

REVIEW

How do they do Wnt they do?: regulation of transcription by the Wnt/ β -catenin pathway**H. C. Archbold,¹ Y. X. Yang,² L. Chen² and K. M. Cadigan^{1,2}**¹ Program in Cell and Molecular Biology, University of Michigan, Ann Arbor, MI, USA² Department of Molecular, Cellular and Developmental Biology, University of Michigan, Ann Arbor, MI, USA

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Correspondence: K. M. Cadigan,
Department of Molecular, Cellular
and Developmental Biology,
University of Michigan, Ann Arbor,
MI 48109-1048, USA.
E-mail: cadigan@umich.edu

Abstract

Wnt/ β -catenin signalling is known to play many roles in metazoan development and tissue homeostasis. Misregulation of the pathway has also been linked to many human diseases. In this review, specific aspects of the pathway's involvement in these processes are discussed, with an emphasis on how Wnt/ β -catenin signalling regulates gene expression in a cell and temporally specific manner. The T-cell factor (TCF) family of transcription factors, which mediate a large portion of Wnt/ β -catenin signalling, will be discussed in detail. Invertebrates contain a single TCF gene that contains two DNA-binding domains, the high mobility group (HMG) domain and the C-clamp, which increases the specificity of DNA binding. In vertebrates, the situation is more complex, with four TCF genes producing many isoforms that contain the HMG domain, but only some of which possess a C-clamp. Vertebrate TCFs have been reported to act in concert with many other transcription factors, which may explain how they obtain sufficient specificity for specific DNA sequences, as well as how they achieve a wide diversity of transcriptional outputs in different cells.

Keywords C-clamp, high mobility group domain, lymphoid enhancer-binding factor 1, T-cell factor, Wnt, β -catenin.

Wnts are a family of secreted proteins that can exert profound influences on cell behaviour through activation of several signalling pathways. In this review, we focus on the best-understood Wnt signalling pathway, sometimes called 'canonical' Wnt signalling but hereafter referred to as Wnt/ β -catenin signalling. This particular Wnt pathway acts by increasing levels of nuclear β -catenin, which then serves as a co-regulator for transcription factors that can recruit β -catenin to specific regulatory elements (Cadigan 2008, Cadigan & Peifer 2009, MacDonald *et al.* 2009). This pathway is known to play many pivotal roles in animal development (Logan & Nusse 2004, Grigoryan *et al.* 2008, Petersen & Reddien 2009, Niehrs 2010) and adult tissue maintenance (Polakis 2007, Nusse *et al.* 2008, Haegebarth & Clevers 2009, Wend *et al.* 2010). In addition, aberrant Wnt signalling has been linked to

several human diseases, most notably (but not restricted to) several cancers (Clevers 2006, Polakis 2007). This review covers a few examples of Wnt/ β -catenin signalling in normal and pathological contexts, where some information is known about the transcriptional targets. The ability of this pathway to activate diverse transcriptional programs in different contexts is remarkable, and we discuss some of the mechanisms that contribute to this diversity of transcriptional output.

Most of our attention will focus on the T-cell factor/lymphoid enhancer-binding factor 1 (TCF/LEF1) (TCF) family of high mobility group (HMG) domain proteins, which act with β -catenin to regulate numerous Wnt targets. But we also discuss other DNA-binding proteins that utilize β -catenin to regulate gene expression. It is unlikely that TCFs possess enough DNA-binding specificity to account for their ability to find specific Wnt

response elements (WREs) among the huge excess of genomic DNA sequences. How TCFs interact with other transcription factors to increase DNA-binding specificity will be discussed. These interactions likely contribute to the differences in target gene expression that the Wnt/ β -catenin pathway achieves in different cellular contexts.

β -catenin is most commonly thought of as a transcriptional co-activator, and this is reflected in our current knowledge of direct targets of Wnt/ β -catenin regulation, which are predominately activated in response to Wnt signalling. But we also discuss reports that link β -catenin to direct transcriptional repression, a less common form of Wnt regulation, but one that might be currently underappreciated among Wnt researchers.

Overview of Wnt/ β -signalling

The overall stability and nuclear localization of β -catenin is thought to play a central role in determining the level of Wnt/ β catenin signalling. In the absence of Wnt stimulation, β -catenin is constitutively inhibited by a complex (termed the β -catenin destruction complex) containing glycogen synthase kinase 3 (GSK3) and casein kinase I (CKI), as well as the scaffolding proteins Axin and adenomatous polyposis coli (APC) protein (Cadigan & Peifer 2009, Kennell & Cadigan 2009). Phosphorylation of specific residues in the N-terminus of β -catenin by CKI and GSK3 is followed by ubiquitination and proteosomal degradation (Cadigan & Peifer 2009, MacDonald *et al.* 2009), (Fig. 1). In addition, Axin and APC are thought to sequester β -catenin in the cytosol, and/or to promote β -catenin efflux from the nucleus (Brocardo & Henderson 2008).

Without Wnt signalling, the β -catenin destruction complex keeps the level of β -catenin low, restricting β -catenin to its essential role in supporting cadherin-mediated cell adhesion (Stepniak *et al.* 2009).

When Wnt protein is recognized at the cell surface by members of the Frizzled (Fz) family of proteins and low density lipoprotein receptor related protein 5 or 6 (LRP 5/6), a large complex termed the ‘Wnt signalosome’ is formed (Cadigan & Peifer 2009, MacDonald *et al.* 2009). The signalosome interacts with the β -catenin destruction complex, inhibiting its activity. This results in the accumulation of β -catenin, some of which enters the nucleus (Fig. 1).

Once in the nucleus, β -catenin can bind to several DNA-binding proteins, the best understood of which are the TCFs. Some TCFs are thought to act as a transcriptional switch, repressing Wnt target gene expression in the absence of signalling and activating transcription upon forming a complex with β -catenin (Fig. 1). There is also a wealth of information on other transcriptional co-factors that contribute to TCF repression in the absence of Wnt signalling or are required for β -catenin-dependent activation of Wnt targets. These factors, several of which alter the state of Wnt target gene chromatin, will not be discussed in detail here, but can be explored in several recent reviews (Arce *et al.* 2006, Willert & Jones 2006, Cadigan & Peifer 2009, Mosimann *et al.* 2009).

The TCF family: a historical perspective

The TCF family of transcription factors was first discovered by researchers interested in lymphocyte gene

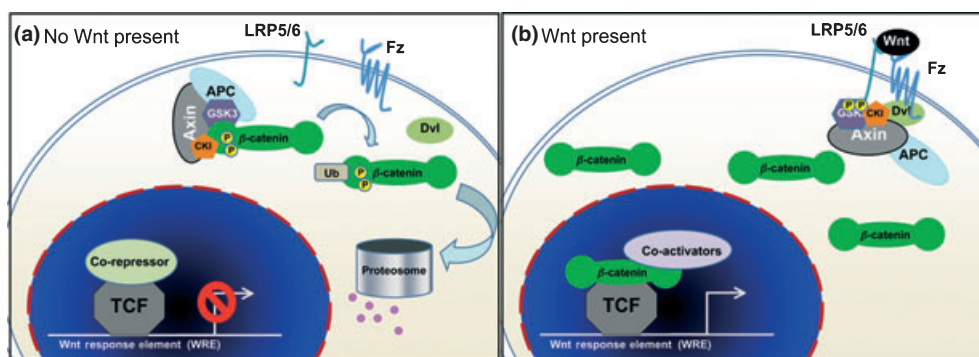


Figure 1 Basic outline of the Wnt/ β -catenin signalling pathway. (a) In the absence of the Wnt ligand, β -catenin is phosphorylated by a ‘destruction’ complex containing APC, Axin and the CKI and GSK3 kinases. These results in ubiquitylation and proteosomal degradation of β -catenin. In the nucleus, TCF can recruit co-repressors to Wnt targets, keeping their rate of transcription very low. (b) When Wnt ligand binds to the Fz and LRP5/6 co-receptors, the destruction complex moves to the plasma membrane through multiple protein–protein interactions with the receptor complex and Dvl. β -catenin is no longer phosphorylated/degraded and newly synthesized/ β -catenin accumulates in the cytosol and the nucleus. Nuclear β -catenin binds to TCFs, displacing co-repressors and recruiting co-activators to increase expression of Wnt targets. See the text for more details. APC, adenomatous polyposis coli; CKI, casein kinase I; GSK3, glycogen synthase kinase 3; TCF, T-cell factor 1; Fz, Frizzled; LRP, lipoprotein receptor related protein; Dvl, Dishevelled.

regulation. A protein originally called TCF1 α or lymphoid enhancer-binding factor 1 (LEF1) was highly expressed in pre-B and pre-T cells and bound a specific DNA sequence in an enhancer controlling the T-cell receptor α (TCR α) gene (Waterman & Jones 1990, Waterman *et al.* 1991) (Travis *et al.* 1991). Another protein enriched in immature T cells called TCF1 bound to a similar sequence in a CD3a enhancer (van de Wetering *et al.* 1991). Both LEF1 and TCF1 were found to contain a single HMG domain, which was sufficient for DNA-specific binding (Giese *et al.* 1991, Oosterwegel *et al.* 1991, Waterman *et al.* 1991). HMG domains are found in animals, plants and fungi, and TCF1 and LEF1 belong to a subgroup within this family, most closely related to the HMG domains of SOX proteins (e.g. SRY) and fungal mating type proteins (e.g. STE11) (Laudet *et al.* 1993).

In addition to sequence specific DNA binding, the HMG domain of LEF1 has been shown to bend DNA

up to 130°, (Giese *et al.* 1992) which was confirmed by solving the crystal structure of a LEF1–DNA-binding site complex (Love *et al.* 1995). This bending has been proposed to play an architectural role in coordinating the binding of several other factors to the TCR α enhancer (Carlsson *et al.* 1993, Giese & Grosschedl 1993, Giese *et al.* 1995). This protein–enhancer complex is sometimes referred to as the ‘T cell enhanceosome’ (Balmelle *et al.* 2004). The high degree of conservation between the HMG domains of the TCF family (Fig. 2) suggests that all members have the ability to bend DNA, though this remains to be tested directly.

Although interest in LEF1 and TCF1 was initially focused on lymphocytes, the finding that mice lacking a functional LEF1 gene displayed defects to several organ systems indicated a much broader role in developmental biology (van Genderen *et al.* 1994). This connection was further solidified by the findings that LEF1 and TCF3 (another member of the family) could bind to

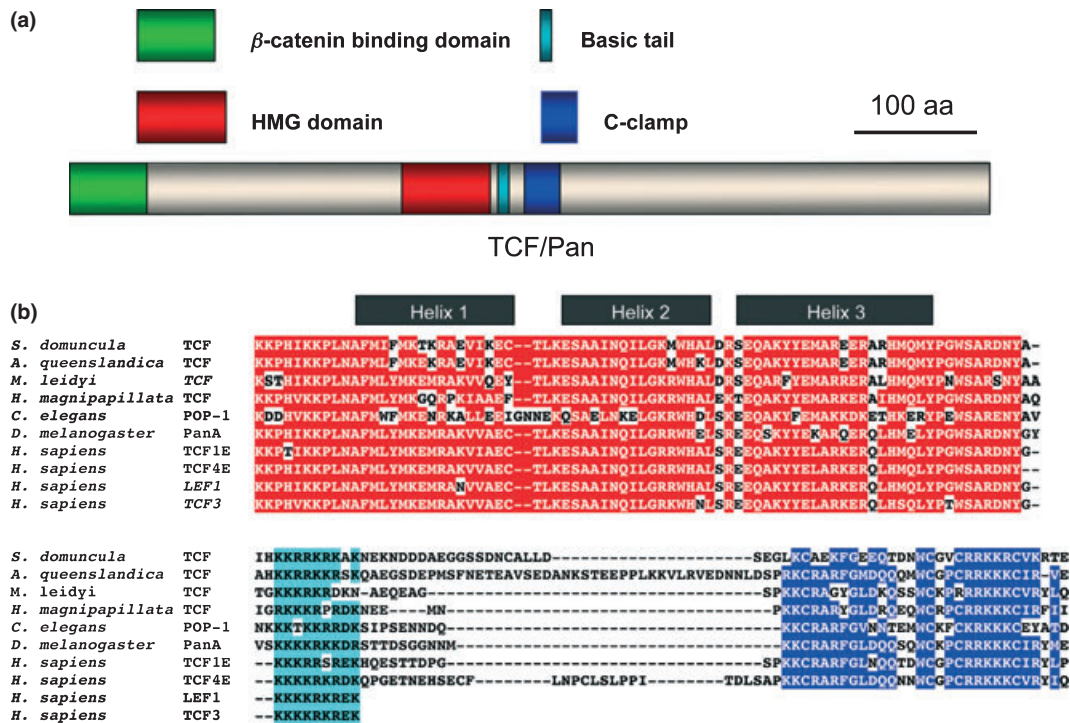


Figure 2 (a) Cartoon depicting the *Drosophila* TCF/Pan (the PanA isoform; 751 aa) showing the location of the β -catenin binding domain (green), the HMG domain (red), the basic tail (aqua) and the C-clamp (blue). (b) Alignment of the HMG domains, basic tails and C-clamps among metazoan TCFs. Non-conserved residues are not coloured in the alignment. The positions of the three α -helices of the HMG domain, based on the structure of LEF1 (Love *et al.* 1995) are indicated at the top of the figure. The six invertebrate TCFs possess all three domains, while only the E box isoforms of vertebrate TCF1 and TCF4 possess C-clamps. The degree of conservation in the HMG domain is quite high, e.g. the TCF of *Suberites domuncula* and human TCF4E are 79.5% identical, 85.9% conserved. The C-clamp is less conserved (55.2% identity; 58.6% for the *S. domuncula*-human TCF4E comparison). The number of non-conserved residues between the basic tail and C-clamps are highly variable. The GenBank accession number of each protein sequence is in parentheses: *S. domuncula* (CAH04889.1); *Amphimedon queenslandica* (ADO16566.1); *Mnemiopsis leidyi* (ADO34164.1); *Hydra magnipapillata* (XP_002159974.1); *Caenorhabditis elegans* (NP_491053.3); *Drosophila melanogaster* (isoform A; NP_726522); human TCF1E (EAW62279.1); TCF4E (CAB97213.1); LEF1 (NP_001124185) and TCF3 (NP_112573.1). TCF, T-cell factor 1; HMG, high mobility group; LEF1, lymphoid enhancer-binding factor 1.

β -catenin (Behrens *et al.* 1996, Huber *et al.* 1996, Molenaar 1996). It was known that mutants in the fly homolog of β -catenin, *armadillo* (*arm*) caused defects very similar to *wingless* (*wg*) mutants, a Wnt gene important in many aspects of fly development (Riggleman *et al.* 1989, Peifer *et al.* 1991, Noordermeer *et al.* 1994, Siegfried *et al.* 1994). In addition, misexpression of β -catenin in ventral blastomeres of *Xenopus* embryos induced a secondary body axis (Heasman *et al.* 1994), reminiscent of misexpression of several Wnt genes (McMahon & Moon 1989, Smith & Harland 1991, Sokol *et al.* 1991).

The N-terminus of TCFs are required for binding to β -catenin, and deletion of this portion of a TCF gene produces a protein that can dominantly inhibit Wnt signalling in several organisms (Behrens *et al.* 1996, Molenaar 1996, van de Wetering *et al.* 1997, Kratochwil *et al.* 2002). In addition, placing multiple copies of high affinity TCF binding sites upstream of a minimal promoter-reporter gene cassette results in reporter gene expression that is highly activated by Wnt/ β -catenin signalling (Molenaar 1996, Korinek *et al.* 1997, van de Wetering *et al.* 1997). These now classic observations contribute to the current working model of Wnt target gene activation depicted in (Fig. 1).

Wnt/ β -catenin signalling in development

Stimulation of the Wnt/ β -catenin pathway can result in a diverse array of cellular outcomes, depending on the context. For example, in *Drosophila*, the fly Wnt protein Wg acts through Arm to promote cell stem identity (Lin *et al.* 2008, Takashima *et al.* 2008, Sinenko *et al.* 2009). While Wg/Arm signalling stimulates proliferation in several tissues (Baker 2007, Herranz & Milan 2008), the pathway can also repress the cell cycle (Duman-Scheel *et al.* 2004). The same is true for apoptosis, where Wg/Arm signalling can activate (Cox *et al.* 2000, Lin *et al.* 2004) or repress (Cox *et al.* 2000, Giraldez & Cohen 2003) programmed cell death. In some contexts, such as the developing wing, Wg acts as a morphogen, activating distinct targets in a concentration dependent manner (Zecca *et al.* 1996, Neumann & Cohen 1997). Since most transcriptional targets of Wg/Arm signalling are regulated in a cell-type (Lee & Frasch 2000, Knirr & Frasch 2001) or temporally restricted manner (Heemskerk *et al.* 1991), it is not surprising the pathway can effect different cells in dramatically different ways.

The following sections summarize some of the vast literature covering Wnt/ β -catenin signalling in development, spanning the entire metazoan clade. Understanding the molecular basis for the specificity of transcriptional outcome in these different systems requires a detailed understanding of how stabilized β -catenin regulates gene expression, and this review

discusses the field's progress towards realizing this challenging goal.

Wnt/ β -catenin signalling in establishing the primary body axes of metazoans

Wnts and β -catenin are not found in choanoflagellates (King *et al.* 2008), but are present in all metazoans. Three Wnt genes are found in the demosponge *Amphimedon queenslandica* (Adamska *et al.* 2010) and four Wnt genes are present in the *Mnemiopsis leidyi* genome (Pang *et al.* 1999). The sea anemone, *Nematostella vectensis* has 14 Wnt genes, including 12 of the 13 subgroups found in mammals (Kusserow *et al.* 2005). This suggests a radiation of the Wnt family after the cnidarian/bilaterian common ancestor diverged from sponges and ctenophores. For a more comprehensive list of Wnt gene number across metazoan evolution see (Lengfeld *et al.* 2009).

The expression patterns of Wnts in simple metazoans are highly suggestive of important roles in development. In *Amphimedon* larvae Wnt is expressed at the posterior end (Adamska *et al.* 2010). In *Hydra*, Wnt3 is expressed at the prospective oral pole of embryos and larvae, which will give rise to the head of the adult (Hobmayer *et al.* 2000, Plickert *et al.* 2006, Duffy *et al.* 2010). In *Nematostella* embryos, Wnts are expressed in overlapping patterns along the anterior/posterior (A/P) axis, suggesting that they provide positional information along the primary body axis (Kusserow *et al.* 2005). The expression of the four ctenophore Wnts in complementary dynamic patterns during embryogenesis also suggests important developmental roles in this organism (Pang *et al.* 1999).

In addition to the presence of Wnts, simple metazoans possess most of the downstream signalling components identified in bilaterians. For example, sponges, ctenophores and cnidarians all contain one gene encoding β -catenin, GSK3 and TCF, which are well conserved with those in other metazoans. Consistent with the existence of a functional Wnt/ β -catenin pathway, application of GSK3 inhibitors, well known to stabilize β -catenin and TCF transcriptional readouts in mammalian cell culture (Cohen & Goedert 2004), produce dramatic phenotypes in these organisms. In sponges, GSK3 inhibition leads to ectopic formation of ostia (canal openings), which can disrupt feeding (Lapebie *et al.* 2009, Windsor & Leys 2010). GSK3 inhibition in *Hydra* produces multiple head and tentacles along the body (Broun *et al.* 2005, Muller *et al.* 2007, Duffy *et al.* 2010). In the marine cnidarian *Hydractinia echinata*, this multiple head phenotype is suppressed by RNAi depletion of TCF (Duffy *et al.* 2010). In *Hydra*, depletion of β -catenin results in loss of head structures, opposite to the phenotype obtained with GSK3 inhibition (Gee

et al. 2010). These results indicate that the basic outlines of the pathway in these organisms are likely operating in a similar fashion to that outlined in Fig. 1.

The direct transcriptional targets of Wnt/ β -catenin signalling in these simple systems are currently not known but one candidate in cnidarians is the *Brachyury* gene, known to be a direct target of Wnt3a signalling in mouse embryos (Yamaguchi *et al.* 1999). In *Hydractina*, *Brachyury* is expressed at the oral pole (like Wnt3a) and is upregulated by GSK3 inhibition and downregulated by Wnt3a and TCF depletion (Duffy *et al.* 2010). In addition, this report provided evidence that Wnt3 and TCF are positively regulated by the pathway in *Hydractina* (Duffy *et al.* 2010). Regulation of TCFs by the Wnt/ β -catenin pathway is also found in mammalian systems (Hovanec *et al.* 2001). Autoregulation of Wnt gene expression by the pathway is also likely in *Hydra*, where in addition to Wnt3 (Hobmayer *et al.* 2000) six other Wnt genes are expressed at the oral end of the organism (Lengfeld *et al.* 2009). During head regeneration, expression of Wnt3 precedes the other Wnts, suggesting that they may be activated by Wnt3 signalling (Lengfeld *et al.* 2009).

In bilaterians, there is also abundant evidence that Wnt/ β -catenin signalling is critical for axial patterning. In *Planaria*, depletion of β -catenin results in additional head structures in posterior regions (Gurley *et al.* 2008, Iglesias *et al.* 2008, Petersen & Reddien 2008). The pathway is also required for the formation of posterior structures in mouse embryos (Liu *et al.* 1999, Yamaguchi *et al.* 1999, Huelsken *et al.* 2000, Kelly *et al.* 2004) and Wnt/ β -catenin signalling plays a similar role in specifying cell identities along the A/P axis of the CNS of *Xenopus* (Niehrs 2010). When one considers that the oral pole of cnidarians likely corresponds to the posterior end of the body plan (Meinhardt 2002, Guder *et al.* 2006, Niehrs 2010), it appears that the role of high levels of Wnt/ β -catenin signalling in promoting posterior identity may be very ancient and predate the split between cnidarians and bilaterians (Petersen & Reddien 2009, Niehrs 2010) (Fig. 3).

Two notable exceptions to the global role for Wnt/ β -catenin signalling in establishing the A/P body axis are *Drosophila* and *Caenorhabditis elegans*. In flies, Wg/Arm signalling is involved in establishing A/P identity in each segment (Sanson 2001), while the maternally provided transcription factor Bicoid is a major determinant of global A/P patterning (Porcher & Dostatni 2010). This is likely the result of the high degree of specialization that has occurred in dipteran evolution (Riechmann & Ephrussi 2001) and the role of Wnt/ β -catenin signalling in other insects is similar to other metazoans [reviewed in (Niehrs 2010)]. In *C. elegans*, asymmetric cell divisions (mostly along the A/P axis) are controlled by the Wnt/ β -catenin/asymmetry

pathway, in which two β -catenin proteins (WRM-1 and SYS-1) act through distinct mechanisms to regulate the TCF family member POP-1. WRM-1 promotes POP-1 nuclear efflux while SYS-1 acts in a similar manner as β -catenin/Arm (Mizumoto & Sawa 2007). It is not clear how the five *C. elegans* Wnts can provide positional information to regulate all the asymmetric cell divisions controlled by POP-1, but there is evidence that transiently expressed Wnt (MOM-2) in posterior blastomeres can maintain A/P polarity in other cells through a Wnt-dependent relay mechanism (Bischoff & Schnabel 2006). While the rapid generation time and stripped down genomes of *Drosophila* and *C. elegans* have made them powerful genetic systems for understanding the molecular basis of development (including Wnt/ β -catenin signalling) it appears that their overall developmental strategies for axial patterning have diverged from the general metazoan scheme.

Wnt/ β -catenin signalling in establishing the A/P axis of vertebrates

In addition to the Wnt/ β -catenin pathway forming a gradient of Wnt signalling along the A/P axis, a gradient of BMP signalling along the dorsal/ventral (D/V) axis is found in a wide array of bilaterians. Niehrs (2010) has proposed a Cartesian coordinate system of Wnt and BMP signalling to control bilaterian body axes. In *Xenopus* embryogenesis, these perpendicular gradients are initiated by the Spemann organizer (De Robertis & Kuroda 2004, Vonica & Gumbiner 2007). Maternally provided *Wnt5a* and *Wnt11* (Tao *et al.* 2005, Cha *et al.* 2008) and other Wnt signalling components (White & Heasman 2008) are relocated to the future dorsal side of the embryo opposite to sperm entry. Wnt/ β -catenin signalling then activates the expression of two homeodomain transcription factors, *Siamois* and *Twin*, which establish organizer identity (Ishibashi *et al.* 2008). Analysis of the regions upstream of the *twin* and *siamois* transcription start sites (TSSs) demonstrated the existence of functionally important binding sites for TCFs (Brannon *et al.* 1997, Laurent *et al.* 1997, Fan *et al.* 1998), which is supported by more recent data with chromatin immunoprecipitation (ChIP) of TCF3 (Hikasa *et al.* 2010). *Siamois*, *Twin* and Wnt/ β -catenin signalling then act together to activate expression of a variety of BMP antagonists, which emanate from the organizer to set up a gradient of BMP4 signalling activity across the D/V axis (De Robertis & Kuroda 2004, Vonica & Gumbiner 2007).

In addition to expressing BMP antagonists, the Spemann organizer also expresses antagonists of Wnt/ β -catenin signalling, such as *Dickkopf1* (*Dkk1*), *Cerberus* and secreted frizzled related protein 2 (*sFRP2*). *Dkk1* is thought to be directly activated by TCF and

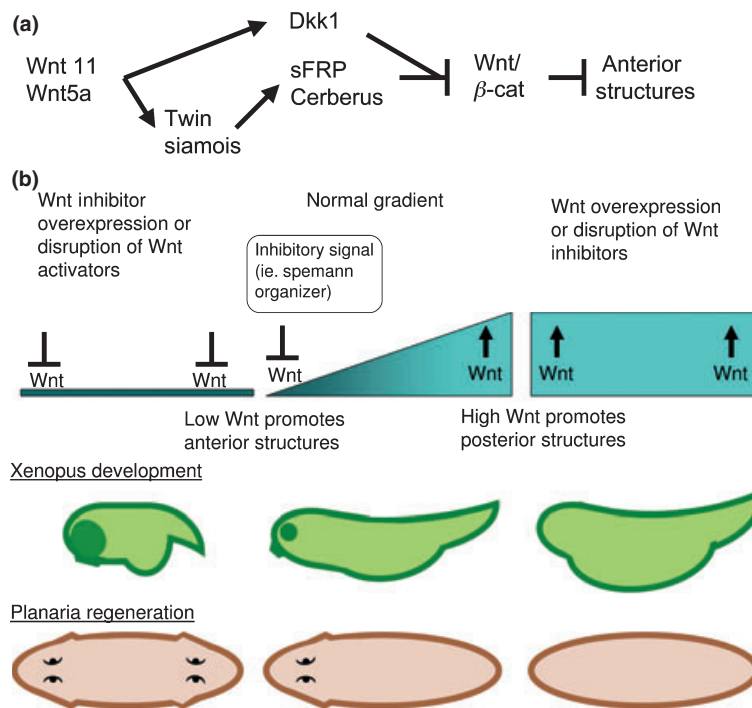


Figure 3 Wnt/ β -catenin signalling in establishing the metazoan A/P axis. (a) Some of the circuitry involved in establishing the A/P axis in *Xenopus*. Maternal Wnt11 and Wnt5a act through β -catenin to directly activate the Spemann organizer genes *siamois* and *twin* (Brannon *et al.* 1997, Laurent *et al.* 1997, Fan *et al.* 1998, Tao *et al.* 2005, Cha *et al.* 2008). Wnt/ β -catenin signalling also activates expression of *Dkk1* (Chamorro *et al.* 2005), and Siamois and Twin are thought to activate *cerberus* and *sFRP* expression (Engleka & Kessler 2001, Yamamoto *et al.* 2003a). These three Wnt antagonists suppress pathway activation in dorsal/anterior structures, allowing anterior structures to form (Niehrs 2006). See the text for more details. (b) Cartoon of an amphibian tadpole or regenerating *Planaria*, illustrating the inhibition of Wnt/ β -catenin signalling promotes an anterior identity, while expression of Wnt3a promotes a posterior identity (centre column). Expression of Wnt antagonists in *Xenopus* or reduction of β -catenin activity in *Planaria* causes an expansion of anterior structures at the expense of posterior structures (Niehrs 2006, Petersen & Reddien 2009). Conversely, inappropriate activation of Wnt/ β -catenin signalling caused loss of anterior structures. See text for further explanation. A/P, anterior/posterior.

β -catenin (Chamorro *et al.* 2005), while Cerberus and sFRP2 are Siamois-dependent (Engleka & Kessler 2001, Yamamoto *et al.* 2003a). These Wnt antagonists contribute to a Wnt/ β -catenin signalling gradient with the highest Wnt signalling in the posterior (De Robertis & Kuroda 2004, Vonica & Gumbiner 2007). Overexpression of *Dkk1* (decreasing signalling levels) expands anterior structures and morpholino depletion of *Dkk1* (increasing signalling levels) results in reduced anterior identity (Niehrs 2006). Consistent with this, disruption of the mouse *Dkk1* gene results in embryos with a loss of head (Mukhopadhyay *et al.* 2001). In *Hydra*, Wnt/ β -catenin signalling inhibits *Dkk* expression, indicating the existence of an ancient Wnt-Dkk axial patterning circuit (Guder *et al.* 2006, Niehrs 2010).

Neural crest: cross-regulation facilitates Wnt-dependent context specificity

One outcome of the Wnt/ β -catenin signalling gradient in vertebrates is the induction of the neural crest (NC).

In *Xenopus*, low BMP4 signalling and other signals in the dorsal portion of the embryo are required for induction of the neural plate (Stern 2005). After the neural plate invaginates to form the neural tube, NC cells are specified near the dorsal-lateral portion of the neural tube (Barembaum & Bronner-Fraser 2005). Wnt/ β -catenin signalling is required for this induction, and the Wnt3a expressed in the overlying epidermis appears to be the ligand (McGrew *et al.* 1997, Li *et al.* 2009). The gradient of Wnt signalling from the posterior of the embryo is required to prevent NC induction in the anterior neural fold (Li *et al.* 2009). After specification, NC cells migrate to different locations to differentiate into a diverse array of tissues, including facial cartilage, bones and smooth muscle cells of the heart (Sauka-Spengler & Bronner-Fraser 2006).

How Wnt/ β -catenin signalling induces the NC is being elucidated in detail. There are over a dozen transcription factors that have been shown to be required for specification of the NC cell fate (Barembaum & Bronner-Fraser 2005, Sauka-Spengler &

Bronner-Fraser 2006). The expression of many of these genes has been shown to be Wnt-dependent, but they also cross-regulate each other, making it difficult to identify the direct targets of the Wnt/ β -catenin pathway. Recently, two reports have used a combination of approaches to provide evidence that *Gbx2* and *Meis3* are important direct Wnt targets in NC induction in *Xenopus* (Li *et al.* 2009, Elkouby *et al.* 2010). Hormone-inducible versions of TCF or β -catenin (fused to the glucocorticoid receptor) demonstrated activation in the absence of protein synthesis. An enrichment of β -catenin at the regulatory regions of these genes was demonstrated via ChIP. Finally, predicted TCF sites in the regulatory regions were mutated, leading to a loss of reporter gene expression (Li *et al.* 2009, Elkouby *et al.* 2010). These results argue that these genes are direct targets of the pathway.

Consistent with the above data, *Gbx2* morphants display a loss of many NC markers (Li *et al.* 2009). Depletion of *Meis3* has a similar phenotype, including a reduction in *Gbx2* expression (Elkouby *et al.* 2010). *Meis3* is clearly a major target of Wnt/ β -catenin signalling, because exogenous addition of the *Meis3* gene can rescue the loss of NC seen in *Wnt3a* morphants (Elkouby *et al.* 2010). These studies suggest that Wnt/ β -catenin signalling initially induces both *Meis3* and *Gbx2*, and *Meis3* contributes to *Gbx2* expression. In a similar manner, *Slug 2* is directly activated by the Wnt/ β -catenin pathway (Vallin *et al.* 2001) but also requires *Meis3* and *Gbx2* for expression (Li *et al.* 2009, Elkouby *et al.* 2010). In addition, *Gbx2* is a transcription repressor, which inhibits the expression of anterior neural fold markers such as *Six1*, restricting NC induction to posterior regions (Fig. 4).

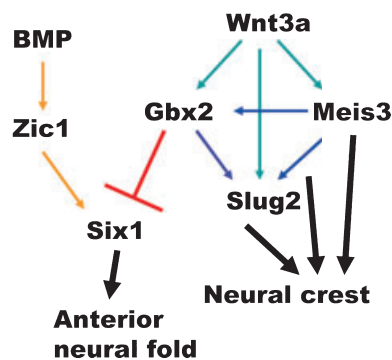


Figure 4 Wnt/ β -catenin signalling induces neural crest. *Wnt3a* from the overlying epidermis induces expression of *Meis3* and *Gbx2* are induced in the neural tube (Li *et al.* 2009, Elkouby *et al.* 2010). *Meis3* also cross regulates *Gbx2* (Elkouby *et al.* 2010) and *Slug2* is activated by a combination of *Wnt3a*, *Gbx2* and *Meis3* (Vallin *et al.* 2001, Li *et al.* 2009, Elkouby *et al.* 2010). See text for further information.

The cross-regulation of Wnt targets *Meis3* and *Gbx2* is one illustration of the complex regulatory networks that are found in development. Such cross-regulation can also occur between the TCF- β -catenin complex and its targets. *Wnt3a* is essential for posterior axial growth in mice (Yamaguchi *et al.* 1999, Aulehla *et al.* 2003, Dunty *et al.* 2008). A similar loss of caudal structures is observed when combinations of the caudal homeobox genes (*Cdx1*, *Cdx2* or *Cdx4*) are mutated in mice or zebrafish (Shimizu *et al.* 2005, Davidson & Zon 2006, Young *et al.* 2009). *Cdx* genes are activated by Wnt/ β -catenin signalling (Ikeya & Takada 2001, Gaunt *et al.* 2003) and in some cases this regulation is direct (Lickert *et al.* 2000, Ikeya & Takada 2001, Pilon *et al.* 2006). However, *Wnt3a* expression also requires *Cdx* activity, and loss of function *Cdx2* and *Cdx4* phenotypes can be rescued by an activated version of LEF1 (Young *et al.* 2009). A similar positive feedback loop between Wnt and *Cdx* has also been reported to be required for posterior development in *Xenopus* (Faas & Isaacs 2009). Interestingly, *Cdx1* autoregulation has been reported to require a physical interaction between LEF1 and *Cdx1* (Beland *et al.* 2004), providing a clue as to how TCF family members and *Cdx* proteins act together. A similar relationship has been demonstrated between the Wnt target *Brachyury* and the Wnt/ β -catenin pathway in promoting posterior mesoderm development (Martin & Kimelman 2008).

Cardiogenesis: reiterative Wnt/ β -catenin signalling required

Beyond helping establish the basic body plan, Wnt/ β -catenin signalling is important for a multitude of developmental decisions. These include limb formation, bone, hair and teeth development as well as formation of every major organ (see (Grigoryan *et al.* 2008) for a comprehensive review of β -catenin-dependent developmental processes in mice). In this review, we will focus on heart formation, which provides a good example of how the Wnt/ β -catenin pathway is used repeatedly to achieve different outcomes in different developmental contexts, even in the same tissue. Further information on the role of canonical and non-canonical Wnt signalling in heart development can be found in additional reviews (Cohen *et al.* 2008, Kwon *et al.* 2008, Gessert & Kuhl 2010).

Although the tube-like insect heart is morphologically different from the multichambered vertebrate heart, they appear to share a common ancestry. One striking example supporting this view is provided by the *tinman/Nkx2.5* gene, which is required for heart formation in *Drosophila* and several vertebrate systems (Bodmer & Venkatesh 1998, Evans 1999). In flies, Wg/Arm signalling is required for *tinman* expression and heart

formation in general (Wu *et al.* 1995, Park *et al.* 1996). In contrast, the first studies in vertebrates found that Wnt/ β -catenin signalling is required to restrict specification of cardiac mesoderm (Marvin *et al.* 2001, Schneider & Mercola 2001, Lickert *et al.* 2002).

This paradox between invertebrate and vertebrates has been resolved by the realization that the Wnt/ β -catenin pathway plays two opposing roles in early vertebrate cardiogenesis. Temporal control of Wnt expression revealed that before gastrulation, the pathway activates *Nkx2.5* expression in zebrafish embryos (Ueno *et al.* 2007). After gastrulation, the previously described inhibitory role was evident. This biphasic relationship was also observed in mouse ES cells, which spontaneously differentiate into cardiomyocytes. Activation of Wnt/ β -catenin signalling in early cultures dramatically enhanced cardiomyocyte differentiation but pathway activation in later cultures reduced cardiogenesis (Ueno *et al.* 2007). These data suggest that the initial pro-cardiogenic effect of the Wnt/ β -catenin pathway in vertebrates is analogous to the positive effect that Wg/Arm signalling has on heart development in the fly.

What are some of the direct targets in the initial regulation of cardiogenesis by the pathway? In flies, the presence of functional TCF binding sites in the enhancers that drive the expression of the transcription factors Sloppy paired 1 (*Slp1*) and Even-skipped (*Eve*) in the cardiac precursors provides strong evidence that these genes are directly activated by Wg/Arm signalling (Halfon *et al.* 2000, Lee & Frasch 2000, Knirr & Frasch 2001, Han *et al.* 2002). In mouse ES cells, gene profiling revealed many potential targets, including *Brachyury*, *Mesp1* and *Sox17* (Liu *et al.* 2007, Ueno *et al.* 2007). In regard to repression of cardiogenesis, the homeodomain protein *Hex* is an important Wnt target that is repressed in the presumptive cardiac mesoderm (Foley & Mercola 2005). In *Xenopus*, *GATA6* expression is repressed by Wnt/ β -catenin signalling, and forced expression of *GATA6* is sufficient to rescue many aspects of heart development that are disrupted by ectopic Wnt pathway activation (Afouda *et al.* 2008). The mechanism by which these genes are repressed by Wnt/ β -catenin signalling is not known.

After the initial specification of the presumptive heart field, a population of cardiomyocytes known as the secondary heart field (SHF) will give rise to the future right ventricle and inflow and outflow tracts of the heart (Dyer & Kirby 2009). Conditional knockout of β -catenin causes a significant reduction in these structures (Ai *et al.* 2007, Cohen *et al.* 2007, Klaus *et al.* 2007, Kwon *et al.* 2007, Tian *et al.* 2010). Removal of β -catenin before SHF specification resulted in a loss of *Islet1* (a LIM homeodomain) expression, a marker of SHF cells (Cai *et al.* 2003). There is some evidence to

indicate that the activation of *Islet1* transcription by Wnt/ β -catenin signalling is direct (Lin *et al.* 2007). Later removal of β -catenin in the developing SHF resulted in normal *Islet1* expression, but the right ventricle and outflow tracts still failed to form (Ai *et al.* 2007, Kwon *et al.* 2007). These results indicate multiple roles for Wnt/ β -catenin signalling in SHF development, both in establishing the SHF and subsequent differentiation of heart tissue.

The complexity of target gene regulation by the Wnt/ β -catenin pathway in the development of the secondary heart field is evidenced by the findings that Wnt/ β -catenin signalling represses *Islet1* expression in differentiating cardiomyocytes (Kwon *et al.* 2009). Likewise, *GATA6*, which is repressed by Wnt/ β -catenin signalling in early heart development in *Xenopus* (Afouda *et al.* 2008), is directly activated by Wnt/ β -catenin signalling in the posterior SHF (Tian *et al.* 2010). The factors that enable the same pathway to both activate and repress the same targets during the cardiomyocyte cell lineage are not known.

There are several additional events in heart development where Wnt/ β -catenin signalling is also required. Mutations lowering pathway activity in cardiac NC cells result in defects to the cardiac outflow tract (Hamblet *et al.* 2002, Kioussi *et al.* 2002). This phenotype is similar to that observed when the gene encoding the bicoid homeodomain protein *PitX2* is mutated (Kioussi *et al.* 2002). *PitX2* is directly activated by LEF1 and β -catenin and then *PitX2* subsequently recruits β -catenin to the *Cyclin D2* regulatory region, activating this cell cycle regulator and promoting proliferation of the cardiac NC cells (Kioussi *et al.* 2002). The Wnt/ β -catenin pathway also promotes endocardial cell proliferation, which contributes to heart valve formation (Gitler *et al.* 2003, Hurlstone *et al.* 2003, Liebner *et al.* 2004, Alfieri *et al.* 2010). Likewise, loss of β -catenin in the developing epicardium results in defects in coronary artery formation (Zamora *et al.* 2007).

Heart development highlights the multiple roles that the Wnt/ β -catenin pathway plays in regulating cell fate and organogenesis (Fig. 5). How can one signalling pathway be utilized so many times to regulate different genes in the cardiac cell lineage? Understanding how Wnt transcriptional output diversity is generated requires a more detailed understanding of how TCF family members and other transcription factors that mediate Wnt/ β -catenin-dependent gene regulation function on target gene chromatin.

Wnt/ β -catenin signalling in stem cell biology and regeneration

In addition to the myriad roles that the Wnt/ β -catenin pathway plays in normal development, it is also a key

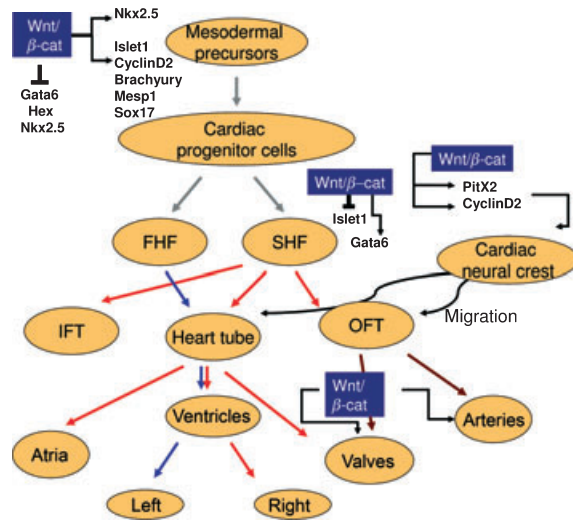


Figure 5 Wnt/ β -catenin signalling (Wnt/ β -cat) has multiple roles in heart development. This schematic depicts a few of the roles the pathway plays in mammalian heart development, both as an activator and as a repressor of gene activity. Prior to gastrulation, *nkx2.5* is positively activated by Wnt/ β -cat signalling, while it is repressed after gastrulation, as are cardiogenic factors GATA6 and Hex (Foley & Mercola 2005, Ueno *et al.* 2007, Afouda *et al.* 2008). Activation of a number of Wnt target genes early in development, such as *Islet1* have been linked to the proliferation of cardiac progenitor cells (Lin *et al.* 2007). These *Islet1*⁺ cells contribute to the second heart field (SHF), inflow tract (IFT) and outflow tract (OFT). The SHF is required for proper formation of the atria and the right ventricle. In the posterior SHF, Wnt activates GATA6 (Tian *et al.* 2010), and represses *Islet1* expression (Kwon *et al.* 2009). Wnt/ β -cat signalling is also instrumental in driving proliferation of cardiac neural crest cells (Kioussi *et al.* 2002), which migrate to the heart tube and OFT, and is important in valve and artery formation (Zamora *et al.* 2007) along with endocardial proliferation (Gitler *et al.* 2003, Hurlstone *et al.* 2003, Liebner *et al.* 2004, Alfieri *et al.* 2010). See text for more information. FHF, first heart field.

regulator of adult tissue homeostasis. The role of Wnt/ β -catenin signalling in stem cell maintenance has been well documented in the intestine (Pinto & Clevers 2005, Barker & Clevers 2010), hair follicles and in the skin (Blanpain *et al.* 2007, Blanpain & Fuchs 2009, Haegbarth & Clevers 2009). In the mouse small intestine, *TCF4* and β -catenin are required for maintenance of the crypt stem cells (Pinto & Clevers 2005). Microarray profiling has identified many transcriptional targets of the Wnt/ β -catenin pathway (van de Wetering *et al.* 2002, Van der Flier *et al.* 2007). One biologically important target is *c-myc*, which is required for normal intestinal crypt development (Muncan *et al.* 2006). As will be discussed in the following section, *c-myc* appears to be a direct target of the pathway. Another gene that is activated by Wnt/ β -catenin signalling encodes the

orphan receptor Lgr5, which has received a great deal of attention, since it marks a population of multipotent cells in the crypt that give rise to all the specialized cells of the intestinal epithelia (Barker & Clevers 2010). Another biologically important Wnt target is the bHLH transcription factor Achaete scute-like 2 (*Ascl2*), which is required for the maintenance of the Lgr5-positive stem cells and can induce crypt hyperplasia upon forced expression (van der Flier *et al.* 2009). Both Lgr5 and *Ascl2* were identified in a genome-wide survey of chromatin enriched for TCF4 binding (Hatzis *et al.* 2008), suggesting they may be direct targets of Wnt/ β -catenin signalling. In addition to intestinal stem cells, Lgr5 and the related protein Lgr6 also appear to mark stem cells populations in several other organs as well (Haegbarth & Clevers 2009, Snippert *et al.* 2010).

Wnt/ β -catenin signalling has also been linked to the ability to replace damaged cells or to regenerate deleted tissues in a wide array of metazoans. In Hydra and Planaria, organisms renowned for their ability to regenerate large portions of their bodies after bisection, the pathway promotes posterior cell fates as described in the previous section. In Hydra, decapitation of the head results in rapid induction of *Wnt3* expression in the epithelial cells which is required for head regeneration (Hobmayer *et al.* 2000, Chera *et al.* 2009, Lengfeld *et al.* 2009). When Hydra is bisected in the midgastric region, a wave of apoptosis among the interstitial cells is coupled to release of *Wnt3* from these dying cells, which results in subsequent activation of *Wnt3* transcription in epithelial and head regeneration (Chera *et al.* 2009, Galliot & Chera 2010).

The coupling of apoptosis to generation of a Wnt signal in regenerating Hydra is reminiscent of a similar phenomenon described in *Drosophila* imaginal discs, where apoptotic cells express *Wg*, which is thought to promote compensatory proliferation of neighbouring cells to maintain the size of the tissue (Fan & Bergmann 2008, Martin *et al.* 2009). While *Wg* is dispensable for disc repair in response to irradiation (Perez-Garijo *et al.* 2009), a functional role for *Wg* has been reported in disc regeneration following expression of a pro-apoptotic signal, where *Wg* induced expression of *myc* and *cyclin E* to promote proliferation (Smith-Bolton *et al.* 2009).

In vertebrates, Wnt/ β -catenin signalling has been shown to be required for tail fin regeneration in zebrafish (Stoick-Cooper *et al.* 2007). *Wnt10a* is induced after fin cutting, and blocking the pathway prevents induction of fibroblast growth factor 20a (*fgf20a*) expression, which is required for regeneration of this tissue (Wills *et al.* 2008). Interestingly, *fgf20a* is directly activated by TCF- β -catenin in cultured human cells (Chamorro *et al.* 2005), though it is not clear whether this is the case in regenerating zebrafish tails. The pathway is also thought

to play a role in the initial step of limb regeneration in *Xenopus* (Yokoyama *et al.* 2007), though the molecular targets remain to be identified.

Wnts gone wrong: misregulated signalling in disease

Given the numerous functions of Wnt/ β -catenin signalling in development and stem cell biology, it is not surprising that misregulation of the pathway would be connected to a large number of diseases. Activation of Wnt/ β -catenin signalling has been linked to many different types of human cancer, including colorectal cancer (CRC) (Polakis 2000, 2007), hepatocellular carcinomas (Armengol *et al.* 2009), cancers of the adrenal gland (El Wakil & Lalli 2011, Morris *et al.* 2010), Wilm's tumour (Tycko *et al.* 2007), breast cancer (Zardawi *et al.* 2009) and several hematological malignancies (Ge & Wang 2010). In some cancers, for example, melanoma, down-regulation of the pathway leads to more aggressive malignancy (Lucero *et al.* 2010). Many of these cancers contain either loss-of-function mutations in the *APC* gene, causing stabilization of β -catenin, or gain-of-function mutations in β -catenin, which interfere with phosphorylation of β -catenin by the destruction complex (Polakis 2000, 2007). Loss of Axin or Axin2 is also correlated with some forms of cancer (Laurent-Puig & Zucman-Rossi 2006). In the next section, we focus on the role of *c-myc* activation by the Wnt/ β -catenin pathway, because of the biological relevance of this regulation in cancer, and as an example of how difficult it can be to identify all the DNA regulatory sequences that mediate Wnt responsiveness (i.e. WRE) of a target gene.

CRC: activation of *c-myc* expression by the Wnt/ β -catenin pathway

The *c-myc* gene encodes a basic helix-loop-helix (bHLH) protein that promotes cell growth in many cell types (Soucek & Evan 2010). Elevated expression of *c-myc* has been linked to numerous human cancers (Eilers & Eisenman 2008). *c-myc* expression is activated by Wnt/ β -catenin signalling in CRC cells (He *et al.* 1998, van de Wetering *et al.* 2002) and in the experimentally induced murine epithelial hyperplasia associated with temporally controlled deletion of *APC* (leading to β -catenin stabilization) (Sansom *et al.* 2004). Strikingly, simultaneous loss of *APC* and *c-myc* in this system completely suppressed the overgrowth phenotype caused by *APC* removal, even though β -catenin levels were still abnormally high (Sansom *et al.* 2007). Microarray analysis suggests that many of the changes in gene expression observed upon removal of *APC* are *c-myc* dependent (Sansom *et al.* 2007),

indicating that *c-myc* is a major factor in mediating the effect of elevated Wnt/ β -catenin signalling in the intestinal epithelia.

When *c-myc* was first identified as a potential transcriptional target of the Wnt/ β -catenin pathway in CRC, examination of the region 5' of the TSS revealed the presence of conserved TCF binding sites (He *et al.* 1998). This region of DNA could activate a minimal promoter/luciferase reporter upon Wnt/ β -catenin pathway activation and thus fits the definition of a WRE. Mutation of two TCF binding sites within this WRE abolished this activation (He *et al.* 1998). ChIP studies demonstrated that this region was bound by LEF1 and β -catenin in CRC cells (Sierra *et al.* 2006). More recently, another site enriched for β -catenin binding in CRC cells was identified downstream of the *c-myc* transcription unit (Yochum *et al.* 2008). This region also conferred Wnt responsiveness in a reporter gene assay and contained functionally important TCF sites (Yochum *et al.* 2008). Chromatin loops between these two WREs and the *c-myc* proximal promoter have been reported (Yochum *et al.* 2010).

While, the data described above suggest that the WREs identified 5' and 3' of the *c-myc* gene contribute to regulation by Wnt/ β -catenin signalling, the large intergenic regions surrounding this Wnt target raised the possibility of other WREs further removed from the TSS (Fig. 6). Indeed, when a genomic fragment containing the 5' and 3' WREs were tested in a transgenic mouse reporter assay, there was little transcriptional activity observed (Lavenue *et al.* 1994). An *in silico* search for WREs identified two regions approx. 26 and 36 kb upstream of the *c-myc* TSS (Hallikas *et al.* 2006). Both regions drove the expression of a reporter in transgenic mice in a subset of the *c-myc* pattern, though it is not clear whether these elements are regulated by Wnt/ β -catenin signalling (Hallikas *et al.* 2006).

Another potentially important WRE controlling *c-myc* has been identified through genome-wide association scans for polymorphisms that increase an individual's risk of developing CRC (Tomlinson *et al.* 2007, Zanke *et al.* 2007). A single nucleotide polymorphism (G/T; known as rs6983267) was linked with an increased occurrence of adenomas and CRC. While control populations had an approximate 50/50 distribution of the G and T allele, the G allele was found at approx. 56% in affected individuals (Tomlinson *et al.* 2007, Zanke *et al.* 2007). This polymorphism is located approx. 335 kb upstream of the *c-myc* TSS (Fig. 6).

The realization that this polymorphism occurs in a putative TCF binding site (GATGAAAGG vs. GATGAAAGT) suggested that this polymorphism resides within a WRE controlling *c-myc* expression. Consistent with this, TCF4 was highly enriched at this site compared to the other 1 Mb of DNA surrounding the

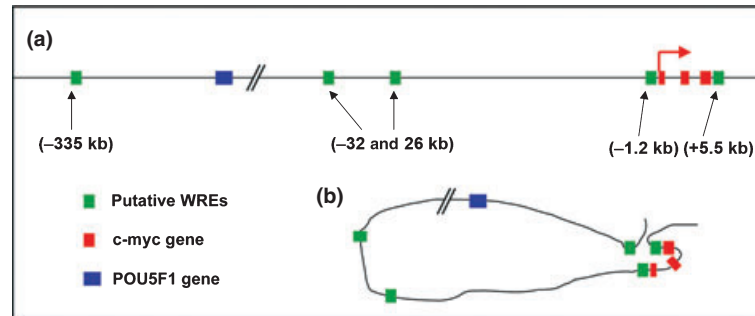


Figure 6 Complex regulation of the Wnt target gene *c-myc*. (a) WREs are located both near and removed from the *c-myc* proximal promoter. The numbers in parentheses refer to the approximate position of the 5' end of the WRE in relation to the *c-myc* TSS. The 5' (-1.2 kb) and 3' (+5.5 kb) WREs respond to Wnt/ β -catenin signalling in cell culture (He *et al.* 1998, Yochum *et al.* 2008). The far upstream (-335 kb) WRE is active in cell culture and transgenic mice (Tuupanen *et al.* 2009) and contains a polymorphism in a TCF binding sites that correlated with increased risk of CRC (Pomerantz *et al.* 2009, Tuupanen *et al.* 2009, Wright *et al.* 2010). The putative WREs at -26 kb and -32 kb are expressed in transgenic mice (Hallikas *et al.* 2006). (b) The presence of chromatin loops between the far upstream and the 3' WRE have been documented in CRC cells with high levels of Wnt/ β -catenin signalling (Pomerantz *et al.* 2009, Wright *et al.* 2010, Yochum *et al.* 2010). These loops are stabilized by protein complexes (data not shown) containing TCF and β -catenin. Presumably, the large chromatin loop allows the upstream enhancer to bypass the *POU5F1* gene, though this has not been experimentally confirmed. WRE, Wnt response elements; TSS, transcription start sites; TCF, T-cell factor; CRC, colorectal cancer.

c-myc locus (Tuupanen *et al.* 2009). This region had WRE activity in cell culture reporter assays (Pomerantz *et al.* 2009, Tuupanen *et al.* 2009, Sotelo *et al.* 2010, Wright *et al.* 2010). More impressively, this region drove expression of a reporter in transgenic mouse embryos in a pattern very similar to the majority of endogenous *c-myc* expression, which was abolished by mutation of the polymorphic TCF site and an adjacent site (Tuupanen *et al.* 2009). Consistent with the increased risk of CRC, the G allele WRE had a greater response to Wnt/ β -catenin pathway activation in cell culture reporter assays (Pomerantz *et al.* 2009, Tuupanen *et al.* 2009, Wright *et al.* 2010). When a CRC cell line with a G/T genotype was examined, TCF4 and β -catenin were enriched on the G allele chromatin, and this allele was more than two times more actively transcribed than the T allele (Wright *et al.* 2010). Taken together, these data support a model where increased recruitment of TCF4 and β -catenin to the G allele WRE results in higher levels of *c-myc* transcription. A chromatin loop between this distal WRE and the *c-myc* proximal promoter has been demonstrated (Pomerantz *et al.* 2009, Wright *et al.* 2010), suggesting a mechanism by which this element can act over such a great distance (Fig. 6B).

The regulation of *c-myc* by the Wnt/ β -catenin pathway illustrates an inconvenient truth about studying gene regulation in higher eukaryotes. The existence of a WRE over 300 kb from the *c-myc* TSS should no longer cause surprise, given the existence of functional elements acting at an even greater distance in regulating *sonic hedgehog* (Jeong *et al.* 2006). In addition, there is a growing realization that the majority of developmen-

tally regulated enhancers are more than 100 kb away from the nearest TSS (Visel *et al.* 2009). The ability to scan the large regions of genome surrounding Wnt targets of interest may often be necessary to identify some functionally important WREs.

Wnts and oxidative stress: diabetes and Alzheimer's disease

In addition to cancers, the Wnt/ β -catenin pathway has been linked to many other diseases, including several pathologies in the kidney (Pulkkinen *et al.* 2008, Hwang *et al.* 2009, Lancaster & Gleeson 2010), bone (Krishnan *et al.* 2006) and cardiac repair (Saraswati *et al.* 2010). In this section, we briefly review the role of Wnt/ β -catenin signalling in metabolic disorders and neurodegenerative diseases. While the direct links between these disorders and Wnt/ β -catenin signalling are not as well established as in cancer, there are some candidate targets where misregulation of the pathway could underlie the pathology.

Emerging evidence has linked several players in Wnt/ β -catenin signalling to metabolic disorders and type 2 diabetes mellitus. Genome-wide association studies demonstrated a strong connection between diabetes type 2 risk and SNPs within the *TCF7L2* (*TCF4*) gene, although the cellular basis of this association is still uncertain (Schinner *et al.* 2009). Mutations in the Wnt receptors LRP5 and 6 have also been implicated in obesity and type 1 diabetes and metabolic syndrome respectively (Jin 2008). Wnt/ β -catenin signalling appears to play roles both in the proliferation of pancreatic β -cells, and in the insulin release from islet

cells. β -cell proliferation in cell culture and a transgenic mouse models appears to be the result of Wnt activation of targets like *cyclin D1* (Liu & Habener 2008, Schinner *et al.* 2008), as well as *cyclin D2* and *PitX2* (Rulifson *et al.* 2007). Consistent with this, knockdown of TCF4 decreases β -cell proliferation and promotes apoptosis (Liu & Habener 2008, Shu *et al.* 2008).

In addition to promoting β -cell proliferation, the Wnt/ β -catenin pathway is also required for efficient insulin secretion (Fujino *et al.* 2003). The pathway may play a role in insulin sensing by activating *glucokinase* transcription (Schinner *et al.* 2008). It is interesting to note that antagonism of Wnt signalling by oxidative stress appears to play an important role in the pathology of insulin resistance and diabetes. The forkhead box DNA-binding protein FOXO, has been shown to compete with TCFs for β -catenin binding (Hoogetboom *et al.* 2008). Upregulation of FOXOs in response to oxidative stress may contribute to insulin resistance by the promotion of gluconeogenesis and/or the promotion of apoptosis and downregulation of TCF-mediated gene expression (Manolagas & Almeida 2007).

The effects of oxidative stress may also be a factor in the neurodegeneration of Alzheimer's disease (AD), where Wnt signalling has been ascribed a neuroprotective role (Manolagas & Almeida 2007). Extracellular accumulation of amyloid- β (A- β) has been shown to bind Fz receptors and downregulate Wnt signalling, and it has been hypothesized that some of the cytotoxicity caused by A- β may be the result of chronic suppression of Wnt/ β -catenin signalling (Inestrosa & Toledo 2008). The protective effects of β -catenin overexpression, but not of transcriptionally inactive β -catenin, indicate that transcriptional activation of target genes plays a role in ameliorating A- β toxicity (Chacon *et al.* 2008). One possibility is that oxidative stress may exacerbate cytotoxicity in AD by increasing FOXO, which then competes with TCF for β -catenin, reducing the expression of anti-apoptotic factors.

Clearly, much remains to be elucidated concerning the role Wnt/ β -catenin signalling plays in neurodegeneration and other diseases. Information gained from the study of Wnt-mediated transcriptional regulation in model systems should facilitate the identification of the important targets in many pathological states where genetics suggests that aberrant Wnt signalling plays a causal role. Given the prominence of TCF family members in regulating numerous Wnt targets, the next few sections will review TCF function in detail.

The TCF family: major regulators of Wnt/ β -catenin transcription

The TCF family of transcription factors is the best characterized DNA-binding regulators of Wnt/ β -catenin

target gene expression. In addition to a β -catenin binding domain at the N-terminus, a hallmark of this family is the presence of a highly conserved HMG domain, followed by a stretch of basic residues (Fig. 2). The TCF subfamily of HMG domains is found throughout metazoans, but not in the sister group choanoflagellates (King *et al.* 2008). The HMG domain contacts DNA largely through minor groove contacts and results in a large bending of the double helix (Love *et al.* 1995). High affinity binding sites for these HMGs have been determined for *Drosophila* TCF/Pangolin (TCF/Pan) and all four mammalian TCFs (Giese *et al.* 1991, van de Wetering *et al.* 1997, van Beest *et al.* 2000, Hallikas *et al.* 2006, Atcha *et al.* 2007). While, these studies show that a site of CCTTTGATS (S = G/C) is bound with highest affinity *in vitro*, as will be described in the following section, many functional TCF binding sites in WREs fit this consensus, while others diverge markedly.

There is a single TCF gene in almost all invertebrate species that have been examined thus far (Figs 2 and 6), with TCF/Pan from flies and POP-1 from *C. elegans* being the most thoroughly characterized. In contrast, amphibians and mammals have four TCF genes. These are most commonly referred to as *TCF1* (*TCF7*), *LEF1* (*LEF1*), *TCF3* (*TCF7L1*) and *TCF4* (*TCF7L2*). The names in parentheses are from the Human Genome Organization (HUGO). In zebrafish, the *TCF7L1* gene is duplicated (*TCF7L1a* and *TCF7L1b*), giving a total of five TCF genes in bony fish (Dorsky *et al.* 2003). As will be discussed below, there is evidence that some of the vertebrate TCFs are more specialized in their function compared to their invertebrate counterparts.

The TCF transcriptional switch

The current working model for regulation of WREs by TCFs proposes the existence of a transcriptional switch, where TCF and co-repressors inhibit the target gene expression in the absence of signalling, and then act with β -catenin and other co-activators to activate transcription of targets (Fig. 1). Evidence for this model was first obtained in the *Drosophila* embryo. *TCF/pan* mutants display mispatterning of the epidermis indicative of reduction of Wg signalling (Brunner *et al.* 1997, van de Wetering *et al.* 1997, Schweizer *et al.* 2003). This defect was not as severe as that of null alleles of *wg*. However, a *wg; TCF/pan* double mutant looked identical to *TCF/pan* mutants (Cavallo *et al.* 1998). In *wg* mutants, there is no activation of targets and TCF repression is intact, resulting in a severe loss of Wg signalling phenotype. But in *wg; TCF/pan* double mutants, loss of repression of Wg targets allows some expression (i.e. derepression), resulting in a less severe phenotype (Cavallo *et al.* 1998). Genetic and physical interactions between TCFs and TLE family co-repres-

sors (Cavallo *et al.* 1998, Roose *et al.* 1998) provided further support for the transcriptional model. The model has also been confirmed in fly cell culture using a combination of RNAi and ChIP of TCF/Pan in the absence and presence of Wg signalling (Fang *et al.* 2006).

In *C. elegans*, the *POP-1* loss-of-function phenotypes indicate both positive and negative roles in regulating Wnt targets (Phillips & Kimble 2009). In some contexts, for example, blocking mesoderm cell fate in the early embryo, repression of Wnt targets is the predominant effect observed (Rocheleau *et al.* 1997, Thorpe *et al.* 1997). But in other stages, for example, QL neuroblast migration and stem cell specification in the somatic gonad, loss of *POP-1* has a similar phenotype as loss of other Wnt/ β -catenin components (Herman 2001, Lam *et al.* 2006). Clearly, *POP-1* and TCF/Pan can both repress and activate Wnt targets.

In vertebrates, some TCF family members are more closely linked to either repression or activation. For example, *headless* (*hdl*) mutants in zebrafish are loss of function *TCF3a* alleles and display a lack of head structures (Kim *et al.* 2000), similar to *Dkk1* knockouts in mice (Mukhopadhyay *et al.* 2001). While *hdl* mutants could be efficiently rescued with a *TCF3a* transgene, adding the VP16 domain, a potent transcriptional activation domain to *TCF3a*, abolished rescue activity of the transgene (Kim *et al.* 2000). This suggests that most of *TCF3a*'s transcriptional activity is repressive. Knockout of *TCF3* in mice also resulted in phenotypes associated with an increase in Wnt/ β -catenin signalling (Merrill *et al.* 2004) and siRNA inhibition of *TCF3* in mouse ES cells largely results in increased expression of target genes (Cole *et al.* 2008). In contrast, *LEF1* knockouts have phenotypes best explained by a loss of Wnt/ β -catenin signalling (van Genderen *et al.* 1994, Reya *et al.* 2000, Kratochwil *et al.* 2002).

In contrast to *TCF3* and *LEF1*, *TCF1* appears to be more versatile. Mouse embryos lacking both *TCF1* and *LEF1* have a loss of caudal somites that is reminiscent of *Wnt3a* mutants (Galceran *et al.* 1999). *TCF1* and *LEF1* also act redundantly to pattern the mesoderm in *Xenopus* embryos, and this activity is linked to transcriptional activation (Liu *et al.* 2005). However, *TCF1* and *TCF3* have also been shown to act redundantly in repressing Spemann organizer genes such as *siamois* in ventral blastomeres (Houston *et al.* 2002, Standley *et al.* 2006). Loss of the *TCF1* gene in mice demonstrated that it was a tumour suppressor in the intestine and mammary gland (Roose *et al.* 1999).

The situation for *TCF4* also indicates both positive and negative roles in regulating Wnt targets. *TCF4* knockouts display a loss of stem cells in the intestinal crypts (Korinek *et al.* 1998), consistent with a loss of

Wnt/ β -catenin signalling. Consistent with this, *TCF4* is required for activation of Spemann organizer genes in *Xenopus* (Standley *et al.* 2006). Conversely, loss of *TCF4* can result in elevated activation of a Wnt/ β -catenin signalling in CRC cells, suggesting a possible role for *TCF4* as a tumour suppressor (Tang *et al.* 2008). Further support for a bi-modal role for *TCF4* comes from studies of *TCF3*; *TCF4* double knockouts in the skin epithelia of mice (Nguyen *et al.* 2009). Loss of both TCFS in the skin epithelia resulted in a dramatic decline in epidermal survival, which was not observed when β -catenin was removed (Nguyen *et al.* 2009). Microarray profiling revealed that many genes were repressed by *TCF3* and *TCF4* in a redundant manner, which were either activated or not regulated by β -catenin (Nguyen *et al.* 2009). These data fit a model where the two TCFS are repressing gene expression in the absence of Wnt/ β -catenin signalling. Like *TCF1*, *TCF4* can activate or repress Wnt targets, depending on the context.

The loss-of-function studies summarized above suggest a model where the transcriptional switch in vertebrate WREs is mediated by two distinct TCFS. For example, in two CRC cell lines, siRNA data fit a model, where *TCF4* represses Wnt targets in the absence of signalling and *TCF1* works with β -catenin to activate targets (Tang *et al.* 2008). Given that these results are not consistent with the phenotype of *TCF1* and *TCF4* knockouts in mice (Korinek *et al.* 1998, Roose *et al.* 1999), follow-up experiments with ChIP and reporter genes are required to confirm this model. In the presumptive Spemann organizer, *TCF3* occupies the WRE upstream of the *siamois* TSS, and its binding to chromatin is reduced by Wnt/ β -catenin signalling (Hikasa *et al.* 2010). It will be interesting to determine the occupancy of *TCF4* on the *siamois* WRE in response to pathway activation, since this TCF is required for *siamois* regulation (Standley *et al.* 2006).

Functional analysis of TCF-DNA recognition in WREs

The DNA sequence motif (CCTTTGATS) that mediates high affinity binding of TCFS *in vitro* (van de Wetering *et al.* 1997, van Beest *et al.* 2000, Hallikas *et al.* 2006) has been shown to be necessary and sufficient for activation of TCF- β -catenin-dependent transcription. This sequence motif has been found in many WREs, that is, regulatory sequences that can activate transcription of promoters in response to Wnt/ β -catenin signalling. Mutation of these motifs in these WREs abolished activation by the pathway (He *et al.* 1998, Yamaguchi *et al.* 1999, Barolo 2006, Chang *et al.* 2008a). In addition, multiple copies of this motif placed upstream of a minimal promoter confer Wnt/ β -catenin responsiveness in cell culture (Korinek *et al.* 1997, Lum *et al.*

2003, DasGupta *et al.* 2005) and in transgenic mice and fish (DasGupta & Fuchs 1999, Dorsky *et al.* 2002, Maretto *et al.* 2003, Nakaya *et al.* 2005). These studies, combined with the TCF loss-of-function studies described in the previous section have led to the view that TCFs are the major transcriptional regulators of Wnt/ β -catenin signalling in most contexts.

Testing the functionality of TCF binding sites in WREs has suggested that the switch model for TCF transcriptional regulation is WRE-specific. In several cases, mutation of the TCF binding sites clearly demonstrated both a repressive and a positive role. When a single TCF site is destroyed in the *END-1* WRE, a reduction in activation by Wnt/ β -catenin signalling was observed, but the reporter was also expressed in cells where it was normally repressed by POP-1 (Shetty *et al.* 2005). Similar phenotypes were observed when TCF sites were mutated in the *siamois* WRE in *Xenopus* (Brannon *et al.* 1997, Fan *et al.* 1998) as well as an *eve* WRE in flies (Knirr & Frasch 2001). In contrast, mutation of TCF sites in a WRE controlling expression of *decapentaplegic* (*dpp*) in the fly visceral mesoderm

resulted in a huge derepression of expression with no loss in maximal activation (Yang *et al.* 2000). It appears that for this WRE, Wg/Arm signalling activates expression by alleviating TCF/Pan repression. At the other extreme, mutation of TCF sites often results in loss of expression of the WRE reporter (Yamaguchi *et al.* 1999, Lee & Frasch 2000, Chang *et al.* 2008b). For these WREs, there appears to be little role for TCF repression, and regulation by the pathway occurs through TCF- β -catenin mediated activation (see Fig. 7A and B for further explanation).

Like many transcription factors, the TCF consensus site is not inviolate. Many functional TCF binding sites in WREs have one or more substitutions from the consensus (Barolo 2006). Systematic analysis of TCF4-DNA binding *in vitro* also demonstrated that single substitutions from the CCTTTGATS consensus reduced, but did not abolish, recognition by TCF4 (Hallikas *et al.* 2006). There is no strict correlation between functional significance and adherence to the TCF consensus site. For example, the TCF site that is polymorphic in the far upstream *c-myc* WRE has either

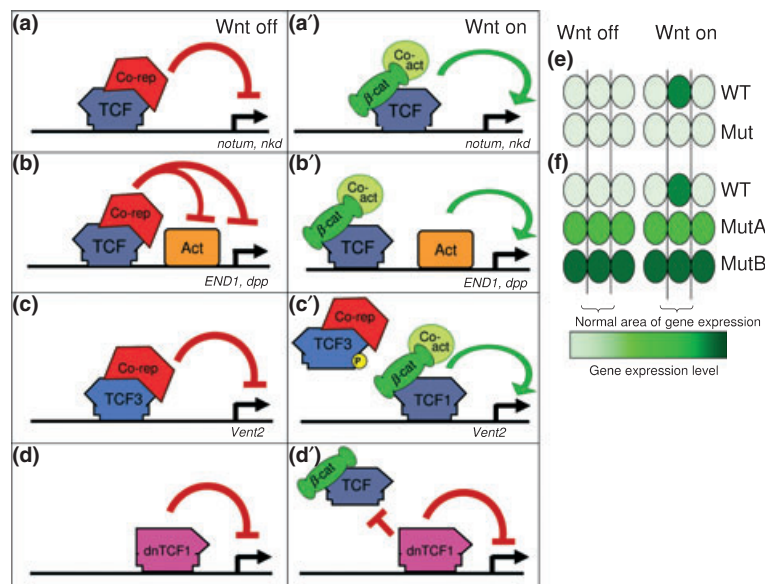


Figure 7 Variations on TCF transcriptional switches. (a & b) In organisms with a single TCF gene, the protein interacts with co-repressors in the absence of signalling, while β -catenin binding to TCF displaces co-repressors and recruits co-activators. In WREs from the *nkd* and *notum* genes, mutation of TCF binding sites results in a loss of activation (e) presumably due to the absence of other transcriptional activators (Chang *et al.* 2008b). In other WREs such as *END1* the loss of a single TCF site reduces activation by Wnt/ β -catenin signalling, but there is also significant derepression of expression in cells where the WRE is not normally active (f, middle row), presumably due to the presence of other transcriptional activators (Shetty *et al.* 2005). In some cases (e.g. the visceral mesodermal *dpp* WRE), mutation of the TCF sites results in full activation of expression in many cells (f, lower row), suggesting that the primary function of β -catenin is to relieve TCF repression (Yang *et al.* 2000). (c) In *Xenopus*, where multiple TCFs reside in the same cells, the transcriptional switch may be accomplished by HIPK2-mediated phosphorylation of the repressive TCF3, which reduces its binding to WRE chromatin, allowing TCF1 to bind and activate transcription (Hikasa & Sokol 2011). (d) The activity of a WRE can also be blocked by dominant negative isoforms of TCF which lack the ability to bind β -catenin (Roose *et al.* 1999, Hovanec *et al.* 2001). See the text for additional examples of WRE regulation by TCFs. WRE, Wnt response elements; TCF, T-cell factor; HIPK2, homeodomain interacting protein kinase 2.

one (CCTTTCATG) or two (TCTTTCATG) changes from the consensus, with the first site having increased affinity for TCF4 (Tuupanen *et al.* 2009, Wright *et al.* 2010). Likewise, binding sites that are highly functional in fly WREs from the *naked cuticle* (*nkd*) locus can have two (GCTTTGTTC) or three (GCTTTGACA) differences from the consensus (Chang *et al.* 2008a). In addition to such high affinity sites in the *eve* and *slp1* WREs, there were also more divergent sites (e.g. ACTTCACAG) that were footprinted by TCF/Pan *in vitro* and contributed to activation in transgenic fly reporter assays (Lee & Frasch 2000, Knirr & Frasch 2001). The heterogeneity of what constitutes a TCF binding site makes locating biologically relevant WREs by sequence analysis alone extremely difficult.

A simple analysis of randomly selected human intergenic DNA helps to illustrate how common predicted TCF binding sites are in the genome (Table 1). Perfect or near perfect sites (CCTTTGAWs) are rare (1 every approx. 22 kb). But if one allows a modest level of degeneracy, for example, SCTTTGAWs or CTTTGWWS, the frequency increases to 1/10 300 or 1/2500 respectively (Table 1). These sequences are well within the range of known functional TCF sites (see preceding paragraph and Chang *et al.* 2008a, Knirr & Frasch 2001, Lee & Frasch 2000). When the consensus is loosened to the level of the polymorphic TCF site found in the upstream *c-myc* WRE (GTTTGWWS; Pomerantz *et al.* 2009, Tuupanen *et al.* 2009, Wright *et al.* 2010), the frequency in random DNA is 1 in 645 bp (Table 1). This analysis suggests that there are millions of potential TCF binding sites in the human genome, and the challenge for researchers is to determine how TCF

can identify functional sites among the sea of irrelevant ones.

Bipartite binding of some TCFs through C-clamp-Helper site interaction

Are TCF sites the only sequence information in WREs that facilitate TCF binding? A distinct sequence motif with a consensus of GCCGCCR (R = A/G) has been shown to be critical for the activation of six WREs in fly cells (Chang *et al.* 2008b). Unlike classic TCF sites, multiple copies of this element (termed the Helper site) are not sufficient for activation of a promoter/reporter cassette. However, these elements greatly enhance the ability of HMG domain binding sites to respond to pathway activation (Chang *et al.* 2008b). While the orientation and spacing of the Helper sites are not fixed in relation to HMG sites, they are predominantly within 6 bp of each other. It appears that the presence of a Helper site nearby provides additional context to the TCF site to facilitate TCF/Pan activation of WREs (Chang *et al.* 2008b).

How do Helper sites facilitate TCF-mediated activation of WREs? The breakthrough came from the identification of an additional DNA-binding domain found in some isoforms of vertebrate TCFs, for example, TCF-1E and TCF-4E (Atcha *et al.* 2007). This domain, coined the C-clamp, contains four highly conserved Cysteine residues (Fig. 2). The presence of a C-clamp downstream of the HMG domain of TCF1 enabled the protein to recognize sequences containing the classic HMG binding site and an additional sequence of RCCG (Atcha *et al.* 2007). WREs from the *LEF1* and *cdx1* genes are only activated by TCFs containing a C-clamp (Atcha *et al.* 2003, 2007, Hecht & Stemmler 2003). These WREs contain RCCG motifs in close proximity to HMG binding sites (Atcha *et al.* 2007). The RCCG motif from vertebrates is similar to the first four nucleotides in the Helper site (GCCG). In flies, the major isoform of TCF/Pan contains a C-clamp, and this domain is required for activation of the WREs containing Helper sites (Chang *et al.* 2008b). Furthermore, recombinant TCF/Pan had a dramatic increase in affinity for HMG sites if a Helper site was present, and this enhanced binding was C-clamp dependent (Chang *et al.* 2008b). These results suggest a model where TCF/Pan, TCF1E and TCF4E recognize DNA through a bipartite mechanism involving HMG domain-HMG-site and C-clamp-Helper site interactions.

In contrast to vertebrates, where only some of TCF1 and TCF4 isoforms contain a C-clamp, almost all invertebrate genomes examined contain only one TCF gene with a C-clamp. The fly *TCF/pan* locus is subject to alternative splicing but the RNA-seq profiling indicates that the two most abundant isoforms expressed

Table 1 The frequency of TCF sites depends on how one defines a TCF site. An open source algorithm called Target Explorer (Sosinsky *et al.* 2003) was used to create a weighted matrix of high to lower quality TCF sites. This matrix was used to search several stretches of human intergenic DNA totalling 134 kb for potential TCF sites. While TCF sites matching the high affinity consensus site (CCTTTGAWs) are relatively rare, allowing increasing degrees of degeneracy causes a rapid increase in the number of potential sites. See text for further explanation

TCF site	Frequency in random intergenic DNA (bp)
CCTTTGAWs	~1/22 300
SCTTTGAWs	~1/10 300
CTTTGWWS	~1/2 500
SCTTTGWW, SGTTTGWWS or SCTTTCWWS	~1/1 175
CTTTGWW, GTTTGWW or CTTTCWW	~1/645

TCF, T-cell factor.

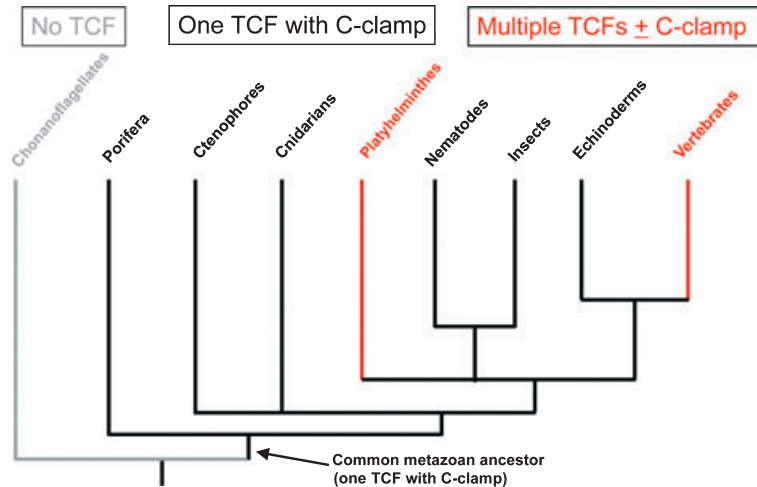


Figure 8 Phylogenetic tree showing the evolution of the TCF family. The genome sequence of *Monosiga brevicollis* (choanoflagellate) reveals no TCF family member, while that of *Amphimedon queenslandica* (porifera), *Mnemiopsis leidyi* (ctenophore), *Nematostella vectensis* (cnidarian), *Caenorhabditis elegans* (nematode), *Drosophila melanogaster* (insect) and *Strongylocentrotus purpuratus* (echinoderm) have a single TCF gene encoding a protein with HMG domain, basic tail and a C-clamp. *Schistosoma mansoni* (Platyhelminthes) has three TCF genes; two with a C-clamp and one without. Mammals and amphibians have four TCF genes, two of which have C-clamp containing isoforms. These patterns suggest that the last common ancestor of all metazoans contained a single TCF gene with a C-clamp. In the two lineages where the TCF family increased in number, the C-clamp became dispensable in some family members. TCF, t-cell factor; HMG, high mobility group.

throughout development (van de Wetering *et al.* 1997), possess both a HMG and a C-clamp domain (see modencode website: <http://modencode.oicr.on.ca/fgb2/gbrowse/fly/?name=4:87956..131430>). While such detailed analysis of TCF isoforms in other invertebrates has not yet been performed, it appears that the ancestral TCF gene contained both domains, and that after amplification during the vertebrate lineage, the C-clamp was lost (LEF1 and TCF3) or partially retained through alternative splicing (TCF1 and TCF4) (Fig. 8). This model makes the prediction that Helper sites will play an important part in specifying invertebrate WREs, as has been found in *Drosophila* (Chang *et al.* 2008b), while additional mechanisms exist for target location of vertebrate TCFs lacking a C-clamp. It should be pointed out that despite the high degree of similarity among C-clamps, some of the invertebrate domains have non-conservative changes at some positions. For example, the *M. leidyi* C-clamp has an arginine in place of the third cysteine (Fig. 2). Direct analysis of these C-clamps will be required to determine whether they enhance TCF binding, as is the case for TCF/Pan, TCF1E and TCF4E.

Genome-wide analysis of TCF binding and targets

In an attempt to define the Wnt/ β -catenin transcriptome, microarray based screens have been performed in a variety of cell types. The number of genes whose

expression is altered in the cells varies from hundreds to thousands (van de Wetering *et al.* 2002, Jackson *et al.* 2005, Klapholz-Brown *et al.* 2007, Van der Flier *et al.* 2007). A list of these microarray studies can be found on the Wnt homepage curated by the Nusse Lab at <http://www.stanford.edu/group/nusselab/cgi-bin/wnt/> and also at the Stanford Microarray Database website: <http://genome-www5.stanford.edu/>. One simple message from these data sets is the limited amount of overlap between Wnt targets in different cell types. It has been estimated that as few as 5% of targets are identified in all studies (Vlad *et al.* 2008). While variations in experimental protocols and signal detection may contribute to this low number, most of the cell-specific differences likely reflect the existence of discrete transcriptional programs. In one study, microarray analysis of PC12 and NIH3T3 cells identified 129 and 355 genes with alteration of expression in response to Wnt3a treatment respectively (Railo *et al.* 2009). Only two genes were commonly activated in both cell lines, one of which was *axin2*, often considered a universally induced feedback antagonist of Wnt/ β -catenin signalling (Jho *et al.* 2002).

While some classes of Wnt targets such as Wnt pathway components, proliferative genes or anti-apoptotic genes are found in multiple studies (Longo *et al.* 2002, Chen *et al.* 2007, Klapholz-Brown *et al.* 2007, Van der Flier *et al.* 2007, Railo *et al.* 2009), other classes may be more restricted in their expression domains. For instance, angiogenic (Masckauchan *et al.*

2005) or osteoblastic and adipogenic targets (Jackson *et al.* 2005) are most likely Wnt responsive only in permissive tissue specific contexts.

One limitation of microarrays is that they do not distinguish between direct and indirect targets. ChIP-based genomic surveys offer the potential to identify regions of the genome that are enriched for a particular TCF or β -catenin. How many of these bound regions actually correspond to a functional WRE? For example, in one study of TCF4 binding sites in LS174T cells, a CRC cell line, over 6 800 high quality binding peaks were identified using a ChIP-microarray approach (Hatzis *et al.* 2008). More than 70% of the identified peaks were over 10 kb from the nearest TSS, highlighting the tremendous amount of genomic real estate that must be examined when searching for WREs. In many cases, several TCF4-bound regions were found near a single gene, such as the 11 peaks surrounding the *Axin2* gene. Four of these regions had WRE activity in a reporter assay, while 10 out of 22 other TCF4 bound regions from other locations tested positive in this assay (Hatzis *et al.* 2008). It remains to be seen whether the regions that tested negative in the reporter assay are simply non-functional binding sites for TCF4 or are WREs that are not active in a simple reporter assay. The relatively small degree of overlap (12.5–20.5% depending on how the comparison is made) between whether a TCF4-bound region was found within 100 kb of a TSS from a gene upregulated in adenomas suggests that many of these Wnt targets may be indirectly regulated (Hatzis *et al.* 2008).

Another study using ChIP followed by high throughput sequencing (ChIP-seq) identified over 20 000 TCF4 bound regions in the human CRC cell line HCT116 (Blahnik *et al.* 2010). Over 6000 of these motifs map to putative enhancer regions with 10–100 kb of a TSS, while over 9000 mapped within 2 kb of a TSS. Using the same cell line, over 2100 regions enriched for β -catenin binding were also reported (Bottomly *et al.* 2010). In this study, only 47% of the peaks contained at least one consensus TCF motif in the vicinity of the peak. The remaining peaks may represent TCF-independent β -catenin targets, peaks containing TCF binding motifs which diverge from the consensus, or false positives.

The above studies indicate that TCF4 can bind to regions far removed from the TSS of target genes. This suggests that the most common way of determining whether a developmental gene is directly regulated by Wnt/ β -catenin signalling, that is, scanning the region immediately upstream of the candidate gene's TSS for conserved TCF binding sites, followed by site-directed mutagenesis, may miss many WREs. While continued genome-wide surveys of TCF-bound chromatin in interesting developmental contexts is desirable, an

alternative is to use computational approaches to detect WREs within entire genomes. For example, an algorithm called the enhancer element locator utilized binding site affinity matrixes and motif clustering conservation between two or more species to identify potential WREs (Hallikas *et al.* 2006). Several putative elements were tested in a transgenic mouse assay and found to be expressed in patterns that were consistent with positive regulation by Wnt/ β -catenin signalling, though this was not directly confirmed by site-directed mutation of the conserved TCF sites (Hallikas *et al.* 2006). While this method is likely to identify some WREs, the challenge of sorting through the entire genome requires stringent screening parameters which likely miss many biologically relevant elements. While one of the benefits of this algorithm is the reliance on conservation of motif clusters rather than strict sequence conservation, in some instances, enhancer elements in divergent species have been shown to retain functional conservation while losing motif clustering or locational conservation (Kalay & Wittkopp 2010), and thus may elude this type of analysis.

Given the likelihood that many targets of the Wnt/ β -catenin pathway are cell specific, it seems that a combination of transcriptome analysis, physical localization assays (e.g. ChIP-seq) and further refinement of computational approaches will be needed to efficiently identify WREs in all the interesting contexts where the pathway plays important roles. The existence of WREs acting over great distances makes this undertaking even more challenging. These efforts should be aided by the realization that many Wnt targets are controlled by TCFs in combination with other transcription factors, often in direct contact with each other. Some of the literature on this topic is covered in the following section.

TCF isoforms: a complicated situation gets more so

The existence of alternative splicing and promoter selection in vertebrate TCF genes results in a highly complex and varied inventory of TCF isoforms. For example, the mouse *TCF4* locus contains 17 exons, and more than a dozen *TCF4* isoforms resulting from alternative splicing have been identified (Weise *et al.* 2010). Four isoforms (E isoforms) contained a C-clamp, though the protein sequence differed at 3 positions depending on whether exon 14 or 15 was used. Three isoforms contained a truncated C-clamp (after the third cysteine – see Fig. 2) and three others contained new protein sequence after position 20 of the motif (Weise *et al.* 2010). These six isoforms were collectively referred to as S isoforms, while isoforms completely lacking the C-clamp were labelled M isoforms (Fig. 9). Representatives from each group were compared in

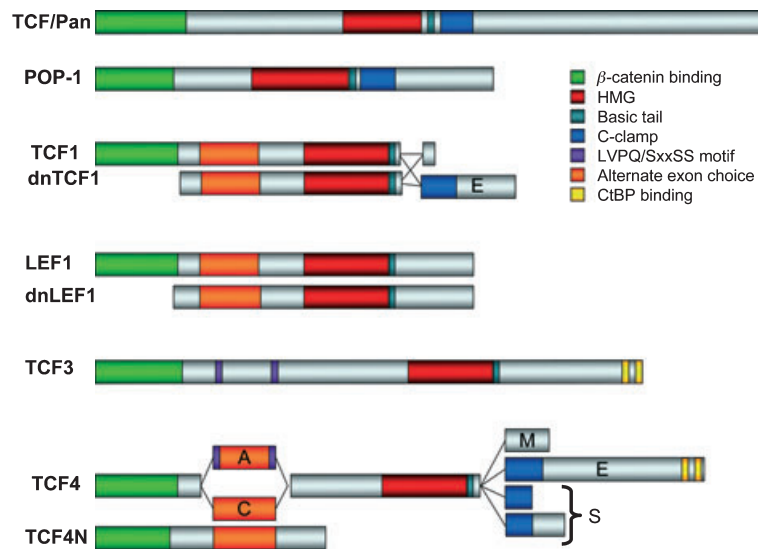


Figure 9 Diversity of TCF/LEFs. Invertebrates contain a single TCF member containing the β -catenin binding (green), HMG (red), basic tail (turquoise), and C-clamp (blue) domains. Pictured here is the most abundant isoform in *Drosophila* (Pan A) and the *C. elegans* POP-1. In vertebrates, alternate promoter usage and alternative splicing result in a myriad of TCF isoforms with diverse functional properties. Alternate usage of downstream promoters can result in isoforms which lack the β -catenin binding domain, and function as natural dominant negatives, such as dnTCF1 and dnLEF1 (Roose *et al.* 1999, Hovanes *et al.* 2001). Alternate exon usage (orange) occurs in all family members except TCF3, and the LVPQ/SXXSS motif (purple) which is invariant in TCF3 confers repressive activity on TCF4 isoforms which contain it (as in TCF4A) (Liu *et al.* 2005). Inclusion of the C-clamp motif is seen in E-tail containing isoforms TCF1E and TCF4E. M isoforms lack the C-clamp, while S isoforms contain truncated C-clamp domains (Weise *et al.* 2010). Some TCF3 and TCF4 isoforms also contain CtBP binding sites. TCF, T-cell factor; LEF, lymphoid enhancer-binding factor; HMG, high mobility group.

several assays and significant differences were found. For example a TCF4E isoform could bind to and regulate a *cdx1* WRE to a far greater extent than TCF4S or TCF4M isoforms (Weise *et al.* 2010). The biological role of the TCF4S isoforms merits further study but there is data suggesting that TCF4E isoforms are preferentially required for increased growth and proliferation in some CRC cell lines (Atcha *et al.* 2007) and the presence of TCF4E isoforms is correlated with increased disease progression in renal cell carcinoma (Shiina *et al.* 2003).

Another class of TCF isoforms lacks the β -catenin binding domain normally found at the N-terminus (Fig. 9). These isoforms are predicted to block Wnt/ β -catenin signalling, since experimentally derived TCFs lacking this domain act as potent dominant negatives (Behrens *et al.* 1996, Molenaar 1996, van de Wetering *et al.* 1997, Kratochwil *et al.* 2002). For example, high expression of truncated TCF1 (*dnTCF1*) isoforms have been proposed to explain the tumour suppressor effect of TCF1 in mice (Roose *et al.* 1999). In human intestinal epithelial cells, LEF1 can be expressed from two distinct promoters. Transcription from the second promoter produces a LEF1 lacking the β -catenin binding domain (Hovanes *et al.* 2001). In human CRC, this truncated LEF1 is suppressed, which is predicted to

contribute to higher levels of Wnt/ β -catenin signalling (Hovanes *et al.* 2001, Yokoyama *et al.* 2010). In T-helper cells, dnTCF1 is thought to regulate cell polarization via IL4 signalling. TCF1E activates GATA3 expression, which then suppresses dnTCF1 transcription in an IL4 dependent manner. This positive feedback loop promotes T helper cell polarization (Maier *et al.* 2011).

Other TCF isoforms influence the ability of the proteins to act in repression or activation. In *Xenopus*, TCF4A contains two motifs in the central portion of the protein (LVPQ and SXXSS) that are missing in TCF4C (Fig. 9). TCF4A can rescue embryos depleted of TCF3, suggesting that it can act as a repressor, while TCF4C can rescue embryos depleted of TCF1 or LEF1, suggesting a role in transcriptional activation (Liu *et al.* 2005). In CRC, expression of TCF4E isoforms lacking a binding site for CtBP (a transcriptional co-repressor) has been correlated with increased malignancy (Cuilliere-Dartigues *et al.* 2006). Furthermore, these isoforms have decreased repressive activity in cell culture reporter assays (Cuilliere-Dartigues *et al.* 2006, Tang *et al.* 2008).

Given the complexities of understanding even a single TCF isoform, comprehending how the entire TCF isoform repertoire is orchestrated to influence Wnt/ β -

catenin signalling is a long-term objective for the field. For further information on TCF isoforms, see the following review (Arce *et al.* 2006).

Post-translational modifications of TCFs

Post-translational modifications of TCFs are known to influence their subcellular localization, stability and their ability to bind to DNA and cofactors. These modifications can have a stimulatory or inhibitory effect, depending on the context. For example, the SUMO E3 ligase PIASy has been shown to facilitate sumoylation of LEF1, resulting in sequestration of the protein into nuclear bodies, where it cannot activate Wnt targets (Sachdev *et al.* 2001). Conversely, PIASy sumoylates TCF4, which increases its affinity for β -catenin, promoting target gene activation (Yamamoto *et al.* 2003b). The physiological role for PIASy in the Wnt/ β -catenin pathway is not clear, given that disruption of this gene in mice resulted in no morphological defects and only mild effects on some Wnt targets (Roth *et al.* 2004).

TCFs have also been shown to be acetylated by the invertebrate homologs of CBP and p300. These histone acetyltransferases are normally associated with histone acetylation and gene activation (Ogryzko *et al.* 1996). Indeed, CBP/p300 is known to be recruited to WREs through binding to β -catenin/Arm and is required for activation of Wnt targets (Mosimann *et al.* 2009). Paradoxically, partial loss of fly *CBP* gene activity elevated Wg/Arm signalling (Waltzer & Bienz 1998). This was shown to be the result of CBP acetylation of TCF/Pan on K25, a conserved lysine in the β -catenin/Arm binding domain. This modification weakens binding of TCF/Pan to Arm (Waltzer & Bienz 1998). An inhibitory role for CBP/p300 has also been reported in mammalian cells, though the exact mechanism is not clear (Li *et al.* 2007). In *C. elegans*, POP-1 can be acetylated by human p300 at K185 just N-terminal of the HMG domain (Gay *et al.* 2003). Acetylation at this site also occurs in worms, and was required for nuclear localization and biological activity of POP-1 (Gay *et al.* 2003). This region of POP1 is not well conserved in other TCFs, so it is not clear whether this mechanism occurs in other organisms.

Phosphorylation has also been linked to POP-1 nuclear localization. In Wnt receiving cells, a complex of the MAP kinase LIT-1 (NLK) and WRM-1 (a worm β -catenin) binds to POP-1 and phosphorylates it (Rocheleau *et al.* 1999). This results in nuclear export of POP-1, which is mediated by PAR-5, a 14-3-3 protein (Lo *et al.* 2004). This export lowers the level of nuclear POP-1, facilitating the switch of POP-1 from a repressor to a β -catenin (SYS-1 or BAR-1) bound transcriptional activator (Phillips & Kimble 2009).

Interestingly, a Wnt and CaMKII-dependent efflux of dnTCF1 has also been reported in human CRC cells (Najdi *et al.* 2009).

Is the NLK-mediated phosphorylation and nuclear export found in *C. elegans* operating in other organisms? In *Xenopus*, NLK phosphorylation of TCF4 and LEF1 promotes ubiquitylation and degradation (Yamada *et al.* 2006). In *Drosophila*, overexpression of Nemo, the fly homolog of NLK, inhibits Wg/Arm signalling and reduction of *nemo* activates the pathway (Zeng & Verheyen 2004). Whether this occurs through nuclear efflux or degradation of TCF/Pan is not yet known.

Several other kinases have been shown to influence TCF activity through direct phosphorylation. CKII can promote Wnt target gene activation by phosphorylating LEF1, which reduces its affinity for TLE co-repressors (Wang & Jones 2006, Sun & Weis 2011). The Traf2- and Nck-interacting kinase (TNIK) is required for activation of TCF4- β -catenin in mammalian cells and can form a complex with TCF4 and β -catenin (Shitashige *et al.* 2008, Mahmoudi *et al.* 2009). High levels of TNIK have also been shown to be required for maximal growth of CRC cell lines with elevated Wnt/ β -catenin signalling (Shitashige *et al.* 2010). The positive relationship between the pathway and TNIK is conserved in *Xenopus*, where primary axis formation and activation of Spemann organizer genes are dependent on TNIK and its kinase activity (Satow *et al.* 2010). This study demonstrated a β -catenin-dependent recruitment of TNIK to the *siamois* and other organizer WREs, consistent with a direct role in Wnt target gene activation (Satow *et al.* 2010).

At least one other TCF phosphorylation event occurs at *Xenopus* Wnt targets in early embryogenesis. In *Xenopus* embryos, TCF3 is phosphorylated in response to Wnt/ β -catenin signalling (Hikasa *et al.* 2010). Homeodomain interacting protein kinase 2 (HIPK2) is a major mediator of this Wnt-dependent phosphorylation and requires β -catenin for this activity. Reduction of HIPK2 reduces TCF3 phosphorylation and inhibits induction of the Spemann organizer (Hikasa *et al.* 2010). Importantly, expression of a TCF3 variant that cannot be modified by HIPK2 also blocks activation of Wnt targets. Interestingly, HIPK2-dependent phosphorylation of TCF3 by Wnt/ β -catenin signalling results in a reduction of TCF3 on *siamois* WRE chromatin (Hikasa *et al.* 2010). These data support a model where β -catenin promotes HIPK2 modification of TCF3, which results in removal from the WRE, alleviating the repressive influence of TCF3. HIPK2 also phosphorylates TCFs in human cells (Hikasa & Sokol 2011), but the functional consequence of this modification awaits further study. In flies, HIPK2 promotes Wg signalling

through stabilization of Arm (Lee *et al.* 2009), though modification of TCF/Pan has not been excluded.

One interesting speculation is that HIPK2 and TNIK act in concert to activate Wnt targets in the *Xenopus* embryo. β -catenin allows HIPK2 to phosphorylate TCF3, removing it from the WRE (Hikasa *et al.* 2010). At the same time, β -catenin recruits TNIK to TCF4, which somehow allows the TCF4- β -catenin complex to activate transcription (Satow *et al.* 2010). In this way, β -catenin could affect a swap of the TCFs that mediate repression and activation respectively. Recently, it has been shown for *Vent2*, a Wnt target in ventral *Xenopus* blastomeres, that pathway activation results in a replacement of TCF3 by TCF1 on the *Vent2* regulatory region (Hikasa & Sokol 2011). Since TCF3 represses and TCF1 activates *Vent2* expression (Hikasa & Sokol 2011), this study provides the first direct support for a ‘TCF switch’ model where Wnt/ β -catenin signalling promotes a physical change of distinct TCFs (Fig. 7C). Whether other kinases such as TNIK are involved in this switch remains to be examined.

Combinatorial interactions of TCFs and other transcription factors

Given the flexibility of what constitutes a HMG binding site, it is likely that this interaction is not sufficient for TCFs to distinguish WREs from non-functional binding sites that occur throughout the genome. Indeed, this appears to be the case in *Drosophila*, where bipartite binding to WREs occurs through HMG domain-HMG site and C-clamp-Helper site interactions (Chang *et al.* 2008b). While the conservation of the C-clamp among invertebrates (Figs 2 and 8) suggests that this strategy may be prevalent in these organisms, what about the situation in vertebrates, where most vertebrate TCF isoforms do not possess a C-clamp? In this section, several transcription factors are discussed that interact with TCFs and/or β -catenin and appear to act cooperatively with TCFs to bind to regulatory elements.

One family of transcription factors that interact with TCFs on cis-regulatory elements are the Smads, which mediate many aspects of TGF- β signalling (Moustakas & Heldin 2009). The Wnt/ β -catenin and TGF- β pathways cross-talk at several levels (Eivers *et al.* 2009, Itasaki & Hoppler 2010) and this review will limit the discussion to reports where both pathways appear to assemble Smad-TCF- β -catenin complexes on cis-acting regulatory elements. This was first shown for the regulatory region of the *twin* gene in *Xenopus* embryos (Nishita *et al.* 2000) and mammalian cells (Labbe *et al.* 2000). In both contexts, both Smad and HMG binding sites were required for maximal activation of reporter constructs by Wnt/ β -catenin and TGF- β signalling. The HMG domain of LEF1 can directly interact with Smad

3 or Smad 4 (Labbe *et al.* 2000, Nishita *et al.* 2000). These results suggest a model where a combination of protein-DNA interactions and protein-protein interactions can promote the formation of a Smad-TCF- β -catenin complex in a signalling-dependent manner (Fig. 10B).

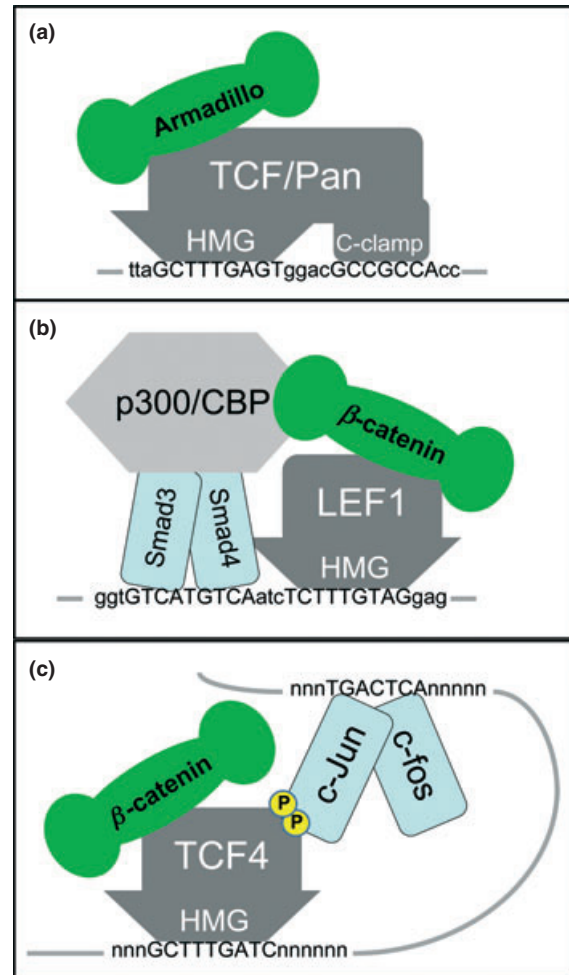


Figure 10 Three different mechanisms that contribute to TCF target selection in the nucleus. (a) Bipartite binding of TCF/Pan with HMG domain-HMG site and C-clamp-Helper site interactions at a binding site in the intronic WRE from *nkd* (Chang *et al.* 2008b). This strategy increases the TCF recognition site to approx. 16 basepairs. (b) Combinatorial binding between LEF1 and a Smad heterodimer on the *twin* WRE in *Xenopus* (Labbe *et al.* 2000, Nishita *et al.* 2000). The adjacent location of the Smad and TCF binding site again increases the amount of basepairs required for binding. Smads and β -catenin are also thought to cooperate in recruiting p300/CBP to TGF β regulated WREs (Lei *et al.* 2004). (c) In the case of the *c-jun* and *c-myc* regulatory regions, the TCF and AP-1 sites are not near each other (Nateri *et al.* 2005, Yochum *et al.* 2008), suggesting a model where DNA looping is stabilized by interactions between c-Jun and TCF. TCF, T-cell factor; HMG, high mobility group; WRE, Wnt response elements; LEF, lymphoid enhancer-binding factor.

Since these initial reports, other genes have been identified that are co-regulated by TCFs and Smads. Most of these studies are in the context of cell culture, with regulatory elements from the *Msx2* (Hussein *et al.* 2003), *c-myc* (Hu & Rosenblum 2005), *gastrin* (Lei *et al.* 2004, Chakladar *et al.* 2005), *Sm22a* (Shafer & Towler 2009), *TMEPA1* (Nakano *et al.* 2010) and several osteogenic genes (Rodriguez-Carballo *et al.* 2011). But the existence of functional Smad and TCF binding sites in close proximity to each other has also been found in *Emx2* elements active in the developing CNS of the mouse (Theil *et al.* 2002, Suda *et al.* 2010). While these studies mostly relied on reporter constructs, there is some ChIP data to suggest that Wnt/ β -catenin signalling can increase Smad recruitment to regulatory chromatin (Hussein *et al.* 2003, Shafer & Towler 2009). Conversely, TGF- β signalling can recruit LEF-1 or TCF4 to chromatin as well (Hussein *et al.* 2003, Nakano *et al.* 2010). The presence of both Smad and β -catenin on the chromatin has been proposed to increase binding for the histone acetyltransferases CBP/p300, leading to increased histone acetylation and transcription (Lei *et al.* 2004, Rodriguez-Carballo *et al.* 2011) (Fig. 10B).

Another transcription factor linked with TCF- β -catenin transcriptional activation is c-Jun, a basic leucine zipper domain protein that can bind DNA specifically as a homodimer or as a heterodimer with c-fos (constituting AP-1) (Shaulian & Karin 2002). In CRC cells, c-Jun, TCF4 and β -catenin cooperated in activating the c-Jun expression (Nateri *et al.* 2005). Phosphorylated c-Jun was found to associate with TCF4 and both transcription factors occupy the c-Jun regulatory region (Nateri *et al.* 2005). These data complement genetic interaction studies in the mouse intestine to support a model, where Wnt/ β -catenin signalling acts with c-Jun in a positive feedback loop to promote carcinogenesis (Nateri *et al.* 2005, Sancho *et al.* 2009). In contrast to most of the elements co-regulated by TCF and Smad, the distance of the functional TCF and AP-1 site suggests the existence of a DNA loop stabilized by protein-protein and protein-DNA interactions (Fig. 10C).

While, the Wnt/ β -catenin-c-Jun autoregulatory loop may be crucial for intestinal cancer in mice and CRC in humans, additional evidence suggests that many Wnt transcriptional targets in CRC cells are co-regulated by TCF4 and c-Jun. The *c-myc* WRE located downstream of the *c-myc* gene (Fig. 6) contains a functional AP-1 site that is required for synergistic activation between the Wnt/ β -catenin pathway and serum-derived mitogens (Yochum *et al.* 2008). A genome-wide survey of chromatin sites with β -catenin enrichment revealed that 40% of the β -catenin bound regions contain both TCF and AP-1 binding sites (Bottomly *et al.* 2010). More

than a dozen sites were bound by TCF4, β -catenin and c-Jun. As previously shown for *c-myc*, the activation of several Wnt targets were enhanced by serum in CRC cells arrested in G₀/G₁ (Bottomly *et al.* 2010). The connection between Wnt/ β -catenin signalling and cell cycle progression has also been noted further upstream in the pathway (Davidson & Niehrs 2010).

The Wnt/ β -catenin pathway-c-Jun connection has also been observed outside the context of intestinal cells and CRC. Regulatory elements controlling either the *matrilysin* gene in kidney or the *versican* gene in melanoma require both TCF and AP-1 sites (Rivat *et al.* 2003, Domenzain-Reyna *et al.* 2009). In addition, interactions between TCF4 and c-Jun (Gan *et al.* 2008) or β -catenin and the AP-1 complex (Toualbi *et al.* 2007) can regulate Wnt targets independent of AP-1 binding sites, though whether this type of regulation occurs under physiological conditions is not clear.

The Smad and c-Jun/AP-1 studies described above are examples where distinct signalling pathways and Wnt/ β -catenin signalling converge on regulatory elements to activate transcription. Such combinatorial control of Wnt targets can also occur through interactions with transcription factors not directly controlled by cell-cell signalling. One candidate for such factors is the Cdx family of homeodomain proteins. As outlined in a previous section, *cdx1* and *cdx4* are known to be direct transcriptional targets of the Wnt/ β -catenin pathway (Lickert *et al.* 2000, Ikeya & Takada 2001, Pilon *et al.* 2006). In addition, there is some developmental genetic data suggesting that the TCF- β -catenin complex may functionally interact with Cdx proteins (Young *et al.* 2009). Indeed, Cdx1 autoregulation has been shown to require a Cdx1-LEF1- β -catenin complex, through direct interactions between the homeodomain and HMG domains (Beland *et al.* 2004). More recently, a genome-wide survey of Cdx2 binding in intestinal cell lines revealed a significant overlap between Cdx2 and TCF4 chromatin bound regions (Verzi *et al.* 2010). TCF4 binding to chromatin was found to be partially dependent on Cdx2 at several locations (Verzi *et al.* 2010). Interestingly, nested TCF-Cdx binding sites have been shown to be required for an intronic *raldh2* enhancer that is active in the dorsal spinal cord of the chick (Castillo *et al.* 2010).

There are other examples of TCFs interacting with other transcription factors to regulate gene expression. LEF1 and microphthalmia-associated transcription factor are thought to physically interact to regulate gene expression in melanocyte differentiation (Yasumoto *et al.* 2002). Likewise, LEF1 and the homeodomain protein Pitx2 may interact in the developing dental epithelium and other tissues (Amen *et al.* 2007). The short list of transcription factors that interact with TCFs discussed here is likely only the tip of the iceberg. The

genome-wide studies of TCF4 binding patterns have found an enrichment for many other transcription factor binding sites besides AP-1, including NF1, PPAR γ , HNF4, Elk-1, GATA3, c-Ets-1, Bach-1 and FoxD-1 (Hatzis *et al.* 2008, Blahnik *et al.* 2010). TCF-protein interactions may be the normative mechanism to locate WREs in the information rich nucleus, though the binding partner is likely to be different for different targets. It should also be noted that while the mechanisms described above have been assumed to operate for TCFs lacking C-clamps, they may also function to facilitate target gene location of C-clamp containing TCFs. It seems likely that multiple mechanisms acting in concert are required for TCFs to locate WREs in the genome.

TCF-independent recruitment of β -catenin to Wnt targets

TCFs are clearly the best characterized transcription factors that mediate the regulation of gene expression via Wnt/ β -catenin signalling. In addition to TCFs, there are several other DNA-binding proteins that have been shown to bind to β -catenin and effect transcriptional responses. Many nuclear receptors, including the oestrogen and androgen receptors bind β -catenin and co-regulate targets of these hormones, as reviewed elsewhere (Mulholland *et al.* 2005, Beildeck *et al.* 2010). In this section, we discuss other transcription factors that serve as recruiters for nuclear β -catenin and how they may contribute to the enormous diversity of transcriptional outputs that are generated by the Wnt/ β -catenin pathway.

To conclude that a transcription factor is sufficient to recruit β -catenin to a regulatory element, the factor must bind and co-regulate targets with β -catenin. This is the case for Sox17, which regulates endodermal genes during vertebrate gastrulation. β -catenin augments Sox17's ability to activate target genes and the two proteins physically interact (Sinner *et al.* 2004). However, a role for TCFs in this process was not ruled out and in CRC cells, Sox17 has also been shown to interact with TCFs and promote their degradation (Sinner *et al.* 2007). In another case, the homeodomain protein Prop1 and β -catenin are both required for Pit1 expression in the developing mouse pituitary gland (Olson *et al.* 2006). These proteins directly interact and are co-localized on Pit1 regulatory chromatin. LEF1 is not required for Pit1 expression, but other TCF family members were not examined (Olson *et al.* 2006). PitX2 can also bind to β -catenin and bring it to the *cyclin D2* promoter in mouse myoblast cells (Kioussi *et al.* 2002). A synthetic enhancer containing multimerized PitX2 sites is activated in a β -catenin-dependent manner, suggesting that PitX2 is sufficient for recruiting β -catenin to DNA

(Kioussi *et al.* 2002). It should be pointed out that PitX2 can also bind LEF1, suggesting that it sometimes works with TCFs to regulate Wnt targets (Vadlamudi *et al.* 2005, Amen *et al.* 2007).

One approach to rule out a role for TCFs in the activation of target genes by β -catenin is the use of dominant negative constructs of TCF (dnTCF) that cannot bind β -catenin. When expressed at sufficient levels, such constructs should prevent the binding of endogenous TCFs to regulatory elements (Behrens *et al.* 1996, Molenaar 1996, van de Wetering *et al.* 1997, Kratochwil *et al.* 2002). In cultured myoblasts, β -catenin is required for the bHLH protein MyoD to activate muscle specific genes (Kim *et al.* 2008). The presence of high levels of β -catenin enhanced MyoD binding to myogenic elements, presumably through direct binding. This regulation was not affected by expression of dnTCF (Kim *et al.* 2008). dnTCF also did not block the ability of β -catenin to augment transcriptional activation of hypoxia inducible factor 1 α (HIF1 α) (Kaidi *et al.* 2007).

In CRC cells with high endogenous levels of β -catenin, hypoxia (which dramatically increases HIF1 α levels) results in a shift of β -catenin away from the classic Wnt target *c-myc* towards HIF1 α targets (Kaidi *et al.* 2007). It should be noted that this effect is cell specific, since in several stem cells, hypoxia activates Wnt/ β -catenin signalling by HIF1 α -dependent induction of LEF1 and TCF1 transcription and β -catenin protein levels (Mazumdar *et al.* 2010). In these cells, sufficient β -catenin is thought to allow both types of transcription factors to activate target genes (Mazumdar *et al.* 2010).

Another example where a stress-induced factor can divert β -catenin away from TCFs is the FOXO family of Forkhead domain transcription factors. FOXO proteins can directly bind β -catenin and use it to activate gene expression in *C. elegans* and mammalian cells (Essers *et al.* 2005). Induction of FOXO protein levels during oxidative stress reduces TCF-dependent gene expression, due to competition for limiting levels of β -catenin (Almeida *et al.* 2007, Hoogeboom *et al.* 2008).

How important are non-TCFs in mediating Wnt/ β -catenin signalling during normal development? In cnidarians, the only functional data thus far on TCFs suggests that in Hydra, TCF is absolutely required for Wnt-dependent head regeneration (Duffy *et al.* 2010). In *Drosophila*, *Tcf/pan* mutants clearly are qualitatively similar to *wg* mutants (Brunner *et al.* 1997, van de Wetering *et al.* 1997, Schweizer *et al.* 2003), but it is not clear that it is required for all Wg signalling. In vertebrates, the role of TCFs is much more difficult to assess, given the likely redundancy between the four TCFs. While the available data suggests that vertebrates TCFs play a major role in mediating Wnt/ β -catenin

signalling, the sum of all other transcription factors recruiting β -catenin to Wnt target genes may ultimately be much more significant than currently appreciated.

Direct transcriptional repression by β -catenin

This review has focused exclusively on the ability of β -catenin to activate transcription, which is reasonable considering the large pile of evidence for such a role. But it is worth noting that Wnt/ β -catenin signalling leads to down-regulation of gene expression about as often as activating genes (van de Wetering *et al.* 2002, Jung & Kim 2005, Naishiro *et al.* 2005, Klapholz-Brown *et al.* 2007). Because of the pathway's obvious role in activation, it is usually assumed that these genes are *indirectly* repressed by Wnt/ β -catenin signalling, that is, the pathway activates a repressor that then inhibits another genes expression. While this no doubt occurs, there are a growing number of cases where β -catenin is thought to directly mediate repression through TCF family members. This is distinct from the well-known repressive role of TCFs in the absence of signalling that has been previously discussed.

What are the experimental criteria for determining that a target is directly repressed by Wnt/ β -catenin signalling? A straightforward approach is to monitor target gene expression when the pathway is activated in cells treated with protein synthesis inhibitors. While, this is straightforward for many pathways, for example, TGF β (Kang *et al.* 2003), it is problematic for Wnt/ β -catenin signalling, given the requirement for new synthesis of β -catenin once its degradation is blocked (see Fig. 1). There are hormone-inducible versions of β -catenin, created by fusing portions of a nuclear receptor to β -catenin, and these have been used to determine activation of targets in the presence of protein synthesis inhibitors (Li *et al.* 2009, Elkouby *et al.* 2010). But these reagents can be tricky to utilize for identifying direct targets of repression (Blauwkamp *et al.* 2008).

In lieu of this approach, researchers have used a combination of site-directed mutagenesis of TCF binding sites in repressed cis-regulatory elements to provide support for direct regulation. In flies, such studies have shown that WREs from the *stripe* and *dpp* genes contain TCF sites that are absolutely required for repression (Piepenburg *et al.* 2000, Theisen *et al.* 2007). In cell culture, a luciferase reporter has been used in a similar way to demonstrate that Wnt/ β -catenin signalling directly represses *E-cadherin* in keratinocytes (Jamora *et al.* 2003), and *p16INK4a* in melanoma (Delmas *et al.* 2007). In these cases, ChIP was also employed to demonstrate that TCF and β -catenin were physically present on the repressed WREs (Jamora *et al.* 2003, Delmas *et al.* 2007).

What is known about the mechanism of these examples of TCF- β -catenin repression? For the *stripe* WRE, the functional TCF binding site overlaps with a binding site for Cubitus Interruptus (Ci), which is crucial for activation of *stripe* in embryos (Piepenburg *et al.* 2000). This suggests that TCF/Pan-Arm may displace the Ci activator. In the case of *dpp*, Brinker binds to the WRE and somehow acts with TCF/Pan-Arm to mediate repression in the leg imaginal disc (Theisen *et al.* 2007). In keratinocytes, LEF1- β -catenin works additively with the Snail repressor to inhibit E-cadherin transcription (Jamora *et al.* 2003). It is not clear in these cases or that of pINK16a where the specificity lies that allows TCF- β -catenin to repress, rather than activate, gene expression.

Another example of direct repression by the pathway occurs in cultured hemocytic cells in *Drosophila*, where Wg/Arm signalling represses expression of *Ugt36Bc*, an enzyme deposited in the extracellular matrix (Blauwkamp *et al.* 2008). TCF/Pan is enriched in the same region upstream of the *Ugt36Bc* TSS that contains a WRE that is repressed by the pathway. This WRE was localized to a 178 bp region that contained no classic TCF binding sites. However, footprinting revealed the existence of three TCF sites, which share a consensus of AGAWAW. These sites are functional, mediating activation of the WRE in the absence of signalling and Arm-dependent repression (Blauwkamp *et al.* 2008). Thus, *Ugt36Bc* is regulated by a 'reverse transcriptional switch' compared with the classic switch depicted in Fig. 1.

The existence of novel TCF binding sites in the *Ugt* reporter suggested that the nature of the binding site contained the specificity for the transcriptional output. Support for this came from an experiment where the novel sites were converted to classic ones. Strikingly, this altered WRE was now activated in response to Wg/Arm signalling (Blauwkamp *et al.* 2008). The possibility of allosteric regulation of TCFs by DNA is supported by structural analysis of the LEF1 HMG domain alone and when complexed with a high affinity binding site. When the HMG is not bound by DNA, it is partially unstructured, particularly helix 1. Upon DNA binding, the HMG domain is converted to a well-folded, highly ordered state (Love *et al.* 2004). It would not be surprising if different DNA-binding sites could influence the conformation of TCF, which could then influence the recruitment of co-activators or co-repressors by β -catenin/Arm.

Another clue to the mechanism of TCF/Arm direct repression was found through mutagenesis of the Arm gene. Deletion or mutation of portions of Arm known to be involved in transcriptional activation created a variant called DisArmed. Consistent with the alterations, DisArmed could not regulate several WREs that

are activated by the pathway (Blauwkamp *et al.* 2008). However it could still efficiently repress *Ugt36Bc* expression and the *Ugt* reporter. In addition, DisArmed could repress several other downregulated Wg targets in cultured cells and fly hemocytes, suggesting that they also are directly repressed by TCF/Pan-Arm (Blauwkamp *et al.* 2008).

As is the case with activated Wnt targets, there are also reports of non-TCF proteins utilizing β -catenin to repress gene expression. The best studied is the homeodomain protein Prop1, discussed earlier as a transcription factor that binds β -catenin to activate transcription (Olson *et al.* 2006). Like TCFs, it can also do the opposite, recruiting β -catenin to a regulatory element controlling another transcription factor called Hesx1 (Olson *et al.* 2006). This repression requires *reptin*, a transcriptional co-repressor (Olson *et al.* 2006). The antimetastasis gene KAI1 is also repressed by a complex containing β -catenin and Reptin, but the DNA-binding transcription factor is not known (Kim *et al.* 2005). Interestingly, Reptin is known to bind to β -catenin/Arm in a region that is not altered in the DisArmed protein (Bauer *et al.* 2000), though it is not known whether fly Reptin is involved in *Ugt36Bc* repression by Wg/Arm signalling.

As with other variations from the classic regulatory mechanism outlined in Fig. 1, it is not yet clear how often direct repression by β -catenin occurs, either through TCFs or other transcription factors. Is it a rarity that plays only a small role in transcriptional regulation by the Wnt/ β -catenin pathway? Or has the focus on transcriptional activation and classic TCF binding sites obscured a currently underappreciated aspect of Wnt gene regulation? Clearly more research in this area is required to find out.

Conclusions

The first detailed characterizations of Wg/Arm signalling already revealed what is now a common assumption, that this single pathway has a remarkable number of roles during fly development (Baker 1988, Peifer *et al.* 1991). The appreciation of Wnt/ β -catenin signalling diversity has only grown in the past twenty years, and this review has only covered a small fraction of the established roles of the pathway in metazoan development. How is this diversity achieved? In vertebrates, differences among the TCF family members and the large number of other transcription factors that recruit β -catenin to WREs is likely part of the answer. But in flies, it thus far appears that a single TCF mediates most, if not all of the Wg/Arm pathway's effects (Brunner *et al.* 1997, van de Wetering *et al.* 1997, Schweizer *et al.* 2003). Combinatorial inputs from other signalling pathways certainly provide an answer

for why some WREs are activated in a cell-specific manner, for example, *eve* in cardiac mesoderm (Halfon *et al.* 2000, Lee & Frasch 2000, Knirr & Frasch 2001, Han *et al.* 2002). Other mechanisms, for example, cell-specific chromatin accessibility, are thought to occur (Wohrle *et al.* 2007), but remain relatively unexplored.

Despite the challenge of understanding the complexities of Wg target gene regulation, our current knowledge is detailed enough so that it can be exploited to further understand how this pathway controls development. The realization of an ancient posterior Wnt signalling gradient throughout metazoans (Petersen & Reddien 2009, Niehrs 2010), provides one example. For cnidarians, we think it likely that the potentially important targets (e.g. *Brachyury*) will be bound by TCFs by a similar bipartite mechanism as has been shown to operate in *Drosophila* (Chang *et al.* 2008b), given the presence of highly conserved HMG and C-clamp domains in cnidarians (Figs 2 and 8). Using information from other systems may allow the dissection of the genetic networks in great detail, complementing the careful analysis of regulatory factors that are expressed temporally downstream of Wnts, exemplified by the study of the amphibian NC (Li *et al.* 2009, Elkouby *et al.* 2010) (Fig. 4). Perhaps in the near future, a combination of genome-wide surveys, WRE identification through bioinformatics and informed examination of candidate target genes can identify important targets relatively quickly in many developmental systems.

This dynamic can be observed in the study of the Wnt/ β -catenin pathway in CRC. The identification of a WRE more than 335 kb removed from the *c-myc* TSS could have only been achieved with a combination of genomic approaches and a recognition of what constitutes a TCF binding site (Pomerantz *et al.* 2009, Tuupanen *et al.* 2009, Sotelo *et al.* 2010, Wright *et al.* 2010) (Fig. 6). Optimization of these approaches should facilitate the identification of relevant targets for the many other pathologies that Wnt/ β -catenin signalling has now been linked to.

Despite intense effort over the past 15 years, there is much to learn about how TCFs (and other transcription factors) transform elevated levels of nuclear β -catenin into the appropriate transcriptional responses in a cell, tissue and temporally specific manner. The vast size of metazoan genomes and the great distances over which WREs can operate make this task even more daunting. Given the importance of this pathway in normal development and physiology as well as numerous pathologies, it's clear that further investigation is worth the effort.

Conflict of interest

The authors declare that they have no conflict of interest.

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