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A tight control over Wnt action

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ABSTRACT Here, we review the WNT pathway and its regulation at different levels. We focus on the transcriptional regulation of WNT target genes, in light of the recently identified negative regulators, i.e. relatives of groucho and CBP.

KEY WORDS: *WNT, β-Cat, TCF, groucho related gene*

Wg/Wnt gene family

In 1980, Nüsslein-Volhard and Wieschaus reported a *Drosophila* null mutation of the gene *wingless (Wg),* first identified as a weak mutation disrupting wing patterning (Sharma and Chopra, 1976; Nüsslein-Volhard and Wieschaus, 1980). The null mutation leads to embryonic lethality and severe patterning defects.

A few years later, Nusse and Varmus discovered that inappropriate activation of the *Int*-1 gene could induce tumours in the murine mammary gland. *Int*-1 turned out to be the orthologue of *Wg* and was later on called *Wnt*-1 (Nusse and Varmus, 1982; Rijsewijk *et al.*, 1987). *Wg*/*Wnt*-1 relatives now form a multigene family with at least 20 different members identified in man, mouse, chicken, *Xenopus laevis*, *Drosophila* and *C. elegans* (for overview and references, see the Wnt webpage (http://www-leland.edu/~rnusse/wntwindow.html). In vertebrates, *Wnt* genes are expressed largely in the nervous system and mesoderm derivatives, and appear to be essential players in embryogenesis and carcinogenesis. Wnts can be divided into two main classes, the Wg/Wnt-1 (WNT) and the Wnt-5A class, based on their different functions and downstream signalling pathways (reviewed in Moon *et al.*, 1997).

Based on the epistatic interactions known in *Drosophila*, several vertebrate orthologues for the different components of the *Wg* signalling pathway were identified and assayed for their functional interactions. A number of recent reviews have discussed the WNT signalling pathway (Miller and Moon, 1996; Cadigan and Nusse, 1997; Cavallo *et al.*, 1997; Clevers and van de Wetering, 1997; Han, 1997; Dale, 1998). Here, we will focus on the recently discovered genes that regulate transactivation of WNT target genes. A schematic view of the WNT pathway is shown in Figure 1.

WNT perception

Genes of the Wnt family encode secreted glycoproteins, which probably act as ligands that bind to specific receptors. The candidate receptors for WNTs belong to the family of Frizzled (Fz) proteins (Bhanot *et al.,* 1996). Frizzled genes encode seven transmembrane proteins with an extracellular cysteine-rich domain (CRD), which is responsible for interaction with the WNT ligand. Once secreted, the availability of WNTs for their receptor is regulated. First, glycosaminoglycans are able to enhance WNT activity, possibly by regulating the level of reactive protein and/or adjusting the affinity for Fz (reviewed in Cumberledge and Reichsman, 1997). Second, there is a competition for interaction with WNT between Fz receptors and Frzb's. The *Frzb* genes encode a CRD, which is related to the extracellular domain in Fz receptors as well as a short stretch of charged residues. For instance, *Xenopus* Frzb-1 binds XWnt-8, preventing it from interacting with its receptor and antagonising its ventralising effect (Leyns *et al.*, 1997; Wang *et al.*, 1997).

Regulation of cytosolic β-catenin levels

Once WNT molecules have bound to their receptors, the cytosolic phosphoprotein Dsh becomes activated, which, in its turn, leads to inactivation of GSK-3 β. Inhibition of GSK-3 leads to elevated levels of cytosolic β-Cat. GSK-3 inhibits the WNT pathway, by phosphorylating N-terminal β-Cat residues, directing β-Cat towards the degradation pathway.

In regulating the stability of cytosolic β-Cat, GSK-3 is accompanied by at least three different molecules: APC, Axin (also

Abbreviations used in this paper: wg, wingless; WNT, Wg/Wnt-1; Fz, Frizzled; CRD, Cysteine Rich Domain; Dsh, Dishevelled; GSK-3, Glycogen Synthase Kinase 3; β -Cat, β -Catenin; APC, Adenomatous Polyposis Coli; GBP, GSK-3 Binding Protein; TCF, T Cell Factor; β -TrCP, β -Transducin repeat Containing Protein; HMG, High Mobility Group; LEF, Lymphoid Enhancer Factor; Pan, Pangolin; Pop-1, Posterior Pharynx defective 1; Ubx, Ultrabithorax; Sia, Siamois; CREB, cAMP Responsive Element Binding; CBP, CREB Binding Protein; Grg, Groucho related gene; TLE, Transducin-like Enhancer of split; AES, Amino Enhancer of split; nr-3, nodal related 3.

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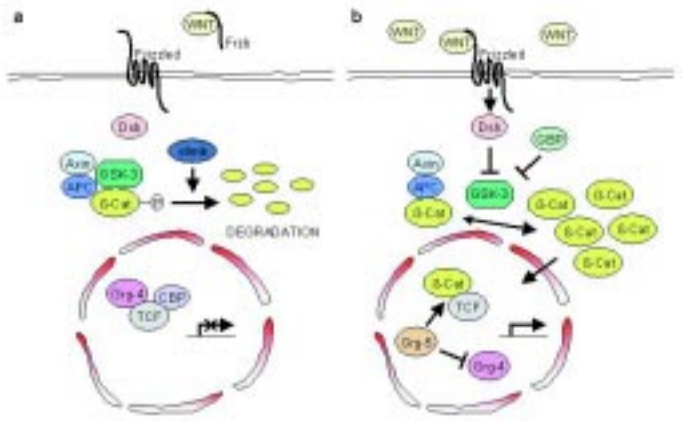

Fig. 1. Model for WNT signalling. **(a)** *When no WNT signal is perceived, GSK-3 phosphorylates cytosolic β-Cat. Phosphorylated β-Cat is degraded via the ubiquitination pathway. In the nucleus, WNT target genes are repressed.* **(b)** *When WNT binds the receptor, GSK-3 is inhibited, cytosolic β-Cat levels rise and β-Cat translocates to the nucleus. β-Cat interacts with TCF to activate WNT target genes. Fz, Frizzled; Frzb, Frizzled b; Dsh, Dishevelled; GSK-3, Glycogen Synthase Kinase 3; APC, Adenomatous Polyposis Coli; β-Cat, β-Catenin; TCF, T Cell Factor; Grg, Groucho related gene; CBP, CREB binding protein; GBP, GSK-3 Binding Protein.*

known as conductin) and GBP. APC contains a β-Cat as well as a GSK-3 binding domain.

APC functions as a tumour suppressor (Polakis, 1997). Colorectal carcinoma cell lines containing mutant APC showed elevated levels of β-Cat and constitutively activated a synthetic TCF reporter (Munemitsu *et al.*, 1995; Korinek *et al.*, 1997). Overexpression of wt APC in these cell lines significantly reduced the level of free β-Cat and suppressed the transactivation of the reporter. Overexpressing wt APC in *Xenopus* embryos and disruption of the function of an APC-related gene in *C. elegans* produced a phenotype as expected for a positive regulator of the WNT pathway (Rocheleau *et al.*, 1997; Vleminckx *et al.*, 1997). Recently, APC2 has been identified in mammals and flies (Nakagawa *et al.*, 1998; van Es *et al.*, 1999). APC2 resembles APC in its structure, and can downregulate the level of free β-Cat both in $APC^{-/-}$ colon carcinoma cells (van Es *et al.*, 1999) and in the early *Xenopus* embryo (unpublished results).

Besides a role in transcriptional activation, β-Cat has a function in cell adhesion. A large proportion of the total content of β-Cat in a cell is associated with cadherin, a molecule that has a role in establishing cell-cell contacts. The rest of the β-Cat pool is captured in a multimolecular complex with APC, GSK-3 and the scaffold protein axin (Zeng *et al.*, 1997; Behrens *et al.*, 1998; Hart *et al.*, 1998; Itoh *et al.*, 1998; Hamada *et al.*, 1999). Without axin, the complex cannot form; β-Cat is no longer phosphorylated and therefore stabilised, which can lead to e.g., the formation of additional axes in mice (Zeng *et al.*, 1997).

To date, only one molecule has been described, that can directly inhibit the action of GSK-3 and that is GSK-3 binding protein (GBP) (Yost *et al.*, 1998). GBP is a maternal protein that inhibits the *in vivo* phosphorylation by GSK-3. Ectopic expression of GBP caused stabilisation of β-Cat leading to the induction of an ectopic axis in *Xenopus* embryos. Depletion of maternal GBP mRNA demonstrated that GBP is required for endogenous axis formation (Yost *et al.*, 1998).

Recently, another regulator of cytosolic levels of β-Cat was described, both in *Drosophila* and in *Xenopus.* Loss of function of the *Drosophila* gene *slimb* and its vertebrate orthologue *β-TrCP* resulted in a cell-autonomous accumulation of high levels of β-Cat and the expression of ectopic WNT-responsive genes (Jiang and Struhl, 1998; Marikawa and Elinson, 1998). *Slimb* and *β-TrCP* genes encode a conserved F-box/WD-40 repeat protein related to yeast cdc4, a protein that targets cell-cycle regulators for degradation by the ubiquitin/proteosome pathway. Overexpression studies of β-TrCP constructs in *Xenopus* embryos, placed β-TrCP at the level of GSK-3, facilitating the degradation of β-Cat (Marikawa and Elinson, 1998).

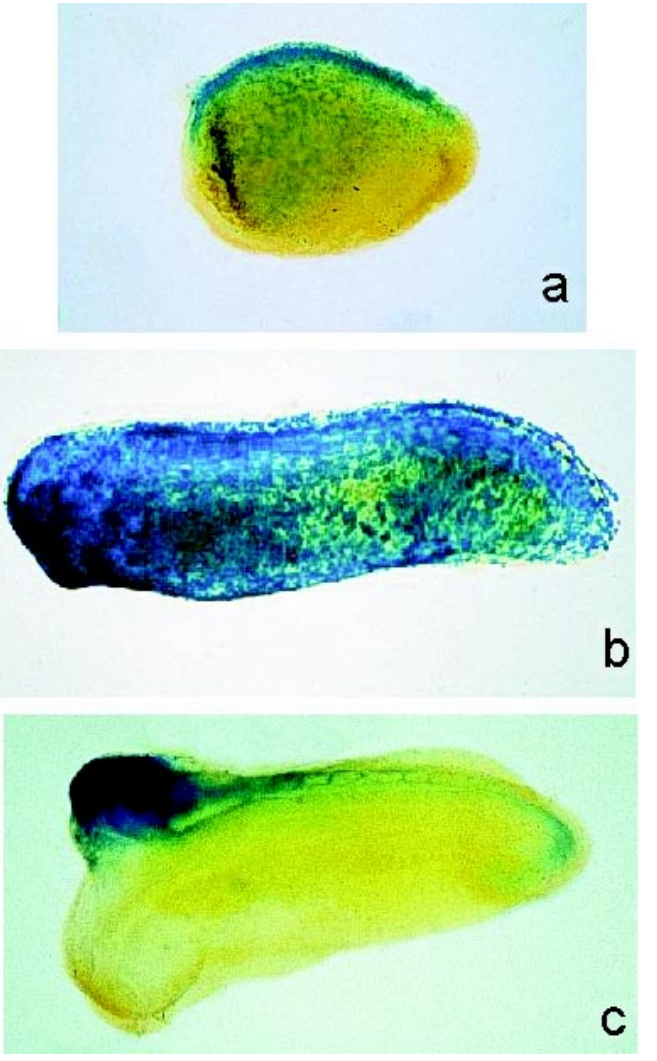

Fig. 2. ΔN-XTcf-3 suppresses, while β-Cat induces axis formation in *Xenopus* embryos. *Injection of mRNA in 4-cell stage embryos. LacZ mRNA was co-injected to trace the cells that received the mRNA. At stage 25, the embryos were fixed and stained for β-galactosidase activity.* **(a)** *Injection of ΔN-XTcf-3 into two dorsal blastomeres caused complete ventralisation of the embryo.* **(b)** *Injection of LacZ mRNA alone has no phenotypic effect.* **(c)** *Injection of β-Cat into one ventral blastomere caused axis duplication.*

Interaction of β-Cat with TCF

When a WNT signal causes elevation of cytosolic β-Cat, β-Cat is transported to and accumulates in the nucleus. We and others found that β-Cat interacts with HMG box transcription factors of the TCF/LEF family to activate transcription of WNT target genes (Behrens *et al.*, 1996; Huber *et al.*, 1996; Molenaar *et al.,* 1996). The genes of the *Tcf/Lef* family encode four different proteins: TCF-1, LEF-1, TCF-3 and TCF-4 (TCF). TCF factors were originally identified as lymphoid-specific enhancers and were later on shown to be present in many different tissues during murine embryogenesis. Although *Tcf* genes encode DNA binding proteins, when transfected together with a reporter gene, they fail to activate transcription. In *Xenopus*, the maternally expressed *XTcf*-3 was found to act directly downstream of β-Cat in embryonic axis specification (Molenaar *et al.*, 1996). Transcriptional activation of reporter genes containing recognition sites for TCF, depended on complex formation between XTcf-3 and β-Cat. Deletion of the N-terminus of XTcf-3 abrogated formation of this complex. This dominant negative ΔNXTcf-3, inhibited the activation of transcription mediated by the wt β-Cat/XTcf-3 complex, resulting in suppression of axis formation in *Xenopus* embryos (Fig. 2). To date, XTcf-3 is the only family member found to be expressed maternally (Molenaar *et al.*, 1998).

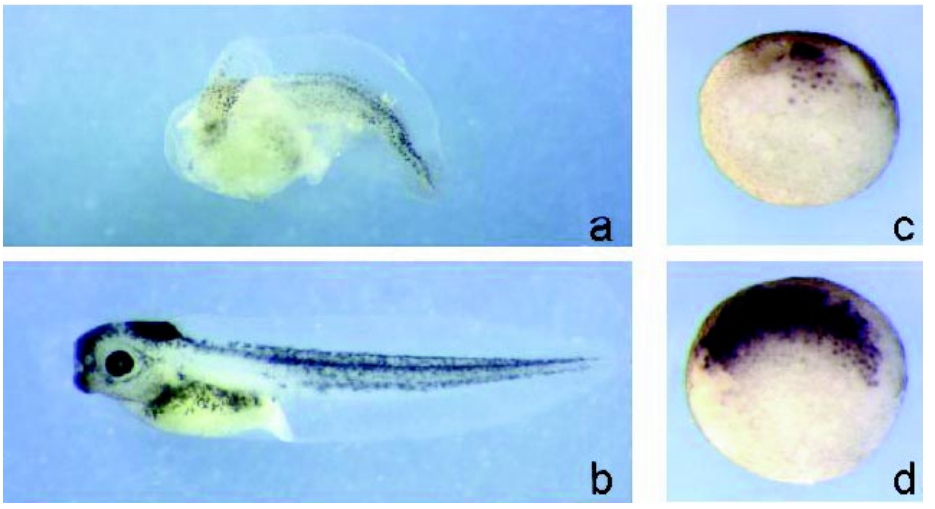

Fig. 3. XGrg-4 suppresses Xnr-3 expression and axis formation in *Xenopus* embryos. *Injection of XGrg-4 mRNA into the two dorsal blastomeres at 4-cell stage resulted in suppression of the endogenous axis* **(a)**. **(b)** *A non-injected sibling of (a). Whole-mount in situ hybridisation with antisense Xnr-3 RNA of stage 9 embryos showed a suppressed expression in embryos injected dorsally with XGrg-4* **(c)** *when compared to non-injected controls* **(d)**.

Similar results, obtained by loss of function genetics, confirmed TCF to be downstream of β-Cat in the WNT pathway. Null mutations of *Drosophila dTcf*, also named *pangolin (pan),* showed a *wg*-like segment polarity phenotype, indicating that dTCF is genetically downstream of Armadillo (ARM) (Brunner *et al.*, 1997; van de Wetering *et al.*, 1997). *Pop*-1, the TCF orthologue in *C. elegans*, is involved in WNT dependent asymmetric cell division of the EMS blastomere. However, unlike *dTcf* in *Drosophila*, *pop-1* has the opposite phenotype to that of the WNT components of the mom class (Lin *et al.*, 1995; Rocheleau *et al.*, 1997; Thorpe *et al.,* 1997). A possible solution to this seeming controversy in function of TCF will be described below.

In the absence of β-Cat, TCF may occupy the regulatory sites of target genes, but cannot activate transcription. The presence of β-Cat in a complex with TCF at the same sites does induce transactivation. Since TCF itself is transcriptionally inert, β-Cat must be the activator. Van de Wetering *et al.* (1997) showed that the C-terminus of β-Cat by itself can act as a transactivation domain, when fused to a GAL-4 DNA binding domain. Moreover, the fusion protein ARM-XTcf-3, a chimera of the C-terminus of ARM and ΔNXTcf-3, was able to activate transcription and induce an ectopic axis in *Xenopus* embryos (Roose *et al.*, 1998). This indicates that the C-terminus of β-Cat is necessary to activate transcription of WNT target genes.

WNT target genes

After the identification of TCF as a class of transcriptional regulators acting downstream from WNTs, a search for direct target genes of WNTs began. The previously identified consensus motif for TCF binding (van de Wetering *et al.*, 1997) is found in a large number of genes. Studies in *Drosophila* and *Xenopus* identified several potential WNT target genes with functional TCF sites in their promoter region. In *Drosophila*, a minimal wingless response element in the midgut enhancer of *Ultrabithorax (Ubx)* is recognised by Lef-1. In complex with ARM, Lef-1 can stimulate transcription of this *Ubx* enhancer (Riese *et al.*, 1997).

In *Xenopus*, one of the events triggered by the WNT pathway is dorsal mesoderm induction. Directly after mid blastula transition (MBT), a number of genes are induced that are responsible for the specification of dorsal mesoderm. In the promoter regions of three of these genes, *Siamois*, *Twin* and *nodal-related*-3 (Lemaire *et al.*, 1995; Smith *et al.*, 1995; Laurent *et al.*, 1997), functional binding sites for TCF factors were identified (Brannon *et al.*, 1997; Laurent *et al.,* 1997; McKendry *et al.,* 1997; Fan *et al.,* 1998). The *XSia* promoter, for example, contains three TCF sites capable of regulating its transcription (Brannon *et al.*, 1997). Mutation of these sites eliminated the β-Cat/XTcf-3 activation of a reporter. The promoter was much more active in the dorsal than in ventral blastomeres. Ventral expression of β-Cat eliminates this difference in transcriptional activity, dependent on the presence of functional TCF/LEF sites. Interestingly, mutating the TCF sites elevated the ventral expression of the reporter gene, compared to the non mutated version (Brannon *et al.*, 1997). This derepression by mutating TCF sites, in the absence of β-Cat, was also shown for the *Ubx* enhancer (Riese *et al.*, 1997). Thus TCF factors can mediate both repression and activation of the same promoter.

TCF as a repressor of transcription

Recently, binding partners have been identified, both in *Drosophila* and *Xenopus*, which are proposed to be responsible for the repressive effects of TCF factors. Waltzer and Bienz (1998) reported that a *Drosophila* CREB binding protein (dCBP) interacted with a region in the HMG box of dTCF. In the midgut, dCBP

loss of function mutants mimic the *wg* gain of function phenotype implying dCBP to be a negative regulator of the WNT pathway. dCBP repressed the *Ubx* midgut enhancer in a dTCF site dependent manner. Acetylation of dTCF by dCBP lowered the binding affinity of dTCF to ARM *in vitro.* The authors proposed that high concentrations of ARM overcome this acetylation block of dTCF and predicted that a balance between ARM and dCBP is particularly critical in cells near a low signalling threshold.

Another binding partner of TCF was identified performing a yeast two-hybrid screen for proteins interacting with human TCF-1 (Roose *et al.*, 1998). This TCF partner is the product of the murine *Groucho-related gene* 5 (*mGrg*-5) and belongs to the Groucho family of transcriptional repressors. *Drosophila* Groucho is a widely expressed co-repressor, proposed to be involved in numerous developmental processes (Hartley *et al.*, 1988; Paroush *et al.,* 1994; Fisher and Caudy, 1998; Parkhurst, 1998). In vertebrates, multiple homologues of groucho have been identified. These are termed TLE 1-4 in man and mGrg-1, 3, 4 in mouse. *mGrg*-5 encodes a naturally truncated product containing only the amino-terminal two domains of the long forms of Grg. Using *mGrg*-5 as a probe, the *Xenopus* orthologues of *Grg*-5 [*XGrg*-5 or *XAES* (Choudhury *et al.*, 1997)] and *Grg*-4 (*XGrg*-4), were cloned. These *Xenopus* Grg's are expressed maternally and throughout embryogenesis (Molenaar *et al.*, 1999). Both XGrg-4 and 5 interact with XTcf-3 in a region upstream of the HMG box, but downstream of the β-Cat binding domain. In transfection assays, the long form (XGrg-4) inhibited β-Cat/XTcf-3 induced activation of transcription of a synthetic TCF reporter and the *XSia* promoter. In contrast, the short version (XGrg-5) enhanced the β-Cat/XTcf-3 induced transcriptional activation. Dorsal injection of XGrg-4 into 4-cell stage *Xenopus* embryos repressed transcription of *XSia* and *Xnr*-3 and suppressed formation of the endogenous axis (see Fig. 3). Ectopic axis formation, induced by a dominant positive ARM-XTcf-3 fusion protein, was inhibited by XGrg-4 and enhanced by XGrg-5.

The functional relevance of this interaction was also shown for *Drosophila* Tcf and groucho (Cavallo *et al.*, 1998). dTcf and Groucho physically interact. A reduction in both dTcf and Groucho expression caused a suppression of the *wg* and *arm* segment polarity phenotype. Hence, dTcf in complex with Groucho acts as a repressor of transcription, in the absence of ARM.

Therefore, the actual transcription of TCF target genes depends on the balance between the constitutive repressive effects, mediated by long forms of Grg's, possibly counteracted by short forms of Grg's, and the activating effects of β-Cat. This dual function of TCF may also explain the observation that the *C. elegans* homologue, Pop-1, has opposite effects to that of WNT. Pop-1 possibly functions as a repressor of transcription in the asymmetric cell division of the EMS blastomere.

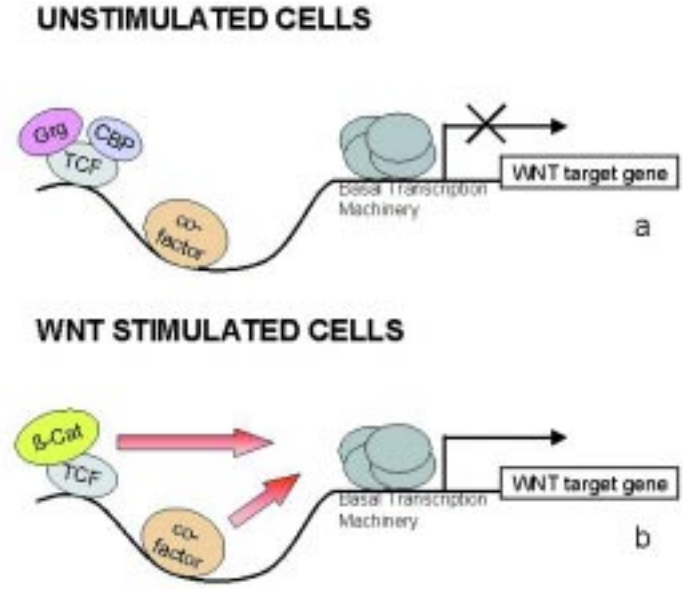

Fig. 4. Model for the action of TCF factors on *WNT* target genes. *(Adapted from M. Bienz; Curr. Opin. Cell Biol. 10, 1998, Fig. 2).* **(a)** *When no WNT signal is received, TCF occupies the binding site on the promoter region of a WNT target gene and represses transcription with the help of transcriptional repressors, such as long forms of Grg and CBP.* **(b)** *When a WNT signal is transduced, β-Cat will enter the nucleus, counteract the repression by interacting with TCF and, subsequently activate transcription of the WNT target gene. Other cell-type-specific co-factors may stimulate transcription in a cooperative fashion. TCF, T Cell Factor; Grg, Groucho related gene; CBP, CREB Binding Protein; β-Cat, β-Catenin.*

The dual role of TCF

The action of TCF factors in transactivation of WNT target genes is illustrated in the model shown in Figure 4. In unstimulated cells, TCF will bind the promoter of WNT target genes, together with other cell-type-specific factors, such as activin for the *XSia* promoter (Crease *et al.*, 1998) or dpp for the *Ubx* enhancer (Riese *et al.*, 1997). Binding of TCF in the absence of β-Cat allows repressors, like Grg or CBP, to interact with TCF and prevent transcription. Following WNT signalling, β-Cat translocates to the nucleus and associates with TCF, thereby activating transcription of WNT target genes, and with the help of short forms of Grg, β-Cat counteracts repression.

The combined repression and activation through TCF secures a tight control over WNT driven developmental decisions. At this point, it is important to learn what signals put the repressors in place, what the molecular events underlying derepression are and which molecules activate and repress the transcriptional machinery.

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