcription machinery. In addition, the CREB binding proteins (CBP) can bind to β-catenin and enhance transcriptional activation via its histone acetylation activity (HAT) (28, 29). This results in a de-condensed chromatin structure which increases the accessibility of other transcription factors to target gene promoters (30, 31). Another β-catenin interacting protein is Brg, a component of the SWI/SNF chromatin remodelling complex (32). β-catenin could, therefore, be viewed as a docking molecule that recruits essential co-activators to target gene promoters. These interaction of β-catenin and a co-activator might be specific for a certain set of promoters.

Recently, two novel nuclear components of the Wnt signaling cascade were identified in *Drosophila*, Pygopus and Legless (33, 34, 35, 36). Mutant flies for Legless or Pygopus have a phenotype similar to *Wingless* mutant, indicating that they are essential for Wg signaling. A direct interaction was observed between Legless and Pygopus, as well as between Legless and Armadillo, the *Drosophila* homolog of β-catenin. Pygopus contains a PHD domain, a motif often found in chromatin remodelling factors. A model was proposed in which Pygopus and Legless are implicated in the transcriptional activation of TCF target genes (33, 34, 35, 36).

Pygopus and Legless are the founding members of two protein families that have been conserved from flies to man. The mammalian genome encodes two homologs of Legless (BCL-9)

and two PYGOPUS proteins. We studied the role of PYGOPUS and BCL9 in the mammalian Wnt signaling cascade. These experiments are described in Chapter 4.

PYGOPUS-2 and BCL-9.2 show an ubiquitous expression pattern in a panel of cell lines, whereas PYGOPUS-1 and BCL-9.1 expression is more restricted. PYGOPUS and BCL-9 together enhance WNT signaling in a TCF reporter assay, only when the cascade is activated by WNT-1, not by any of the other stimuli tested. Cellular localization studies suggest a role of BCL-9 in nuclear transport of β-catenin.

Comparing these results with the published data from *Drosophila* leads to some intriguing differences. In *Drosophila* Legless and Pygopus are downstream of activated Armadillo, as was shown by epistasis experiments using flies mutant for these proteins. In our TCF reporter assays, we only observe an enhancing effect of BCL-9 and PYGOPUS when the WNT signaling cascade is activated with Wnt-1, activation by any other stimulus is unaffected (e.g. β-catenin, LiCl or dominant negative Axin). This difference may be explained by the difference in species studied. Furthermore, we used over-expression of BCL-9 and PYGOPUS in cell lines, whereas in *Drosophila* experiments the result of the absence of both proteins was studied.

Other captivating aspects emerge from this study. Co-transfection of β-catenin with BCL-9 results in enhanced nuclear accumulation of both proteins, but no effect on TCF transcriptional activation is observed in our reporter assay. Apparently, increased nuclear localization of β-catenin by BCL-9 is not enough to enhance TCF transcriptional activation. Perhaps additional cofactors are limiting and/or lacking.

Another question is raised by the activation of the cascade in colon carcinoma cell lines. In these cells β-catenin is not degraded, due to mutations in APC or β-catenin. As a result, the WNT signaling cascade is constitutively activated. However, there is no evidence for up-regulation of BCL-9 and/or PYGOPUS. Are these proteins not limiting in these conditions, do they not function down stream of AXIN or GSK3β, or are they not essential for WNT signal transduction in the mammalian system at all?

Further research will unravel the intriguing role of BCL9 and PYGOPUS in WNT signaling. This will be carried out by using protein interaction screens to identify which proteins are involved in nuclear import of β-catenin, antibodies to elucidate the *in vivo* localization of BCL-9 and β-catenin and knock down in cell lines and mouse knock out models.

Concluding remarks

The highly regulated WNT signaling cascade plays a decisive role during embryonic patterning and cell-fate determination. The inappropriate expression of WNT target genes, resulting from deregulation of this pathway, is also implicated in tumorigenesis. Thus, regulation of this pathway is of paramount importance. The transcriptional status of WNT target genes is not merely determined by a binary code of activation or repression by TCF transcription factors, but is subject to many subtle levels of regulation. Additional factors, such as ΔN-TCF splice variants, Grg-5 proteins natural inhibitors of Grg function and promotor specific β-catenin interacting co-activators, will ultimately determine the transcriptional state of a specific WNT target gene. Understanding this complexity of Wnt signaling regulation is just beginning to be elucidated. The study described in this thesis adds a small but significant step.

Reference list

1. Cadigan, K. M. and Nusse, R. (1997). Wnt signaling: a common theme in animal development. *Genes Dev.* 11: 3286-3305.

2. Van Es, J. H., Barker, N., and Clevers, H. (2003). You Wnt some, you lose some: oncogenes in the Wnt signaling pathway. *Curr.Opin.Genet.Dev.* 13: 28-33.

3. Giles, R. H., van Es, J. H., and Clevers, H. (6-5-2003). Caught up in a Wnt storm: Wnt signaling in cancer. *Biochim.Biophys.Acta* 1653: 1-24.

4. Cavallo, R., Cox, R., Moline, M., Roose, J., Polevoy, G., Clevers, H., Peifer, M., and Bejsovec, A. (1998). *Drosophila* Tcf and Groucho interact to repress Wingless signaling activiy. *Nature* 395: 604-608.

5. Roose, J., Molenaar, M., Peterson, J., Hurenkamp, J., Brantjes, H., Moerer, P., van de Wetering, M., Destree, O., and Clevers, H. (1998). The *Xenopus* Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors. *Nature* 395: 608-612.

6. Levanon, D., Goldstein, R. E., Bernstein, Y., Tang, H., Goldenberg, D., Stifani, S., Paroush, Z., and Groner, Y. (9-29-1998). Transcriptional repression by AML1 and LEF-1 is mediated by the TLE/Groucho corepressors. *Proc.Natl.Acad.Sci.U.S.A.* 95: 11590-11595.

7. Molenaar, M., van de Wetering, M., Oosterwegel, M., Peterson Maduro, J., Godsave, S., Korinek, V., Roose, J., Destree, O., and Clevers, H. (1996). XTcf-3 transcription factor mediates beta-catenin-induced axis formation in Xenopus embryos. *Cell* 86: 391-399.

8. Van de Wetering, M., Cavallo, R., Dooijes, D., van Beest, M., van Es, J., Loureiro, J., Ypma, A., Hursh, D., Jones, T., Bejsovec, A., Peifer, M., Mortin, M., and Clevers, H. (1997). Armadillo coactivates transcription driven by the product of the Drosophila segment polarity gene dTCF. *Cell* 88: 789-799.

9. He, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., da Costa, L. T., Morin, P. J., Vogelstein, B., and Kinzler, K. W. (1998). Identification of c-MYC as a target of the APC pathway [see comments]. *Science* 281: 1509-1512.

10. Van De, Wetering M., Sancho, E., Verweij, C., de Lau, W., Oving, I., Hurlstone, A., van der, Horn K., Batlle, E., Coudreuse, D., Haramis, A. P., Tjon-Pon-Fong, M., Moerer, P., van den, Born M., Soete, G., Pals, S., Eilers, M., Medema, R., and Clevers, H. (10-18-2002). The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. *Cell* 111: 241-250.

11. Brannon, M., Brown, J. D., Bates, R., Kimelman, D., and Moon, R. T. (1999). XCtBP is a XTcf-3 co-repressor with roles throughout Xenopus development. *Development* 126: 3159-3170.

12. Nibu, Y., Zhang, H., and Levine, M. (4-3-1998). Interaction of short-range repressors with Drosophila CtBP in the embryo. *Science* 280: 101-104.

13. Zhang, H. and Levine, M. (1-19-1999). Groucho and dCtBP mediate separate pathways of transcriptional repression in the Drosophila embryo. *Proc.Natl.Acad.Sci.U.S.A* 96: 535-540.

14. Nibu, Y., Zhang, H., and Levine, M. (5-1-2001). Local action of long-range repressors in the Drosophila embryo. *EMBO J.* 20: 2246-2253.

15. Chen, G., Fernandez, J., Mische, S., and Courey, A. J. (9-1-1999). A functional interaction between the histone deacetylase Rpd3 and the corepressor groucho in Drosophila development. *Genes Dev.* 13: 2218-2230.

16. Brantjes, H., Roose, J., van De, Wetering M., and Clevers, H. (4-1-2001). All Tcf HMG box transcription factors interact with Groucho-related co- repressors. *Nucleic Acids Res.* 29: 1410-1419.

17. Pinto, M. and Lobe, C. G. (1996). Products of the grg (Groucho-related gene) family can dimerize through the amino-terminal Q domain. *J.Biol.Chem.* 271: 33026-33031.

18. Van de Wetering, M., Castrop, J., Korinek, V., and Clevers, H. (1996). Extensive alternative splicing and dual promoter usage generate Tcf-1 protein isoforms with differential transcription control properties. *Mol.Cell Biol.* 16: 745-752.

19. Korinek, V., Barker, N., Morin, P. J., van Wichen, D., de Weger, R., Kinzler, K. W., Vogelstein, B., and Clevers, H. (1997). Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma [see comments]. *Science* 275: 1784-1787.

20. Fisher, A. L., Ohsako, S., and Caudy, M. (1996). The WRPW motif of the hairy-related basic helix-loop-helix repressor proteins acts as a 4-amino-acid transcription repression and protein-protein interaction domain. *Mol.Cell Biol.* 16: 2670-2677.

21. Husain, J., Lo, R., Grbavec, D., and Stifani, S. (1996). Affinity for the nuclear compartment and expression during cell differentiation implicate phosphorylated Groucho/TLE1 forms of higher molecular mass in nuclear functions. *Biochem.J.* 317: 523-531.

22. Yao, J., Liu, Y., Husain, J., Lo, R., Palaparti, A., Henderson, J., and Stifani, S. (1998). Combinatorial expression patterns of individual TLE proteins during cell determination and differentiation suggest non-redundant functions for mammalian homologs of Drosophila Groucho. *Dev.Growth Differ.* 40: 133-146.

23. Wiechens, N. and Fagotto, F. (1-9-2001). CRM1- and Ran-independent nuclear export of beta-catenin. *Curr Biol.* 11: 18-27.

24. Fagotto, F., Gluck, U., and Gumbiner, B. M. (1998). Nuclear localization signal-independent and importin/karyopherin- independent nuclear import of beta-catenin. *Curr.Biol.* 8: 181-190.

25. Yokoya, F., Imamoto, N., Tachibana, T., and Yoneda, Y. (1999). beta-catenin can be transported into the nucleus in a Ran-unassisted manner. *Mol.Biol.Cell* 10: 1119-1131.

26. Hecht, A., Litterst, C. M., Huber, O., and Kemler, R. (6-18-1999). Functional characterization of multiple transactivating elements in beta-catenin, some of which interact with the TATA-binding protein in vitro. *J.Biol.Chem.* 274: 18017-18025.

27. Bauer, A., Huber, O., and Kemler, R. (12-8-1998). Pontin52, an interaction partner of beta-catenin, binds to the TATA box binding protein. *Proc.Natl.Acad.Sci.U.S.A* 95: 14787-14792.

28. Hecht, A., Vleminckx, K., Stemmler, M. P., van Roy, F., and Kemler, R. (4-17-2000). The p300/CBP acetyltransferases function as transcriptional coactivators of beta-catenin in vertebrates. *EMBO J.* 19: 1839-1850.

29. Takemaru, K. I. and Moon, R. T. (4-17-2000). The transcriptional coactivator CBP interacts with beta-catenin to activate gene expression. *J.Cell Biol.* 149: 249-254.

30. Ogryzko, V. V., Schiltz, R. L., Russanova, V., Howard, B. H., and Nakatani, Y. (11-29-1996). The transcriptional coactivators p300 and CBP are histone acetyltransferases. *Cell* 87: 953-959.

31. Goldman, M. A. (1997). Executive decision: chromatin structure and gene regulation. *Trends Genet.* 13: 387-388.

32. Barker, N., Hurlstone, A., Musisi, H., Miles, A., Bienz, M., and Clevers, H. (9-3-2001). The chromatin remodelling factor Brg-1 interacts with beta-catenin to promote target gene activation. *EMBO J.* 20: 4935-4943.

33. Kramps, T., Peter, O., Brunner, E., Nellen, D., Froesch, B., Chatterjee, S., Murone, M., Zullig, S., and Basler, K. (4-5-2002). Wnt/wingless signaling requires BCL9/legless-mediated recruitment of pygopus to the nuclear beta-catenin-TCF complex. *Cell* 109: 47-60.

34. Thompson, B., Townsley, F., Rosin-Arbesfeld, R., Musisi, H., and Bienz, M. (2002). A new nuclear component of the Wnt signaling pathway. *Nat.Cell Biol.* 4: 367-373.

35. Belenkaya, T. Y., Han, C., Standley, H. J., Lin, X., Houston, D. W., Heasman, J., and Lin, X. (2002). pygopus Encodes a nuclear protein essential for wingless/Wnt signaling. *Development* 129: 4089-4101.

36. Parker, D. S., Jemison, J., and Cadigan, K. M. (2002). Pygopus, a nuclear PHD-finger protein required for Wingless signaling in Drosophila. *Development* 129: 2565-2576.

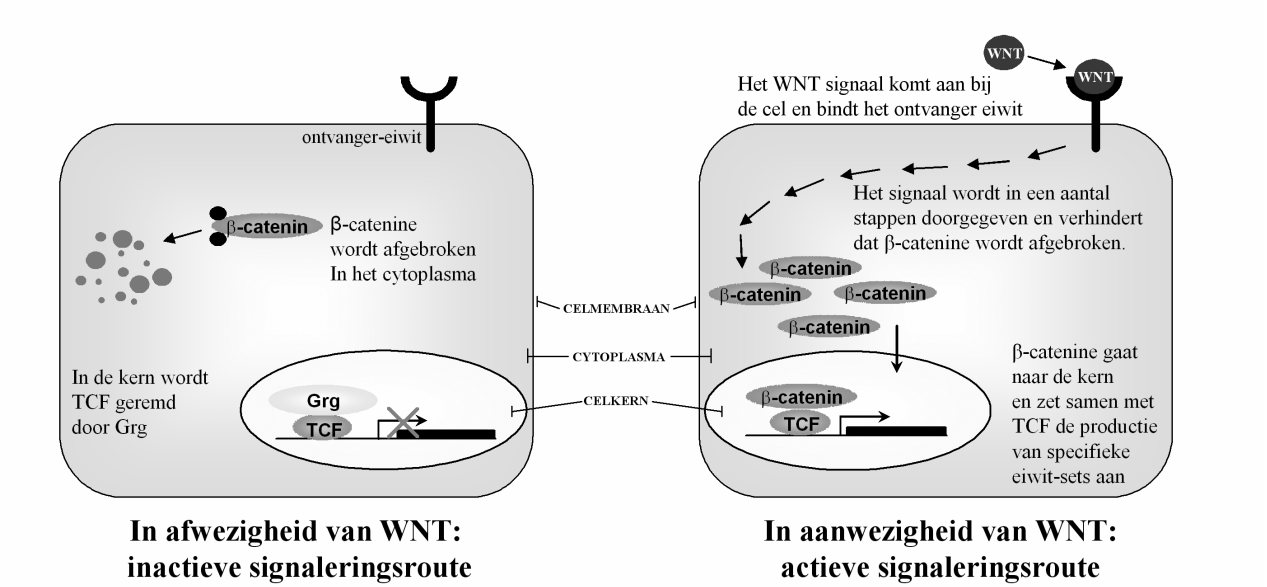
SAMENVATTING IN HET NEDERLANDS

Een organisme bestaat uit verschillende weefsels, die opgebouwd zijn uit cellen. Cellen zijn dus de bouwblokken van elk levend organisme. De ontwikkeling van slechts een cel naar een heel organisme is een zeer complex en nauwkeurig gereguleerd proces. Hierbij is het belangrijk dat de juiste cellen zich op het juiste moment ontwikkelen tot een celtype met een bepaald functie, zoals botcellen of spiercellen. Dit proces van specialiseren noemen we differentiatie. Een volwassen organisme heeft cellen die een heel leven meegaan en niet opnieuw kunnen worden gemaakt, zoals zenuwcellen en hersencellen. Er zijn echter ook cellen die een relatief korte levensduur hebben en dus steeds opnieuw aangemaakt moeten worden, zoals bloedcellen, huidcellen en darmcellen. Ook dit is een zeer gecontroleerd proces). De celtypes die steeds opnieuw worden aangemaakt, hebben 'moeder'cellen ofwel stamcellen, die steeds opnieuw delen, maar zelf niet specialiseren. Slechts de dochtercel differentieert zich tot een cel met een specifieke functie. De stamcellen zijn een continue productiebron van een bepaald type cellen. Zo zijn er onder andere stamcellen te vinden in de darm, in het bloed en in de huid.

Eiwitten zijn de structurele bouwstenen van de cel en tevens de uitvoerders bij verschillende processen, zoals de energiehuishouding en celtype specifieke functies zoals voedselopname en -verwerking in de darm en zuurstoftransport door het bloed. De informatie voor de productie van de eiwitten ligt in het DNA, in de genen. Genen zijn stukjes DNA die expliciet voor één eiwit coderen. Iedere cel bevat exact hetzelfde DNA, maar niet alle DNA wordt vertaald naar eiwit. Verschillende celtypen hebben verschillende functies en gebruiken dan ook verschillende combinaties van eiwitten.

Het onderzoek beschreven in dit proefschrift heeft de regulatie van WNT signalering bestudeerd. De WNT cascade is een van de signaleringsroutes die het proces van delen en specialiseren in goede banen moeten leiden. Een signaleringsroute is een soort door-geef-systeem in een cel, waarbij de cel signalen uit zijn omgeving ontvangt die hem vertellen wat hij moet gaan doen, delen of specialiseren en hoe. Een signaal komt aan bij de cel en wordt herkend aan het oppervlak van deze cel. Daarmee wordt een proces in de cel in werking gezet waarbij het signaal in verschillende stappen wordt doorgegeven naar de celkern, de uitvoerende afdeling van de cel. In de kern wordt het signaal gelezen en de cel ontwikkelt zich in de richting die hem door deze boodschap verteld wordt.

Dit proces van WNT signalering gebeurt door middel van eiwitten. Een eiwit, WNT, komt aan bij een cel, bindt aan zijn ontvanger-eiwit aan de buitenkant van de cel en via aan aantal eiwitten in de cel, wordt dit het signaal doorgegeven aan de celkern. In de celkern wordt het signaal ontvangen en vertaald, wat leidt tot de productie van een bepaalde set van eiwitten die essentieel zijn voor de verdere ontwikkeling van dit celtype. Het aanschakelen van de productie van eiwitten gebeurt door zogenaamde transcriptiefactoren. Transcriptiefactoren zijn als het ware de machientjes die het DNA van genen lezen en de productie van eiwit in gang zetten. Als er dus een WNT signaal van het celoppervlak door de cel naar de celkern wordt doorgegeven, leidt dit uiteindelijk tot de activatie van deze transcriptiefactor die op hun beurt een programma aanzetten dat leidt tot de productie van specifieke eiwitsets. In het geval van activatie van de WNT signaleringscascade wordt een transcriptiefactor aangezet die is opgebouwd uit twee eiwitten, namelijk TCF en β-catenine (zie figuur 1).



Figuur 1. Een schematisch overzicht van de WNT signaleringscascade.
In afwezigheid van een WNT signaal wordt β-catenine afgebroken in het cytoplasma. Wanneer een WNT eiwit de cel bereikt wordt de signaleringscascade geactiveerd. β-catenine wordt niet langer afgebroken en gaat naar de celkern. In de celkern bind β-catenine aan TCF en samen zetten zij de productie van specifieke eiwitsets aan.

Het is belangrijk dat de WNT signaleringsroute alleen in heel specifieke omstandigheden geactiveerd wordt. Dit mag namelijk alleen in de zogenaamde stamcellen die slecht delen en niet specialiseren zodat het een continue bron van nieuwe dochtercellen is. Als de WNT signaleringsroute onverhoopt toch aan komt te staan in een ander celtype, dan zal dit daar ook leiden tot voortdurende celdeling, wat kan leiden tot kanker. Dit kan gebeuren door een mutatie in het DNA waarbij de transcriptiefactor al in zijn actieve staat geproduceerd wordt, zodat er geen signaal van buiten de cel nodig is om dit hele proces aan te zetten. Het is gebleken dat een ontregelde controle over deze signaleringsroute de oorzaak is van meer dan 90 % van de darmkanker in de westerse wereld. Ook speelt deze ontregeling een rol bij huidkanker en bij bepaalde soorten leverkanker. Een beter begrip van deze signaleringsroute zal ons dus meer inzicht geven in het ontstaan van deze soorten kanker en kan mogelijk op de lange termijn leiden tot een medicijn.

Wanneer ontregeling van deze signaleringsroute zulke dramatische effecten heeft en wanneer deze route alleen aan staat in een specifiek soort cellen, dan moet de negatieve regulatie, de manier waarop wordt verzekerd dat deze route UIT staat, wel heel goed gewaarborgd zijn. In cellen die geen WNT signaal ontvangen, is op een aantal manieren verzekerd dat de signaleringsroute niet spontaan geactiveerd wordt. Een van de manieren om dit te bereiken is door de transcriptiefactor β-catenine erg instabiel te maken. Deze wordt namelijk direct nadat hij is aangemaakt meteen weer afgebroken. Alleen wanneer een WNT signaal de cel activeert wordt β-catenine niet langer afgebroken, gaat naar de celkern en bindt daar aan TCF zodat de productie van eiwitten kan beginnen. Ook is er in de kern een negatieve controle waarbij TCF gebonden wordt door het Grg eiwit, zodat TCF niet actief is.

In de eerste twee experimentele hoofdstukken van dit boekje wordt de rol van deze remmers ofwel repressoren, de Grg eiwitten, nader bestudeerd. In zoogdiercellen bestaan vijf Grg genen die erg veel op elkaar lijken, zogenaamde Grg familieleden, Grg-1, Grg-2, Grg-3, Grg-4 en Grg-5. Zo ook voor de TCF genen, die een deel zijn van de transcriptiefactoren die geactiveerd wordt als de signaleringsroute 'aan' staat. Van deze TCF eiwitten zijn er ook vier: TCF-1, LEF, TCF-3 en TCF-4.

In het tweede hoofdstuk hebben we bestudeerd of alle TCF familieleden kunnen binden aan en geremd kunnen worden door ieder Grg eiwit, of dat er misschien alleen bepaalde combinaties van een TCF met een Grg leiden tot remming. Dit hebben we bestudeerd door cellen verschillende combinaties van TCF en Grg eiwitten te laten produceren en dan de activiteit van TCF te meten. Hieruit bleek dat alle Grg eiwitten de activiteit van ieder TCF eiwit kunnen remmen. We hebben ook bestudeerd welke van de TCF en Grg eiwitten voorkomen in verschillende celtypen. Een aantal humane cellijnen werd getest op de aanwezigheid van ieder van de Grg en TCF familieleden. Hieruit bleek dat binnen een cel minstens drie, soms zelfs vier van de Grg en TCF eiwitten aanwezig waren. Dit geeft ook aan dat de negatieve regulatie van de activiteit van de TCF eiwitten erg belangrijk is. Van het Grg-5 eiwit was reeds bekend dat een stuk kleiner is dan de andere Grg familieleden. Het was niet duidelijk of dit eiwit nog dezelfde functie had als de andere Grgs. Wij laten zien dat Grg-5 eiwit functioneert als een anti-repressor, dat wil zeggen dat het wel aan TCF eiwitten kan binden, maar dat het geen remmende activiteit heeft. Wanneer Grg-5 bindt aan een TCF eiwit, dan kunnen de grote familieleden, de repressoren, niet meer binden, en dus niet meer remmen, vandaar de naam anti-repressor voor het Grg-5 eiwit.

In het derde hoofdstuk maken we antilichamen tegen de verschillende Grg eiwitten. Antilichamen zijn een soort vlaggetjes waarmee de aanwezigheid van een bepaald eiwit aangetoond kan worden. Met antilichamen die specifiek een van de Grg eiwitten herkennen, wilden we de aanwezigheid van de verschillende Grg eiwitten in muizenweefsels bestuderen. Na het vrij langdurige proces van het maken van deze antilichamen, werden deze in verschillende types experimenten getest op hun specificiteit. Dit wil zeggen dat het antilichaam tegen Grg-1 alleen Grg-1 mag herkennen en geen van de overige familieleden, hoewel deze eiwitten wel heel erg op elkaar lijken. Hieruit bleek dat we vijf zeer specifieke antilichamen in handen hadden, specifiek voor Grg-1, Grg-2, Grg-3, Grg-4 en Gg-5. Daarna hebben we deze antilichamen getest in een heel ander experiment, namelijk in het kleuren van muizenweefsels om de aanwezigheden van de verschillende Grg eiwitten aan te tonen. Slechts een van de vijf antichamen bleek functioneel te zijn in dit type experiment, namelijk het antilichaam tegen Grg-3. De weefselkleuringen laten zien dat het Grg-3 eiwit in bijna alle weefsels aanwezig is. Dit komt overeen met de resultaten uit het tweede hoofdstuk, waarin Grg-3 ook aanwezig is in alle humane cellijnen die we getest hebben. De hier ontwikkelde antilichamen tegen de Grg eiwitten zouden verder wellicht ook in andere experimenten gebruikt kunnen worden, zoals in biochemische studies om te bestuderen hoe deze eiwitten mechanistisch functioneren. Dit soort experimenten is echter niet uitgevoerd omdat ze buiten de doelstellingen van dit project vallen.

In het vierde hoofdstuk beschrijven we de studie naar twee eiwitten, waarvan pas zeer recentelijk duidelijk werd dat ze een rol spelen in de WNT signalering. Zoals gezegd speelt WNT signalering een belangrijke rol bij de balans van celdeling en differentiatie van cellen, zowel in volwassen organismen als in het zich ontwikkelende embryo. Gebleken is dat deze route zeer geconserveerd is gebleven in de evolutie. Dit wil zeggen dat de eiwitten die een rol spelen bij het doorgeven van het WNT signaal bij allerlei organismen voorkomen, bij mens en muis, maar ook bij kikkers en fruitvliegen. In eerste instantie is de WNT signaleringsroute zelfs ontdekt door

onderzoek dat werd gedaan naar de ontwikkeling van de fruitvlieg. Zo werden ook deze twee nieuwe eiwitten, Pygopus en Legless, voor het eerst geïdentificeerd in de fruitvlieg. Het was al snel duidelijk dat er ook zeer nauw verwante eiwitten bestaan in het zoogdiersysteem, maar hun exacte functie is nog onduidelijk.

Bij zoogdieren zijn er twee eiwitten nauw verwant aan Pygopus van de fruitvlieg, deze zijn PYGOPUS-1 en PYGOPUS-2 genoemd. Ook zijn er twee eiwitten die lijken op het Legless bij fruitvliegen, die reeds eerder ontdekt waren bij ander onderzoek en BCL-9.1 en BCL9.2 heten. In het vierde hoofdstuk hebben we bestudeerd of deze PYGOPUS en BCL-9 eiwitten een rol spelen bij de WNT signaleringscascade. In eerste instantie hebben we bepaald of deze eiwitten een direct effect hebben op de activiteit van de transcriptiefactor TCF-β-catenine, maar dat bleek niet zo te zijn. Alleen wanneer deze signaleringsroute wordt geactiveerd door het WNT signaal zelf, neemt deze activatie toe wanneer extra BCL-9 gecombineerd met PYGOPUS wordt toegevoegd. Hieruit is nog niet duidelijk wat deze eiwitten nu precies voor functie hebben. Dit lijkt ook in contrast te staan met de resultaten van experimenten bij fruitvliegen, waaruit een rol voor deze eiwitten in de celkern en wel een direct effect op de transcriptiefactor TCF-β-catenine lijkt te blijken. Wanneer wij de lokalisatie van deze eiwitten bekijken in zoogdiercellen, dan zien we dat PYGOPUS in de celkern zit, maar dat BCL-9 in het cytoplasma aanwezig is. (het cytoplasma is het deel van de cel tussen celkern en celmembraan) En zodra wij PYGOPUS en BCL-9 samen door een cel geproduceerd laten worden, zien we dat nu beide eiwitten zich in de celkern bevinden en dat BCL-9 niet meer in het cytoplasma aanwezig is. Ook als we BCL-9 met β-catenine samen bekijken zien we dat deze eiwitten samen een voorkeur hebben voor de celkern. Dit suggereert een rol voor BCL-9 en PYGOPUS in het transport van β-catenine naar de kern. Het exacte mechanisme waardoor β-catenine naar de kern gaat na een WNT signaal is nog niet opgehelderd, en vormt dus een interessant onderwerp voor nadere studie. De rol van BCL-9 en PYGOPUS in de WNT signaleringsroute zal onder andere met antilichamen en meer lokalisatie-experimenten ontrafeld kunnen worden.

Curriculum Vitae

Helen Mechteld Brantjes werd geboren op 16 mei 1973 in Amby, Maastricht. Na het behalen van haar Gymnasiumdiploma aan het Jeanne d'Arc College in Maastricht, begon zij in 1991 met de studie Scheikunde aan de Rijksuniversiteit Leiden. Een eerste stage werd doorlopen bij Gist-Brocades in Delft, onder leiding van Dr. R. Baankreis, waar de relatie tussen suikeropname en groeiomstandigheden met betrekking tot osmotische stress en co-fermentatie werd bestudeerd. Haar hoofdvakstage volbracht zij bij de vakgroep Moleculaire Genetica onder de begeleiding van Prof. J. Brouwers. Hier werd de rol van een mogelijk gist homoloog van het humane XP-E eiwit bestudeerd in het mechanisme van nucleotide excisie DNA herstelmechanisme. Een derde stageperiode werd doorgebracht bij het Nederlands Kanker Instituut in Amsterdam, onder begeleiding van Prof. Dr. R. Bernards, waar de rol van PR59, het substraatspecifieke deel-eiwit van het phosphatase PP2A, werd bestudeerd bij de regulatie van p107, een familielid van het retinoblastoma eiwit. Na het behalen van haar doctoraal examen in 1997 werd begonnen met het werk dat resulteerde in dit proefschrift, uitgevoerd onder leiding van Prof. Dr. H. Clevers, eerst aan het Universitair Medisch Centrum en later, na de verhuizing van diens onderzoeksgroep, aan het Hubrecht Laboratorium in Utrecht.

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