A T-box Protein Interacting with the TCF Transcriptional Switch of Wnt Signaling in *Xenopus* Dorsal Axis Development

by

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DEDICATION

To my beloved family

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Wnt/β-catenin signaling plays important roles in dorsal axis development in vertebrates

1.1.1 Overview of the Wnt signaling pathway

The Wnt/β-catenin signaling pathway is a cell-cell signaling pathway highly conserved among all metazoans (Archbold et al., 2012). It plays essential roles in numerous biological processes throughout embryonic development, such as cell-fate specification, proliferation and differentiation (van Amerongen and Nusse, 2009; Archbold et al., 2012; Clevers, 2006; Logan and Nusse, 2004a; Niehrs, 2010). It is also required for maintaining adult tissue homeostasis, where Wnts function as a stem cell niche signal (Archbold et al., 2012). Dysregulation of the pathway has been implicated in a variety of human pathologies, including various cancers, diabetes and bone disorders (Archbold et al., 2012; Cadigan and Peifer, 2009; Clevers and Nusse, 2012; Polakis, 2012).

Wnts are a family of secreted glycoproteins that activate several downstream signaling cascades, among which the best characterized is the Wnt/β-catenin pathway (Archbold et al., 2012). This pathway is transmitted through a critical messenger protein, β-catenin. In the absence of Wnt, β-catenin is sequestered in cytosol by the "degradation complex", consisting of glycogen synthase kinase 3 (GSK3), casein kinase I (CKI) and scaffolding proteins Axin and adenomatosis polyposis coli (APC). CKI and GSK3

consecutively phosphorylate β-catenin. Phosphorylated β-catenin is further poly-ubiquitinated by β-TrCP and sent to proteasomal degradation (Archbold et al., 2012; Cadigan and Peifer, 2009). As a result, β-catenin remains at a low level in the cytosol when Wnt signaling is off (Figure 1.1A).

When Wnt is received at the cell surface by the receptor complex, consisting of Frizzled (Fz) family proteins and low density lipoprotein receptor related protein 5 or 6 (LRP5/6), it results in inhibition of the degradation complex through a mechanism that is not fully understood. The current model suggests that Wnt stimulates phosphorylation of cytosolic Dishevelled (Dsh), promoting its interaction with the intracellular domain of Fz. Accompanying this, Wnt-activated LRP6 also gets phosphorylated at the intracellular domain, which then recruits the degradation complex via directly binding with the scaffolding protein Axin. Assembly of the Fz-LRP6, Dsh and degradation complex is referred as the signalosome (Cadigan and Peifer, 2009; MacDonald et al., 2009). Within the signalosome, Axin undergoes a dephosphorylation-induced conformational change, leading to its dissociation from the signalosome and disassembly of the degradation complex (Clevers and Nusse, 2012; Kim et al., 2013). As a consequence, β-catenin is released from the degradation complex and translocates into the nucleus to participate in transcriptional regulation (Figure 1.1).

In the nucleus, β-catenin is recruited to Wnt-dependent *cis*-regulatory modules (W-CRMs) mainly by transcription factors (TF) of the T Cell Factor (TCF) family through a direct interaction (Figure 1.1). TCFs are the best-characterized DNA-binding effector of the Wnt/β-catenin pathway, which is thought to mediate the majority of Wnt-controlled transcriptional events (Cadigan and Waterman, 2012a; Schuijers et al., 2014).

TCFs act as transcriptional switches, repressing target genes with the aid of co-repressors in the absence of Wnt (Figure 1.1A), and activating target genes with β-catenin and additional co-activators upon Wnt stimulation (Figure 1.1B). As the TCF transcriptional switch is the central topic of this thesis, more details about the mechanisms of the TCF transcriptional switch will be discussed in a later part of this chapter.

1.1.2 The organizer as signaling center to induce dorsal axis

Historical perspective

In 1924, Spemann and Mangold demonstrated that the dorsal blastopore lip of the amphibian gastrula organizes the dorsal axis patterning by influencing the cell fate of neighboring cells, and therefore named the dorsal blastopore lip "the organizer" (Spemann and Mangold, reprinted in 2001). In their famous intra-species transplantation experiments, the dorsal blastopore lip from a light pigmented salamander gastrula was grafted to the ventral side of a dark pigmented species, leading to a complete secondary dorsal axis (Figure 1.2; Spemann and Mangold, reprinted in 2001). The pigment difference allowed them to track the cell fate of donor and host cells. They found the light donor cells kept their original fate to form the notochord whereas the dark neighboring host cells were induced to adopt a dorsal fate, giving rise to dorsal tissues, such as somites and nerve cells (Figure 1.2; Spemann and Mangold, reprinted in 2001). Their discovery elegantly demonstrated the existence and importance of cell-cell communication during development. The organizer or its developmental analogues are also present in other chordates, such as embryonic field in fish, Hensen's node in bird and mice, and they play pivotal roles in patterning overall body plan in each species (Harland and Gerhart, 1997; Joubin and Stern, 2001; Marlow, 2010; Schier and Talbot, 2005).

Molecular nature of the Spemann Organizer

Extensive molecular screens have identified numerous organizer-specific genes (De Robertis, 2006). These genes can be divided mainly into two classes: transcription factors and secreted signaling antagonists.

Siamois and its closely related homologue Twin, two homeodomain transcription factors, are expressed in the precursor cells of the organizer during Mid-blastula transition (MBT), a developmental time point when zygotic transcription initiates (Blythe et al., 2010; Laurent et al., 1997; Lemaire et al., 1995; Newport and Kirschner, 1982). As the earliest organizer genes expressed, Siamois and Twin are redundantly responsible for all aspects of the organizer function. Simultaneous knockdown of Siamois and Twin blocks dorsal axis induction (Bae et al., 2011). Ventral injection of *Siamois* mRNA could induce a complete secondary axis, mimicking the effect of dorsal blastopore lip transplantation (Ishibashi et al., 2007; Lemaire et al., 1995). These data suggested Siamois and Twin play an essential role in promoting the organizer formation in *Xenopus* blastula. At the mechanistic level, they directly activate transcription of several downstream organizer genes, including *Goosecoid, Chordin* and *Cerberus* (Collart et al., 2005; Ishibashi et al., 2007; Laurent et al., 1997; Reid et al., 2012). In zebrafish, the *Siamois* homologue *Bozozok/Dharma* is also required for formation and function of the fish organizer (Ryu et al., 2001).

A hallmark gene expressed in all vertebrate organizers is the transcription factor Goosecoid, a (Rivera-Pérez et al., 1995). It was the first organizer gene identified in *Xenopus* (Cho et al., 1991). It functions as a transcriptional repressor to maintain organizer identity and promote head formation, through counteracting Wnt and bone

morphogenetic protein (BMP) signaling (Sander et al., 2007; Yao and Kessler, 2001). In mice, Goosecoid is not essential for the organizer function; rather, it is important for craniofacial and rib cage formation during later development (Rivera-Pérez et al., 1995).

As a potent signaling center, the organizer was expected to secrete a cohort of new growth factors and signaling molecules. Surprisingly, a plethora of signaling antagonists were isolated (De Robertis and Kuroda, 2004; Robertis and Larrain, 2000). The organizer emits a number of BMP antagonists, such as Chordin, Noggin and Follistatin, as well as Wnt antagonists, such as Frzb1, secreted frizzled-related protein-2 (sFrp2), Crescent and Dickkopf-1 (Dkk-1) (De Robertis and Kuroda, 2004). These antagonists mainly work by blocking ligand-receptor interactions. They play key roles in setting up the perpendicular gradients of Wnt and BMP activities that are essential for patterning the future anterior/posterior (A/P) and dorsal/ventral (D/V) structures (Figure 1.3D; Niehrs, 2010). More specifically, anterior specification requires low Wnt, whereas high Wnt promotes posteriorization of the embryo (Figure 1.3C & D, Hikasa and Sokol, 2013; Robertis and Larrain, 2000). The organizer-secreted Wnt inhibitors antagonize Wnt anteriorly to ensure head induction (Glinka et al., 1997, 1998; Leyns et al., 1997; Piccolo et al., 1999). An ascending BMP gradient along the D/V axis is established by a combined action of BMP antagonists expressed by the dorsal organizer and a ventral BMP center expressing high levels of BMP ligands (Figure 1.3 C&D; Robertis, 2006). Inhibition of BMP dorsally is essential for dorsal trunk development (Khokha et al., 2005).

Wnt/β-catenin signaling is required for the organizer formation

Wnt/β-catenin signaling plays an essential role in promoting the organizer

formation (Harland and Gerhart, 1997; Hikasa and Sokol, 2013). The engagement of Wnt signaling in dorsal axis formation was first suggested by gain of function experiments in *Xenopus* embryos: ventral injection of Wnt mRNA induced an ectopic organizer on the ventral side, resulting in a duplicated axis (McMahon and Moon, 1989; Smith and Harland, 1991; Sokol et al., 1991). Axis duplication could also be induced by other Wnt signaling components, for example, Dsh (Sokol et al., 1995), β-catenin (Guger and Gumbiner, 1995), and a dominant-negative form of GSK3 (Dominguez et al., 1995). Depletion of maternal β-catenin resulted in ventralized embryos, demonstrating maternal β-catenin is required for the organizer formation and dorsal axis formation (Heasman et al., 1994).

It is unclear how the Wnt pathway is activated in dorsal cells initially. It has been thought that the pathway was initiated intracellularly through a GSK inhibitor protein, Frat, which is dorsally enriched and essential for dorsal axis formation (Yost et al., 1998). However, triple knockout of Frat homologs in mice has no phenotype consistent with a Wnt defect, suggesting this mechanism may be not conserved in mammals (van Amerongen et al., 2005). Later, anti-sense oligonucleotide-mediated maternal depletion of Wnt11, Wnt5a and Fz7 indicated involvement of more upstream Wnt pathway components (Cha et al., 2008; Kofron et al., 2007; Tao et al., 2005).

During oogenesis, several maternal dorsalizing factors/components of Wnt signaling are deposited at the vegetal pole of oocytes, including maternal β-catenin (Larabell et al., 1997) and Wnt 11 (Ku and Melton, 1993) (Figure 1.3A). Shortly after sperm entry, these factors are transported to the future dorsal side through cortical rotation, a process during which cortical cytoplasm is moved toward the opposite of

sperm entry point by 30 degree on average (Houston, 2012) (Figure 1.3B). In certain frog species (e.g. *Rana pipiens*), the cortical rotation is visible as the conspicuous gray crescent, marking the future dorsal side of the embryo (Houston, 2012). Cortical rotation is driven by microtubules (Houliston and Elinson, 1991). Fertilized eggs treated with microtubule-depolymerizing agents, such as UV irradiation or nocodzaole undergo ventralization (Jesuthasan and Stähle, 1997; Scharf and Gerhart, 1980). These experiments suggest cortical rotation is essential for establishing asymmetric Wnt activity as well as subsequent organizer formation.

Organizer genes activated by Wnt/β-catenin signaling

Dorsally enriched β-catenin accumulates in the nucleus (Larabell et al., 1997; Schneider et al., 1996; Schohl and Fagotto, 2002) and gets recruited to chromatin by TCFs to activate target genes that delineate the organizer. For example, *Siamois, Twin* and *Xenopus nordal related3 (Xnr3),* another BMP antagonist, are among the earliest zygotic genes activated by TCF in the organizer (Brannon et al., 1997; Laurent et al., 1997; Mckendry et al., 1997). In their *cis*-regulatory modules, functional TCF binding sites have been identified and shown to be required for their Wnt-regulated expression (Brannon et al., 1997; Laurent et al., 1997; Mckendry et al., 1997). *Bozozok/Dharma*, a zebrafish *Siamois* homolog, has also been demonstrated as a direct target of maternal Wnt signaling regulated by TCF proteins (Ryu et al., 2001), indicating a conserved regulatory role of TCF in the organizer. Several other organizer genes are also implicated as Wnt target genes, as their expression are enhanced by overexpression of some TCFs (Standley et al., 2006) and abrogated by maternal depletion of β-catenin (Xanthos et al., 2002). However, evidence is lacking to support the hypothesis that they are direct targets.

As gastrulation begins, a wave of zygotic Wnt signaling initiates on the ventral side, characterized by Wnt8 expression in a crescent territory circling the blastopore outside the organizer zone (Christian and Moon, 1993; Kiecker and Niehrs, 2001). Together with Wnt3a, Wnt5a and Wnt11, these zygotic Wnt proteins sustain high Wnt signaling in the posterior part of the embryo and promotes posteriorization (Hikasa and Sokol, 2013). *Vent1/2, Cdx4, Gbx2* and *Meis3* have been indicated as major targets of the zygotic Wnts involved in posterior specification (Hikasa and Sokol, 2013). TCF binding sites found in their promoters are required for their Wnt responsiveness, suggesting direct Wnt/TCF regulation (Elkouby et al., 2010; Haremaki et al., 2003; Hikasa et al., 2010; Li et al., 2009). Interestingly, the early maternal and the late zygotic Wnt signaling activate distinct sets of target genes (Archbold et al., 2012; Hikasa and Sokol, 2013). This well illustrates the developmental stage and context specific transcriptional regulation by Wnt/β-catenin signaling. However, how Wnts achieve such specificity is not largely unknown.

1.2 TCFs are the major transcription factors of the Wnt/β-catenin pathway

The TCF family

Although β-catenin can bind to several transcription factors (TF) in the nucleus, the T-Cell Factor (TCF) family are the best-studied TF that recruits β-catenin to regulate the majority of target genes of Wnt signaling (Cadigan and Waterman, 2012; Schuijers et al., 2014).

As the name indicates, TCFs were originally discovered as lymphocyte specific TFs. They belong to a large TF family with a featured sequence-specific DNA binding

domain called High Mobility Group (HMG). TCFs were first implicated in the Wnt pathway by yeast two-hybrid screens showing their ability to interact with β-catenin (Behrens et al., 1996; Huber et al., 1996; Molenaar et al., 1996). Early studies also demonstrated that overexpressing LEF1, a member of TCF family, promoted β-catenin nuclear translocation and could mimic secondary axis inducing activity of ectopic Wnt signaling in *Xenopus* embryos (Behrens et al., 1996; Huber et al., 1996). TCF proteins bind with β-catenin via a conserved N terminal domain consisting of approximately 50 amino acids (Figure 1.4). TCF proteins lacking this domain could inhibit Wnt-dependent axis formation, behaving as dominant negative mutants. Genetic loss-of-function studies in several other organisms unequivocally demonstrate the general requirement of TCF in Wnt/β-catenin signaling. For example, mutation of *Drosophila* TCF(Cavallo et al., 1998), RNAi knockdown of *C. elegans* TCF (Herman, 2001) and double knock-out of LEF1/TCF1(two members of mouse TCFs) in mice (Galceran et al., 1999) give rise to phenotypes indicative of disrupting Wnt signaling.

Almost all invertebrates carry a single TCF ortholog, with the best-studied examples *Pangolin* (*Pan*) in *Droshophila* and *POP-1* in *C.elegans* (Figure 1.4A). However, most vertebrate species have four TCF genes, TCF1 (HUGO gene name : TCF7), LEF1, TCF3 (TCF7L1), and TCF4 (TCF7L2) (except in bony fish, including *zebrafish*, TCF1 is duplicated as TCF7L1a and TCF7L1b, thus five TCF genes in total (Dorsky et al., 2003). Except TCF3, all three other TCFs each encodes a variety of isoforms due to alternative selection of promoter and splicing sites (Figure 1.4A; Archbold et al., 2012; Cadigan and Waterman, 2012). These vertebrate TCF members share and vary in many aspects of their functions, which will be discussed in details in

following section.

1.2.1 Multiple functions of TCF

DNA binding by TCF

HMG domain and basic tail

The HMG domain (about 90 amino acids) is highly conserved (95% ~ 99% of similarity) among TCFs across different species (Figure 1.4B), endowing all the members with highly similar DNA binding specificity (Arce et al., 2006). In vitro DNA binding assays reveal that Pan (Drosophila TCF) and mammalian TCFs bind with the highest affinity to the consensus SCTTTGATS (S=G/C) (Atcha et al., 2007; van Beest et al., 2000; Giese et al., 1991; Hallikas et al., 2006; van de Wetering et al., 1991, 1997). This consensus also matches with many functional TCF binding sites found in endogenous W-CRMs (Brannon et al., 1997; He et al., 1998).

A small basic tail region located downstream of and adjacent to the HMG domain is also conserved among different TCFs (Figure 1.4B). This region has also been shown to make contact with the cognate DNA, contributing to the integral DNA recognition (Love et al., 1995). In addition to DNA binding, the HMG domain also bends the bound DNA up to 130 degrees, revealed by a structural analysis of the LEF1 HMG domain (Giese et al., 1992; Love et al., 1995). The DNA bending property has been suggested to play a structural role to coordinate the assembly of a DNA-protein complex on an enhancer of T cell receptor (TCR)-alpha (Love et al., 1995).

C-clamp domain

A distinct secondary DNA binding domain called C-clamp (about 30 amino acids)

has been identified in most invertebrate TCFs and E-tail isoforms of vertebrate TCF1 and TCF4 (refer as TCF1-E and TCF4-E hereafter) (Archbold et al., 2012; Atcha et al., 2007; Bhambhani et al., 2014; Chang et al., 2008b). The C-clamp domain is located downstream of the HMG domain and the basic tail, connected by a linker region (Figure 1.3B). The C-clamp is marked by four highly conserved cysteine residues, which has been recently shown to coordinate a zinc ion in the TCF of *Drosophila* (Ravindranath and Cadigan, 2014). Adding chelating agents could abolish DNA binding of the C-clamp, implying it functions as a novel zinc finger (Ravindranath and Cadigan, 2014). A related C-clamp domain has also been identified in other DNA binding transcription factors including HDBP1/GEF(GlutEF), HDBP2/PBF, and Gig1 (Tanaka et al., 2004). However, these C-clamp-containing TFs have not been implicated in the Wnt pathway so far.

The C-clamp domain specifically binds to a DNA motif termed the Helper site. This interaction enhances the overall DNA binding affinity of fly TCF in vitro (Chang et al., 2008b; Ravindranath and Cadigan, 2014). Many natural W-CRMs in flies, worms and mammals contain helper sites proximal to functional TCF binding sites. Mutagenesis analysis clearly demonstrates both the C-clamp domain and the helper sites are required for activation of these W-CRMs (Atcha et al., 2007; Bhambhani et al., 2014; Chang et al., 2008b). The helper site consensus sequence derived from W-CRMs varies modestly in different organisms but share a GC enriched core. For example, GCCGCCR (R=A/G) in fly, GCCRAnW (W=A/T) in worms and RCCG in human (Atcha et al., 2007; Bhambhani et al., 2014; Chang et al., 2008b). All together, these data suggests a bipartite DNA binding model, involving HMG domain-TCF binding site and C-clamp-Helper site

interactions. This mechanism is proposed to improve the specificity of TCF target selection.

However, the C-clamp domain is missing in the majority of vertebrate TCF members (Archbold et al., 2012), implying alternative mechanisms have replaced C-clamp function. In agreement with this, helper sites are not found in many well-characterized vertebrate W-CRMs, such as *Siamois, Axin2* (Brannon et al., 1997; Jho et al., 2002). How these non-C-clamp vertebrate TCFs locate and operate on helper site-free W-CRMs remains largely uninvestigated.

TCF co-repressors

TCFs themselves possess none or little intrinsic trans-regulatory activity (Cadigan, 2012). They regulate gene expression by recruiting co-regulators to W-CRMs via protein-protein interactions.

In the absence of Wnt signaling, TCF represses gene expression. Several co-repressors participate in this process, such as myeloid translocation gene related-1 (Mtgr1) (Moore et al., 2008), corepressor of Pan (Coop) (Song et al., 2010), and hydrogen peroxide-inducible clone (HIC5) (Ghogomu et al., 2006; Li et al., 2011). The best studied TCF/LEF1 co-repressor is the Groucho/transducin-like enhancer of split (Gro/TLE) repressor family members (Cadigan, 2012; Cavallo et al., 1998; Roose et al., 1998). Gro/TLEs serve as general transcriptional co-repressors for many other TFs (Chen and Courey, 2000), mainly through recruiting histone deacetylases (HDACs) to local targets (Chen and Courey, 2000), which deacetylate histone proteins, thereby altering local chromatin structure and silencing transcription (Narlikar et al., 2002).

All TCFs are able to interact with Gro/TLEs in vitro (Brantjes et al., 2001). A

conserved region called Groucho binding sequence (GBS, Figure 1.4A) had been demonstrated essential for LEF1 binding to TLE1 (Arce et al., 2009; Daniels and Weis, 2005). Although this region is modestly conserved in most TCFs, whether it mediates in vivo Gro/TLEs binding at endogenous W-CRMs awaits further investigation. Structural analysis showed the GBS overlaps with a central region of LEF1, which makes partial contact with β-catenin when β-catenin complexes with LEF1 (Daniels and Weis, 2005). It suggests β-catenin displaces Gro/TLE through competing interaction domain on TCF, providing a molecular basis of the switch from transcriptional repression to activation.

C-terminal binding protein (CtBP) has been reported to specifically interact with TCF3 and certain TCF4 isoforms in vitro (Brannon et al., 1999; Valenta et al., 2003) and negatively influences Wnt taget gene expression (Brannon et al., 1999; Tang et al., 2008; Valenta et al., 2003). The interaction likely occurs through two classic CtBP binding motifs shared only by TCF3 and TCF4, and located at C termini (Figure 1.4; Brannon et al., 1999; Valenta et al., 2003). The presence of the two motifs correlate with increased repressive acitivity of TCF4 (Tang et al., 2008). Therefore, the two CtBP binding sites may confer TCF3/TCF4 specific repression, at least partially. Together, it suggests CtBP act as a TCF3/4 specific co-repressors, accounting for certain context-dependent gene regulation by TCFs. However, the CtBP-TCF interaction was not seen by other studies (Hamada and Bienz, 2004; Valenta et al., 2006). It is worth further characterization to verify if CtBP interacts with TCF3/TCF4 through the two C terminal motifs and to what extent it accounts for TCF3/TCF4 related repression.

TCF co-activators

An array of TCF co-activators are recruited intermediately by β-catenin, mainly

through β-catenin's N terminal and C terminal transactivation domains (Cadigan, 2012; Mosimann et al., 2009). For example, Legless/Bcl9 (Lgs in flies, Bcl9 and Bcl9-2 in vertebrates) and Pygopus (Pygo) are the most important TCF co-activators (Jessen et al., 2008). Lgs binds to the N terminus of β-catenin and it further recruits Pygo via direct interaction (Jessen et al., 2008). The N-terminal homology domain (NHD) of Pygo interacts with several factors involved in transcriptional activation. For example, in flies, Pygo interacts with subunits of the mediator complex, bridging the TCF transactivation complex with RNA polymerase II complex (Carrera et al., 2008; Malik and Roeder, 2010).

Many co-activators interacting with the C terminal transactivation domain of β-catenin are also required for Wnt target gene activation (Cadigan, 2012). The best studied examples include several chromatin modifiers and remodelers. For instance, cAMP-response element-binding protein (CREB)-binding protein (CBP) and its closely related protein p300 catalyze histone acetylation, a common marker for transcriptional activation (Grewal and Moazed, 2003). CBP/p300 are involved in activating many Wnt targets in several contexts (Hecht et al., 2000; Li et al., 2007; Ma et al., 2005; Sun et al., 2000; Takemaru and Moon, 2000). MLL2 histone methyltransferase, responsible for H3K4 trimethylation, is recruited to some Wnt targets and promotes gene activation (Sierra et al., 2006). Chromatin remodeling ATPases, Brahma (Brm) and Brahma related gene 1 (Brg-1), ISWI and components of their associated chromatin remodeling complexes have also been reported for their roles in promoting Wnt target gene activation in flies and vertebrates (Barker et al., 2001; Sierra et al., 2006; Song et al., 2009; Eroglu et al., 2006; Mahmoudi et al., 2010; Major et al., 2008).

1.2.2 Functional specialization of vertebrate TCF proteins

The size and complexity of the TCF family has expanded greatly through gene duplication and isoform diversification in vertebrate lineage. This has led to functional specialization of each TCF factor, meaning unique gene targeting, or engaging specific co-factors for transcriptional activity (Cadigan and Waterman, 2012; Hoppler and Kavanagh, 2007). This appears to be an important strategy to further refine the context-dependent gene regulation of Wnt signaling in higher organisms (Cadigan and Waterman, 2012).

Based on their regulatory activity, the four vertebrate TCFs can be divided into three classes. First, TCF3 is a transcriptional repressor, keeping transcription off in the absence of Wnt signaling (Houston et al., 2002; Kim et al., 2000; Liu et al., 2005; Merrill et al., 2004) . This was first demonstrated in zebrafish. The *headless* mutant, with a truncated mutation of TCF3, displays severe defects in head formation (Kim et al., 2000). As previously discussed, Wnt inhibits head formation. Depletion of the Wnt inhibitor Dkk1 in mice results in a similar headless phenotype, indicating TCF3 acts to antagonize Wnt signaling to promote head formation (Mukhopadhyay et al., 2001). TCF3 attached with the engrailed repressor domain can efficiently rescue the headless mutant. However, replacing the engrailed domain with VP16, a potent transactivation domain, completely abolished the rescue (Kim et al., 2000). These data strongly suggests TCF3 functions as a transcriptional repressor. Deleting the β-catenin binding domain does not alter the rescue efficiency of full length TCF3, suggesting the β-catenin binding domain is dispensable for the repressor activity of TCF3 (Kim et al., 2000).

In *Xenopus*, TCF3 is required as a transcriptional repressor in multiple processes

during early embryogenesis, including patterning D-V, A-P axes (Houston et al., 2002), specifying mesoderm (Liu et al., 2005), as well as promoting head formation (Hikasa et al., 2010). Particularly, TCF3 is essential for the organizer function during dorsal axis formation. Maternal knockdown demonstrated TCF3 inhibits organizer genes in dorsal cells and suppresses ectopic expression of those genes in ventral cells (Houston et al., 2002). For instance, the organizer gene, *Siamois,* is a direct target of TCF3(Brannon et al., 1997; Hikasa and Sokol, 2011; Hikasa et al., 2010). TCF3 has been shown to be associated with *Siamois* W-CRM chromatin (Hikasa et al., 2010). Mutating the TCF sites results in *Siamois* reporter derepression (Brannon et al., 1997; Fan and Sokol, 1997), further supporting the repressing role of TCF3 on *Siamois*. In agreement with findings in Zebrafish and Xenopus, TCF3 knockout mice also display phenotypes linked to increase of Wnt/β-catenin signaling (Merrill et al., 2004). As repeatedly shown in embryonic stem cells (ESC), hair follicle stem cells, and skin stem cells, TCF3 plays a crucial role in maintaining stemness of these cells by transcriptionally repressing Wnt target genes (Cole et al., 2008; Lien et al., 2014; Nguyen et al., 2009; Yi et al., 2011).

In contrast to TCF3, full length LEF1 and TCF1 are always associated with gene activation in response to Wnt stimulation (Cadigan and Waterman, 2012b; Galceran et al., 1999; Hoverter et al., 2012; Kratochwil et al., 2002; Liu et al., 2005; Merrill et al., 2001; Yi et al., 2011). The activator activity of TCF1 and LEF1 absolutely requires an interaction with β-catenin. The isoforms lacking the β-catenin binding domain exhibit dominant negative effect, inhibiting Wnt signaling (Hovanes et al., 2001; Roose et al., 1999; Tiemessen et al., 2012). The only exception which relates full length TCF1 to transcriptional repression was reported in *Xenopus* ventral blastomeres (Standley et al.,

2006). Depletion of maternal TCF1 in *Xenopus* led to decrease of several organizer genes in dorsal blastomeres but increase in ventral cells, arguing that TCF1 being an activator in dorsal cells but a repressor in ventral cells (Standley et al., 2006). Given that the repressor function of TCF1 is contradictory to observations made in all the other contexts examined, it is worth re-exploring if this effect is true, and why TCF1 behaves as such in this particular context.

Full length TCF4 is capable to conduct both activation and repression depending on the contexts and isoforms (Angus-Hill et al., 2011; Lien et al., 2014; Liu et al., 2005; Nguyen et al., 2009; Standley et al., 2006; Tang et al., 2008; Weise et al., 2010; Van de Wetering et al., 2002). In mice, TCF4 knockouts display loss of the stem cell compartment in the intestine (Korinek et al., 1998), consistent with a loss of Wnt/β-catenin signaling. In colon cancer cells, a genome survey study revealed that, TCF4 is the most abundantly expressed TCF, and binds to regulatory regions of target genes that are up-regulated by Wnt signaling, including a common direct target, Axin2 (Hatzis et al., 2008). However, in the same cellular context, TCF4 siRNA results in increased Wnt/β-catenin signaling activity, suggesting repressive role (Tang et al., 2008). These seemingly conflicting results further illustrate the highly context-dependent and target-dependent diverse transcriptional outputs of Wnt signaling. Consistent with this idea, another study focusing on characterizing function of a repertoire of TCF4 splicing isoforms in murine ESCs and neural progenitors, demonstrated different transactivational potential among isoforms (Weise et al., 2010). In mice skin stem cells, TCF4 primarily works in redundancy with TCF3 as transcriptional repressor to keep long-term homeostasis of skin epithelia (Nguyen et al., 2009). In early *Xenopus* embryo, maternal

depletion of TCF4 suggests TCF4 is involved in activating dorsalizing genes, including *Siamois and Chordin* (Standley et al., 2006). However, during gastrulation, the same TCF4 isoform exhibits repressor activity, rescuing mesoderm formation caused by morpholino knockdown of TCF3 (Liu et al., 2005). This repressive function is suggested to be conferred by two small motifs, LVPQ and SXXSS, located in the central region upstream of the HMG domain (Liu et al., 2005). A naturally occurring isoform TCF4C, which lacks these two motifs, was unable to rescue TCF3 knockdown, but could rescue TCF1 knockdown (Liu et al., 2005). Interestingly, these two motifs are only shared by TCF3 and TCF4A. However, whether these two motifs are essential for the repression mechanism of TCF3/TCF4A and how they mediate the repression is not known. It is fundamental to understand the molecular basis of the specialized functions of vertebrate TCFs, however so far it remains largely unknown.

Apparently, in vertebrate systems, multiple TCFs have to participate in the transcriptional switch, with a distinct TCF executing each specialized functions. An interesting question is how Wnt signaling coordinates multiple TCFs to convert transcriptional repression into activation. It has been suggested that Wnt/β-catenin releases TCF3 repression by reducing TCF3 target occupancy through distinct mechanisms depending on the contexts (Atlasi et al., 2013; Hikasa et al., 2010; Shy et al., 2013). For example, in ESCs, Wnt stimulation inhibits TCF3 transcription (Atlasi et al., 2013) and β-catenin promotes TCF3 degradation (Shy et al., 2013). Wnt-stimulated TCF3 removal from chromatin has been demonstrated on some Wnt targets (such as *Siamois* and *Vent2*) in early *Xenopus* development (Hikasa and Sokol, 2011; Hikasa et al., 2010). The same studies also showed activating TCF1 displaces TCF3 to bind to

targets after TCF3 is removed from chromatin (Hikasa and Sokol, 2011; Hikasa et al., 2010). This TCF exchange model offers a framework of how multiple TCFs coordinate in the vertebrate transcriptional switch. This part will be further discussed in details in the vertebrate TCF transcriptional switch section.

1.2.3 TCF transcriptional switch

Using the same transcription factor to flip a switch to repress or activate target gene expression is a common strategy of cell-cell signaling pathways, such as Su (H)/CBF1 in the Notch pathway, and Ci/Gli in the Hh pathway (Barolo 2002). In the case of the Wnt pathway, the TCF transcriptional switch comes in several forms. In invertebrate systems, the single TCF operates both sides of the switch with the aid from co-repressors for repression, and β-catenin and co-activators for activation (Figure 1.5A&B; Cadigan and Waterman, 2012). In vertebrates, TCF/LEF1 heterogeneity and functional specialization allow the possibility of multiple TCF factors engaged in the switch. A recent study demonstrated Wnt stimulates an exchange of repressive TCF for activating TCF on W-CRM (Figure 1.5C&D; Hikasa and Sokol, 2011; Hikasa et al., 2010). Although this model has only been experimentally validated in *Xenopus* embryos for limited number of Wnt targets, it fits well with the observations in many other vertebrate contexts, which imply divided labor in regard to repression and activation among TCFs (Cadigan and Waterman, 2012). This model sheds light on a mechanistic understanding of the vertebrate TCF transcriptional switch.

TCF transcriptional switch in invertebrates

The switch model of the single TCF in invertebrates was supported by several lines of evidence (Cadigan and Waterman, 2012). For example, in flies, TCF mutation

caused defects in embryonic epidermal patterning that is similar to Wingless (Wg, a fly Wnt ligand) mutants, but less severe (Brunner et al., 1997; van de Wetering et al., 1997). Interestingly, TCF/Wg double mutants display the identical phenotype as single TCF mutants (Cavallo et al., 1998). This TCF suppressing Wg effect suggests a dual role of TCF. In brief, loss of TCF's repressive function derepresses Wg targets, compensating the loss of activation by Wg. Loss of TCF's activating function reduces gene activation, mimicing compromised Wg signaling. In fly cell culture, a study using RNAi and ChIP of TCF demonstrated that the TCF switch is operated on several Wg targets, lending more mechanistic insights for the model (Fang et al., 2006)

In *C. elegans*, the TCF switch model is slightly different. The current view proposes that the switch occurs involving nuclear efflux of POP-1 (the single worm TCF) in addition to β-catenin/co-activator recruitment (Sawa, 2012). The model is best supported by the action of POP-1 in specifying endoderm cell fates. POP-1 is kept low in the nucleus of prospective endoderm cell (E cells) as a result of efflux driven by high Wnt signaling (Sawa, 2012). Low level of nuclear POP-1 somehow stabilizes POP-1-β-catenin complex on chromatin, leading to activation of endoderm genes (Shetty et al., 2005). On the other hand, POP-1 remains high in the nucleus of prospective mesoderm cells (MS cells) due to low Wnt signaling. In MS cells, POP-1 represses those endoderm genes. Depletion of POP-1 causes derepression of these genes in MS cells (Shetty et al., 2005). Agreeing with the model, mutagenesis analysis of the W-CRM of *end-1*, one important E cell specific gene, reveals that the single TCF binding site in this W-CRM is sufficient to mediate both activation of end-1 reporter in E cells as well as repression of the reporter in MS cells (Shetty et al., 2005).

TCF transcriptional switch in vertebrates

Mutating TCF sites in some vertebrate W-CRM reporters support that they are both negatively and positively regulated by TCFs (Brannon et al., 1997; Hikasa et al., 2010). The best example is the *Xenopus Siamois* W-CRM (Brannon et al., 1997; Fan et al., 1998). It has been shown that TCF3 represses *Siamois* (Brannon et al., 1997; Houston et al., 2002), and TCF1 contributes to *Siamois* activation in the dorsal organizer region (Standley et al., 2006).

Recent studies in *Xenopus* showed that Wnt promotes the phosphorylation of LEF1, TCF3, and TCF4 by homeodomain interacting protein kinase 2 (HIPK2; Hikasa and Sokol, 2011; Hikasa et al., 2010), which is a highly conserved serine/threonine kinase, and functions in transcriptional regulation, cell growth and apoptosis (D'Orazi et al., 2002, 2012). The phosphorylation disengages the chromatin binding of TCFs. Upon Wnt stimulation, TCF1, lacking the HIPK2 phosphorylation sites, displaces TCF3 to re-occupy W-CRM chromatins (Figure 1.5C&D; Hikasa and Sokol, 2011; Hikasa et al., 2010). The Wnt-stimulated TCF3 exchange for TCF1 was demonstrated on *Vent2* and *Siamois* W-CRMs in *Xenopus* early embryos. Consistent with the exchange model, in mice, it has been observed that Wnt3a-stimulated ESC self-renewal requires TCF1 to antagonize target gene suppression by TCF3 (Yi et al., 2011). In the same context, another study showed β-catenin binding to TCF3 promotes TCF3 degradation, leading to decreased TCF3 chromatin binding; HIPK2, however, is not involved in this process (Shy et al., 2013). It would be very interesting to investigate whether an exchange for TCF1 accompanies TCF3 removal in this context.

The exchange model assumes TCF3 occupies targets by default, which is

puzzling, given that TCF3 has similar/identical DNA binding specificity as other TCF members. What determines TCF3 to outcompete TCF1 in DNA binding when Wnt signaling is off? Understanding this fundamental question will help us better understand the operation of the switch and improve our ability to identify novel Wnt targets.

1.2.4 Target selection by TCFs

Insufficiency of TCF binding site

In general, TFs locate at "correct" target *cis*-regulatory modules (CRMs) via their DNA binding domain recognizing cognate sequences (Levine, 2010). Specific target recognition is essential to ensure proper gene expression, however it is not easy for eukaryotic TFs, because they recognize very short sequences (typically 6-12bp), which are highly degenerate and do not contain sufficient information to specify CRMs in large genomes (Todeschini et al., 2014).

In the case of TCFs, the HMG domain mediates the primary DNA binding by recognizing a high affinity consensus "SCTTTGATS, S=G/C". Synthetic reporters driven by concatemerized optimal TCF binding sites (3 to 12 copies of 'CCTTTGAT'), such as TOPFLASH, have been shown to specifically respond to Wnt signaling in various contexts, including mammalian cell cultures, transgenic zebrafish and mice (Barolo, 2006). This is generally used as an argument that the TCF binding site is sufficient to locate TCF to genomic sites in vivo. But such a dense cluster of high affinity sites is rare in natural W-CRMs (Archbold et al., 2012; Bhambhani and Cadigan,2014). Functional TCF sites tend to be randomly scattered across natural W-CRMs. Also, the sites, judged by their in vitro affinity to HMG, is often less optimal than the sites in synthetic reporters (Hallikas et al., 2006; Bhambhani and Cadigan,

2014). In addition, the multimerized TCF site reporters do not give complete Wnt signaling pattern, meaning they are only active in a subset of Wnt –positive territories in transgenic animals, such as in *Xenopus* and mice (Barolo, 2006). In sum, given the quality and the organization of TCF binding sites in endogenous W-CRMs, they appear insufficient to determine TCF recognition specificity.

In vitro DNA binding profiling experiments revealed that the HMG domain of TCFs could recognize a broad spectrum of degenerate sequences (Badis et al., 2009). In vivo, functional TCF sites can deviate from the consensus quite far (Bhambhani and Cadigan, 2014). For instance, divergent sites (e.g., CTTTTGAAG, CTTTATAG, GTTTGATG, CCTTTTTTC) have been suggested to contribute to Wnt responsiveness (Hilton et al., 2003; Lam et al., 2006). The high extent of degeneracy makes it extremely difficult to identify authentic TCF binding sites from excess DNA. Genome-wide studies have found that a significant number of regions occupied by TCFs do not contain recognizable TCF sites (Blahnik et al., 2010; Frietze et al., 2012; Hatzis et al., 2008; Junion et al., 2012; Wu et al., 2012). This may be due to indirect recruitment of TCF via protein-protein interactions. But it is also possible that TCF binds to divergent sites in those areas. Taking degenerate sites into account (within the range of known functional divergent sites), millions of potential TCF sites exist in the human genome (Archbold et al., 2012). How TCF distinguishes functional sites among all the irrelevant sites is a challenging question to the field.

Bipartite binding of some TCF through C-clamp and Helper site interaction

Direct evidence demonstrating HMG-TCF site interaction is insufficient for TCF locating endogenous targets comes from studies on C-clamp and helper site in flies,

worms and mammalian cells (Atcha et al., 2007; Bhambhani et al., 2014; Chang et al., 2008b; Hoverter et al., 2012). As discussed earlier, the bipartite DNA binding by HMG and C-clamp allows TCF to recognize a composite DNA code with extended sequence information, thereby increasing target selectivity (Figure 1.6A; Chang et al., 2008b). A recent study in flies showed recombinant HMG-C-clamp protein binds to a DNA probe containing only a TCF site worse than when it binds to a probe containing a TCF site and a helper site, suggestive of inhibitory effect of C-clamp (Ravindranath and Cadigan, 2014). This paper also showed direct interaction between C-clamp and HMG. This interaction is suggested to auto-inhibit individual DNA binding of each domain and promotes bipartite binding when possible (Ravindranath and Cadigan, 2014). This also explains how the C-clamp assists TCF to select specific W-CRMs.

Cooperative DNA binding with other transcription factors

TFs often recognize DNA in cooperation with additional TFs via protein-protein interactions (Levine, 2010; Todeschini et al., 2014). This could occur as a homo-oligomer, as seen with p53 binding to repeated half sites as a tetramer (Brandt et al., 2009). Many nuclear hormone receptors form heterodimers on DNA in different combinations partially depending on the half sites organization (Todeschini et al., 2014). To accommodate cooperative TF binding, the CRM has to harbor multiple cognate codes, which decreases the probability of random occurrence and increasing the selectivity of target sequence. In regard to TCF, several TFs have been suggested to act cooperatively with TCF to bind to regulatory regions.

Two members of the Smad family, which is the major TF family of the TGF-β pathway, Smad3 and Smad4, directly interact with LEF1 (Labbé et al., 2000; Nishita et

al., 2000). In the regulatory region of the *twin* gene of *Xenopus*, TCF and Smad binding sites are paired in close proximity and both are required for full activation of a *twin* reporter by Wnt and TGF-β signaling in the contexts of cell culture and *Xenopus* embryos (Figure 1.6B; Labbé et al., 2000; Nishita et al., 2000). Several other genes have also been reported to be co-regulated by TCF and Smad proteins, such as *Msx2* (Hussein et al., 2003), *c-myc* (Hu and Rosenblum, 2005), and *Emx2* (Suda et al., 2010; Theil et al., 2002). Consistent with this, ChIP data demonstrates β-catenin increases Smad recruitment on LEF1 bound regulatory region of *Msx2* (Hussein et al., 2003).

Another example of a cooperative binding partner of TCF is c-Jun, a TF that binds DNA as a homodimer or a heterodimer with c-Fos (constituting AP-1) (Shaulian & Karin 2002). In colorectal cancer (CRC) cells, c-Jun activates itself in cooperation with TCF4 and β-catenin, forming a forward feedback loop to promote carcinogenesis (Nateri et al., 2005). Phosphorylated c-Jun could interact with TCF4, and both associates with the regulatory DNA of c-Jun (Nateri et al., 2005). In contrast to co-regulation of Smad-LEF1, where the binding sites of each factor are closely linked, the TCF and AP-1 sites are far apart, suggesting formation of a DNA loop stabilized by protein-protein and protein-DNA interactions (Figure 1.6C). On a genome level, a chromatin binding survey of β-catenin in CRC cells revealed that 40% of β-catenin bound regions contain TCF4 as well as AP-1 sites (Bottomly et al., 2010). More than a dozen sites are bound by TCF4, c-Jun and β-catenin (Bottomly et al., 2010).

TCF also cooperates with transcription factors not directly controlled by cell–cell signaling. The best example is the Cdx family of homeodomain proteins. *Cdx1* and *Cdx4* are direct targets of Wnt signaling themselves (Archbold et al., 2012). Cdx1 auto-

regulation requires Cdx1-LEF1-β-catenin complex to assemble on *Cdx-1* W-CRM via direct interaction between the homedomain of Cdx1 and the HMG domain of LEF1 (Béland et al., 2004). Interestingly, a genome-wide survey of Cdx2 binding in intestinal cell lines revealed a significant overlap between Cdx2 and TCF4 bound regions and TCF4 chromatin binding decreases in Cdx2 mutant cells, suggesting DNA binding of TCF4 depends on Cdx2 in intestinal cells (Verzi et al., 2010).

Genome-wide survey and Transcription Factor collective

Comparing genome binding profiles of TCF4 from different types of cells, an emerging theme is TCF binding pattern largely depends on the cell type (Bhambhani and Cadigan,2014). For example, Freitze, et al. 2012 measured TCF4 binding in 6 distinct human cell lines. The overlap of TCF bound regions ranged from 18% to 46% in pairwise comparisons (Frietze et al., 2012). Interestingly, the same study also discovered cell type-specific transcription factors tend to co-localize with TCF4. In liver-derived HepG2 cells, TCF4 was found to occupy genomic loci with liver specific factors HNF4 and FoxA2, and with GATA3 in the breast cancer cell line MCF7 (Frietze et al., 2012). This suggests cell type specific transcription factors play a role in specifying context-dependent target genes of TCFs.

Another common observation made by genome-wide studies is a large portion of TCF bound loci contain no consensus sites, indicating TCF could be recruited to chromatin indirectly (Bhambhani and Cadigan,2014). In a study surveying the genomic locations of five TFs, TCF, Tinman, pMad, Pannier and Dorsocross, all known to be genetically required for cardiac specification in the fly embryo, the five TFs were demonstrated to act as a collective unit, cooperatively binding to regulate heart CRMs

(Junion et al., 2012). However, areas co-occupied by five TFs were strikingly devoid of predicted TCF binding sites but enriched with binding sites of Doc and Pannier (Junion et al., 2012), suggesting TCF is tethered by other DNA binding proteins. Supporting this, interactions between all five factors have been observed in both flies and vertebrates (Brown et al., 2004; Bruneau et al., 2001; Durocher et al., 1997; Gajewski et al., 2001; Garg et al., 2003; Nishita et al., 2000; Zaffran et al., 2002). Functionally, the five TF co-occupancy signature strongly correlates with enhancer activity (Junion et al., 2012). 92% of 28 five-TF co-bound regions tested in transgenic flies exhibited expression in the mesodermal lineage (Junion et al., 2012). This study illustrates a distinct TCF recruitment mechanism relying less on direct DNA binding and also demonstrates physically mapping the TF collective is an effective way to identify novel CRMs. However, other TF collectives involving TCF have largely not been explored.

Identifying direct target gene of Wnt signaling is key to better understand Wnt biology. Over 100 genes have been found as Wnt target genes (see Wnt homepage for a full list, http://web.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes), however only half have been confirmed as direct target using a candidate approach. We still lack a systematic method to identify new Wnt targets because of our limited understanding of the TCF target selection and the "grammar" of W-CRMs.

1.3 VegT is a master regulator of mesoendoderm development in *Xenopus*

My main thesis work (chapter 3) focused on the role of the maternal T-box protein, VegT, in the TCF transcriptional switch regulating *Siamois* in *Xenopus*. In the following section, VegT will be introduced in detail.

1.3.1 VegT governs endoderm and mesoderm specification

VegT (also known as *Xombi, Antipodean,*or *Brat*) was identified as a strong endoderm and mesoderm inducer from a series of functional screens in *Xenopus* by four separate groups (Horb and Thomsen, 1997; Lustig et al., 1996; Stennard et al., 1996; Zhang and King, 1996). VegT is maternally expressed at high levels in oocytes and fertilized embyros, where it is localized to the vegetal hemisphere (Figure 1.7; Zhang and King, 1996). In embryos with maternal VegT depeleted by an antisense oligonucleotide, neither endoderm nor mesoderm forms (Kofron et al., 1999; Xanthos et al., 2001). Overexpressing VegT in the ectoderm (animal cap cells) induces a full spectrum of markers of both endoderm and mesoderm (Horb and Thomsen, 1997; Stennard et al., 1999). Together, it suggests maternal VegT plays an essential role in determining endoderm and mesoderm formation.

At mid-blastula stage, VegT drives zygotic expression of numerous genes, composing a regulatory network instructing vegetal cells to differentiate into endoderm (Wylie et al., 1987; Xanthos et al., 2001). Maternal depletion of VegT blocks expression of the majority of endoderm genes (Xanthos et al., 2001). Among these genes, the ones that have been demonstrated to be direct VegT targets include transcription factors *Bix1, Bix4, Sox17α* and TGFβ growth factors *Xnr1, 2 and 4* (Casey et al., 1999; Howard et al., 2007; Hyde and Old, 2000; Xanthos et al., 2001). Endoderm cells send a diffusible signal (mainly TGFβ growth factors) to overlying marginal cells to induce mesoderm formation (Smith, 2009). VegT has also been suggested to play a direct role in mesoderm induction via directly activating several mesoderm marker genes, such as *derrière* (White and Heasman, 2008) and *Gsd* (Sudou et al., 2012).

1.3.2 VegT is also required for the organizer formation

Maternal VegT is involved in establishing the dorsal organizer via two distinct mechanisms. First, VegT directly activates expression of *Xenopus* nodal related (Xnr) proteins, giving rise to high nodal signaling (a subset of TGFβ signaling) in the dorsal vegetal zone of the early blastula (Agius et al., 2000; Rex et al., 2002; Takahashi et al., 2000; Yang et al., 2002), where is also known as Nieuwcoop center (Figure 1.7). High nodal signaling derived from the Nieuwcoop center induces overlying cells (dorsal marginal cells) to form the organizer in combined action with maternal Wnt signaling (Robertis and Larrain, 2000) (Figure 1.7). Within the organizer, several hallmark genes, such as *Gsd, Chordin* and *Cerberus* are controlled by transcriptional cooperation of Wnt signaling and Nodal signaling (Reid et al., 2012).

The Nieuwcoop center corresponds to the area where VegT overlaps with dorsally distributed β-catenin (Figure 1.7). In fact, β-catenin has also been implicated in initiating the zygotic nodal signaling. The pre-MBT expression of Xnr5 and Xnr6 (two nodal ligands expressed earliest) depends on both VegT and β-catenin (Agius et al., 2000; Rex et al., 2002; Takahashi et al., 2000; Yang et al., 2002). Maternal depletion of VegT or β-catenin abolished Xnr5 and Xnr 6 transcription and disrupted the organizer-inducing activity of the Nieuwcoop center (Skirkanich et al., 2011; Yang et al., 2002), demonstrating the interplay of VegT and β-catenin is necessary for establishing the Nieuwcoop center and inducing the organizer.

The second mechanism is maternal mRNA of VegT stabilizes the localization of maternal Wnt11 message at the vegetal pole (Heasman et al., 2001). When maternal VegT mRNA is degraded by anti-sense oligonucleotide, the vegetal pole distribution of

Wnt 11 mRNA is disrupted (Heasman et al., 2001). The VegT depleted embryos lack apparent axis structures (Kofron et al., 1999; Xanthos et al., 2002), consistent with disruption of maternal Wnt11 function (Tao et al., 2005). Interestingly, morpholino knockdown of VegT, which only blocks translation but not affects mRNA of VegT, did not affect Wnt 11 mRNA localization (Heasman et al., 2001), and the morphants display no dorsal axis defects (Ishibashi et al., 2007). Together, these results uncover a novel function of VegT mRNA in regulating localization of maternal Wnt11 and affecting the organizer formation. To avoid the caveat of disrupting Wnt11 localization, we chose to use morpholino knockdown to perform VegT loss of function experiments in my study.

1.3.3 T-box proteins in *Xenopus*

VegT belongs to a large TF family featuring a conserved T-box DNA binding domain. 18 family members have been identified in mammals so far, functioning in a wide range of developmental processes, including primary germ layer specification (e.g. Bra), limb development (e.g. TBX4/5) and heart development (TBX1,2,3,5,6,18,20) (Showell et al., 2004).

In *Xenopus*, besides VegT, three other well-characterized T-box factors, Brachyury (Bra), Eomesodermin (Eomes), and TBX6, also play important roles in early embryogenesis of *Xenopus* (Showell et al., 2004). Unlike maternal VegT, they are all zygotically expressed, and function in patterning the mesoderm downstream of VegT (Showell et al., 2004). The VegT gene also encodes a zygotic isoform, lacking 25 amino acids at the N terminus of the maternal VegT protein. It is expressed in dorsal marginal cells at the early gastrula stage and it is involved in paraxial mesoderm patterning (Fukuda et al., 2010). It is unknown if the N terminal difference causes the functional

difference of the two isoforms.

T-box and transactivation domains

The T-box DNA binding domain (about 200 amino acids) is modestly conserved among VegT, Bra and Eomes (Showell et al., 2004). In vitro, they bind to the same core sequence, TCACACCT, but slightly differ in the preference of flanking sequence (Conlon et al., 2001). VegT, Bra and Eomes have all been shown to induce mesoderm genes in isolated ectoderm cells when overexpressed, however the resulting mesoderm type in response to each factor is qualitatively different. For example, VegT and Eomes induce the entire spectrum of mesoderm types, whereas Bra induces mainly the posterior mesoderm (Conlon et al., 2001; Messenger et al., 2005). It has been inferred that the flanking sequence of the core motif accounts for target selection specificity of each T-box factor. However, this idea was only based on limited functional tests with overexpressed proteins (Conlon et al., 2001). Thus, the physiological relevance of flanking sequence variance remains to be determined.

Functional T-box sites identified in vivo exhibit varying degrees of degeneracy in the core motif and flanking sequences, making the endogenous T-box sites indistinguishable for each factor. In fact, a genome-wide binding survey of zygotic VegT, Bra and Eomes reveal that three factors are recruited to the same genomic loci for the majority of the bound peaks (Gentsch et al., 2013). Indirect recruitment via interactions between each other is unlikely, because so far no evidence for direct physical interaction among the three proteins is found (Showell et al., 2004), arguing they may recognize and bind to the same T-box motifs in vivo (Gentsch et al., 2013). To achieve specific regulation, they may cooperate with distinct co-factors, utilizing unique domains

outside of the T-box domain. The best example illustrating this idea comes from the study addressing why VegT is able to induce Gsd in ectoderm whereas Bra cannot. They found Bra activates *Xom*, which is a strong repressor of *Gsd* (Messenger et al., 2005). Induction of *Xom* requires specific interaction between Bra and Smad1 (a component of the BMP signaling pathway). This interaction is mediated by the N terminus upstream of T-box domain of Bra. Switching this domain with the corresponding region of VegT, converts Bra into a *Gsd* activator (Messenger et al., 2005).

It has been well established that most T-box factors function as transcriptional activator, except Tbx2 and Tbx3 (Showell et al., 2004; Singh and Kispert, 2010). Generally, the C terminal region of most T-box factors are correlated with their transactivation activity (Showell et al., 2004). Consistent with this, when the C terminal region downstream of the T-box domain of VegT fused to the Gal4 DNA- binding domain, it was able to activate reporter gene in yeast, suggesting the C terminal region of VegT has transactivation function (Zhang and King, 1996).

Although VegT primarily acts as a transcriptional activator, it has been previously linked to repression of *Siamois* and *Xnr3*, both are major organizer genes primarily activated by TCF/β-catenin in response to Wnt signaling (Hyde and Old, 2000; Ishibashi et al., 2007). However, whether the repression is directly mediated by VegT has not been examined before. Further study is needed to elucidate the mechanism of the repressive action of VegT.

1.3.4 Interaction between T-box proteins and Wnt signaling

As discussed previously, Xnr5 and Xnr6 expression in the Nieuwcoop center requires combined inputs from VegT and TCF/β-catenin, however, the molecular

mechanism of this cooperation is not known (Yang et al., 2002). Another study demonstrated that VegT physically interacts with TCF3 (Cao et al., 2007), providing a possible mechanism for the combinatory action of VegT and TCF. In this study, using GST pull down assay with truncated proteins, they have roughly mapped the interaction domain to the N-terminal half of VegT (1-230 amino acid) containing the T-box domain and a TCF3 fragment (190 – 551 amino acid) encompassing the HMG domain and entire C terminal region (Cao et al., 2007).

Other T-box family members have also been suggested to cooperate with TCF in other systems. For example, *Dll1, Msgn1, Hes7* are important genes involved in patterning presomitic mesoderm in mouse. Clusters of TCF sites and T-box sites found in regulatory regions were necessary for their full transcriptional activation in mice (González et al., 2013; Hofmann et al., 2004; Wittler et al., 2007). In these cases, TCF and Tbx seem to cooperate by forming a complex on DNA, yet no direct evidence for TCF and Tbx interaction have been shown.

RATIONALE AND SPECIFIC AIMS

Wnt/β-catenin signaling controls diverse biological events mostly through regulating target gene expression. The TCF family members are the primary transcriptional regulators of the Wnt pathway. The major focus of my dissertation research is to understand certain aspects of the vertebrate TCFs, including the C-clamp independent target selection and the transcriptional switch involving exchange of specialized TCF members. My thesis comprises two data chapters:

Chapter 2: The goal was to explore how non-C-clamp vertebrate TCFs select specific targets

Invertebrate TCFs (e.g. fly TCF) bind two distinct DNA motifs, the TCF and the Helper sites, via two DNA binding domains, the HMG and the C-clamp domain. This bipartite recognition enhances the target selection specificity and is essential for regulation of various W-CRMs. However, the C-clamp is absent in most vertebrate TCFs (e.g. LEF1 and TCF3), indicating they require other mechanisms to achieve specific targeting. I have investigated two hypotheses: First, if the HMG domain of non-C-clamp TCFs is sufficient to recognize endogenous targets on its own. I found that LEF1 needs C-clamp to activate endogenous fly targets, suggesting the HMG domain of LEF1 still requires help in selecting specific targets. Second, if other transcription factors cooperate with non-C-clamp TCFs to assist their target selection. I examined the TCF3-regulated *Siamois* W-CRM. Through a mutagenesis screen, a variety of novel DNA motifs that control Wnt-dependent activation of *Siamois* reporter were identified, indicating additional transcription factors are involved in TCF regulation of the *Siamois* W-CRM.

Part of this work has been published in Bhambhani et al., 2014.

Chapter 3: to characterize VegT's role in the TCF transcriptional switch regulating *Siamois* transcription in *Xenopus*

A set of repressive T-box sites identified from the aforementioned mutagenesis screen of the *Siamois* W-CRM, and their binding factor VegT have been characterized in detail in this chapter. I found that VegT functions in the TCF transcriptional switch with two distinct roles. In the absence of Wnt signaling, VegT cooperates with TCF3 to repress *Siamois* via physical interaction. Upon Wnt stimulation, HIPK2 phosphorylates TCF3 as well as VegT, and disrupts the interaction between VegT and TCF3, leading to TCF3 removal from chromatin and subsequent exchange for TCF1 occupying the *Siamois* locus. In contrast, VegT remains bound to *Siamois* locus and switches to participate in activating *Siamois* transcription in a TCF1-independent manner. My findings provide a resolution for the controversy of VegT's function in *Siamois* regulation in the literature. Also, they add a new level to our understanding of how the TCF transcriptional switch regulates Wnt targets.

The data presented in this chapter is a major portion of the manuscript: "VegT plays a dual role in the TCF transcriptional switch regulating Siamois expression in *Xenopus*", Yaxuan X. Yang, Ann L. Miller, Robert J. Denver and Ken M. Cadigan. This manuscript has been submitted to Developmental Biology, and is currently in revision.

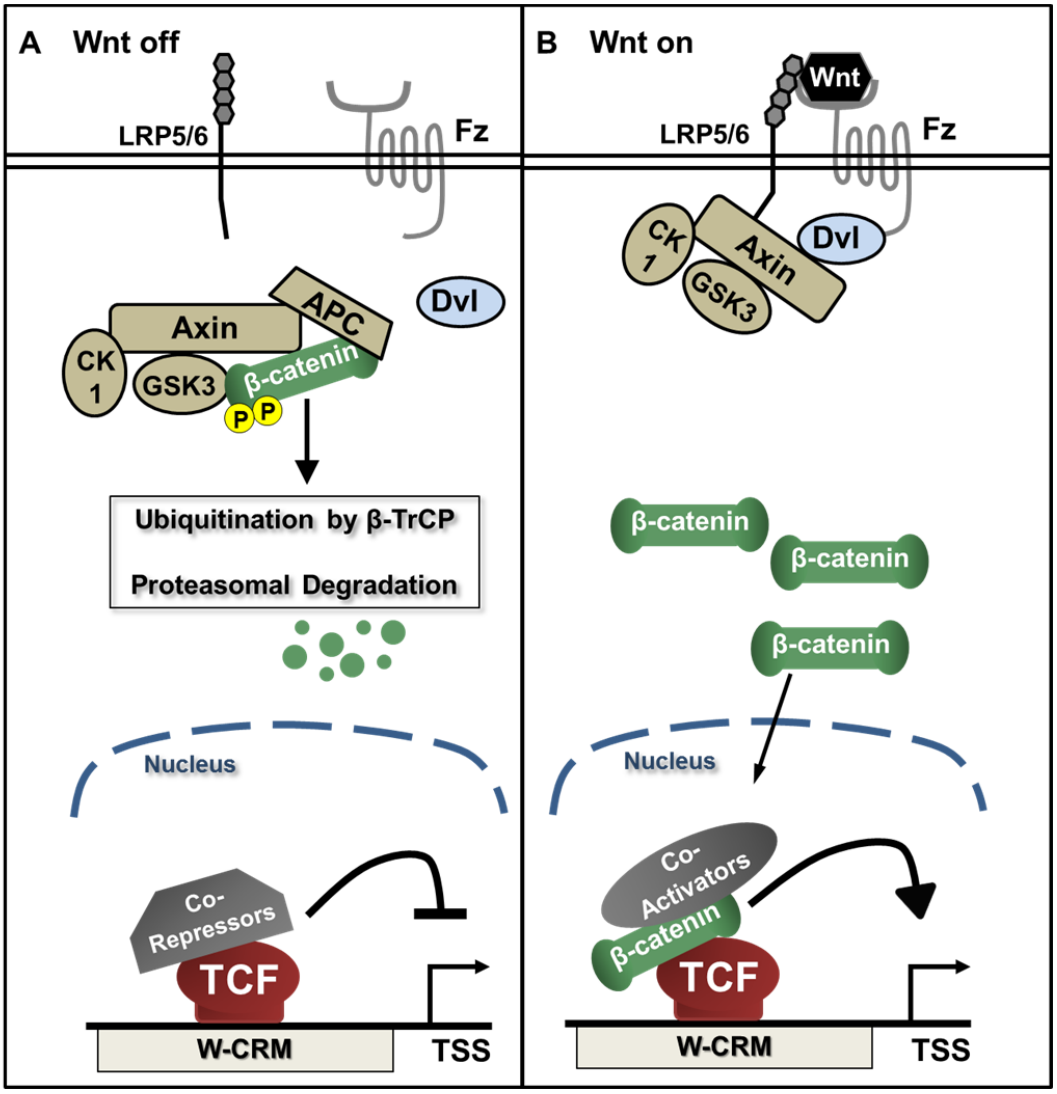

Figure 1.1 Simplified model of the Wnt/β-catenin signaling cascade.

(A) In the absence of Wnt, β-catenin is kept at low levels in the cytosol due to constant degradation. In the nucleus, TCFs are localized at Wnt-dependent *cis*-regulatory elements (W-CRMs) and repress transcription of Wnt target genes with co-repressors. (B) Wnt stimulation inhibits the activity of the degradation complex, which includes Axin, APC, CK1, and GSK3, thereby stabilizing β-catenin in the cytosol. β-catenin then translocates into the nucleus, and binds to TCF by replacing co-repressors. Additional co-activators are recruited to the TCF/β-catenin complex, turning on expression of target genes.

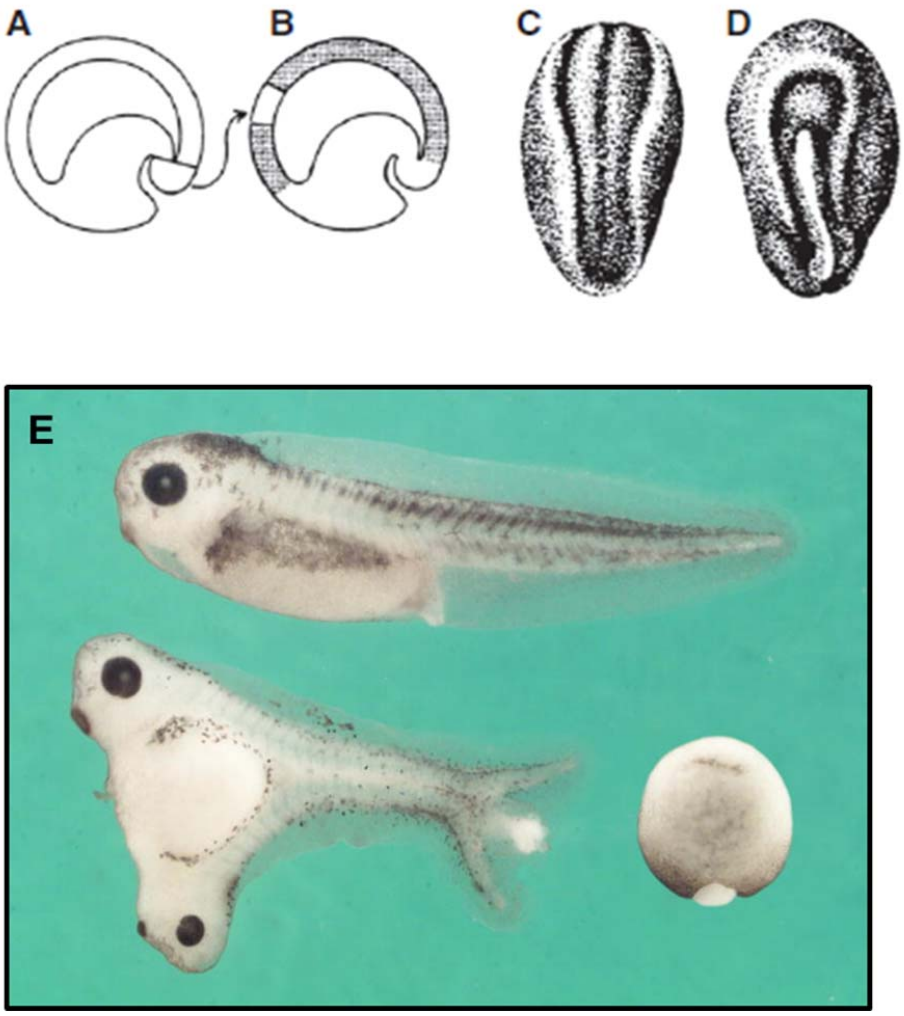

Figure 1.2 The Spemann-Mangold experiment and discovery of the organizer.

(A-D) Taken from (Niehrs, 2010), reprinted, with permission, from Development: dev.biologists.org. (A,B) Transplantation of the upper blastopore lip (the organizer) of a gastrula of Triturus cristatus (A) to the ventral side of a gastrula of T. taeniatus (B). (C) Neural plate of host embryo. (D) Secondary induced neural plate. Note the white donor cells in the midline. (E) Taken from (Kuroda et al., 2004), reprinted, with permission, from the Annual Review of Cell and Developmental Biology, Volume 20 (c) 2004 by Annual Reviews www.annualreviews.org. The Spemann–Mangold experiment reproduced in *Xenopus laevis*. A graft of albino dorsal lip was transplanted into the ventral side of the gastrula (bottom right), resulting in a siamese twin (bottom left). Note that the D–V and A–P axes are perfectly integrated; this can be seen, for example, in the perfect alignment of somites (segments) of the duplicated axes.

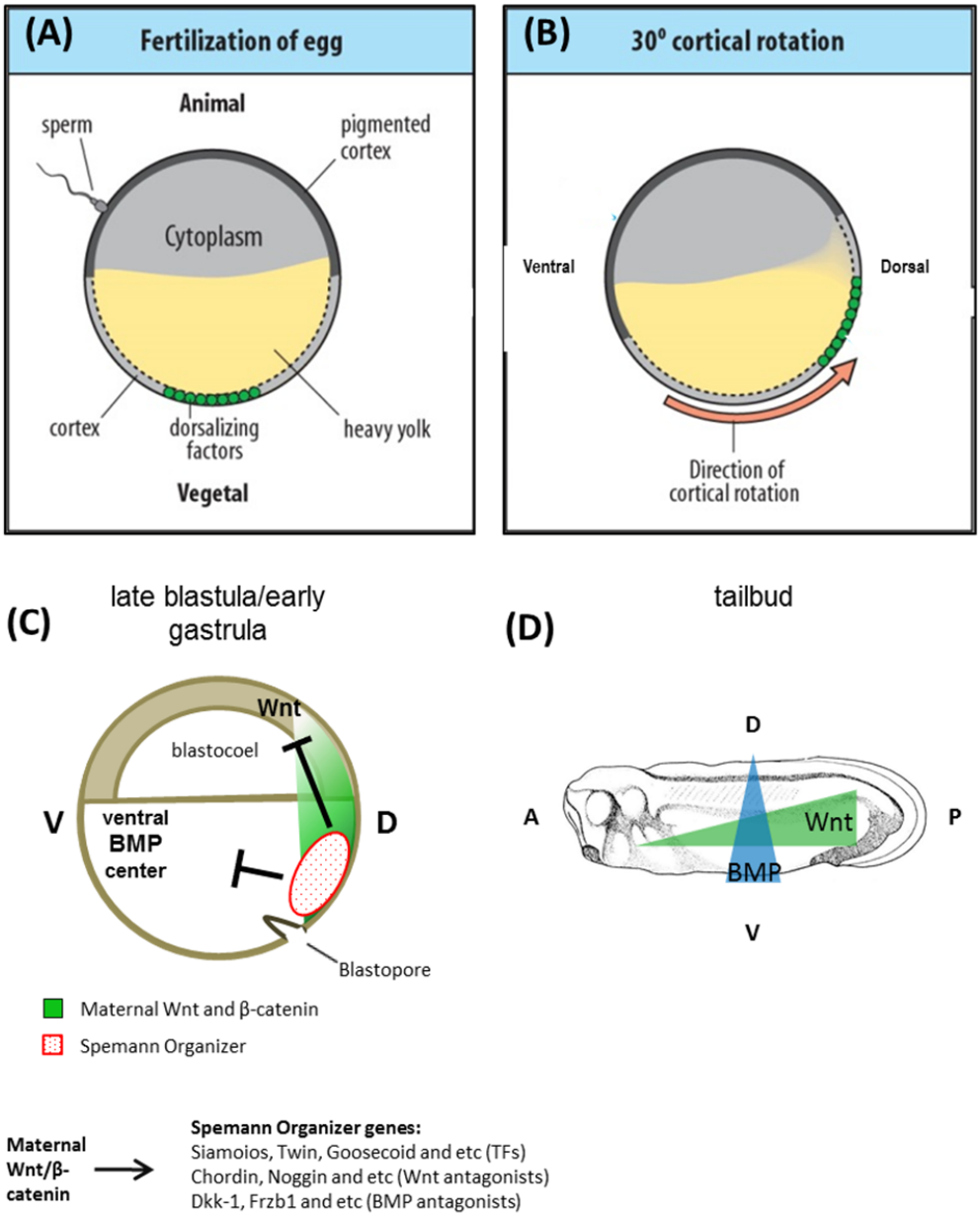

Figure 1.3 Simplified model for dorsal axis formation in *Xenopus*.

(A&B) Taken from
http://www.mun.ca/biology/desmid/brian/BIOL3530/DB_03/DBNVert1.html
(A) During oogenesis, dorsalizing factors, including maternal Wnt11, β-catenin, and β-catenin stabilizing factors, are localized to the vegetal pole. (B) Cortical rotation. Following fertilization, the cortex of the egg rotates relative to the inner cytoplasm using a parallel array of microtubules. Movement generally occurs opposite to the sperm entry point, and transports cortical and subcortical dorsalizing factors into the equatorial region of the embryo. (C) At late blastula and gastrula, the Spemann organizer is localized above

the dorsal blastopore lip (red dotted oval). Dorsally distributed β-catenin is indicated by green shade. The organizer expresses a pool of genes listed below the cartoon. Among those, diffusible Wnt inhibitors, such as Dkk-1, Frzb1, antagonizes Wnt activity toward the future anterior zone of the embryo. Diffusible BMP inhibitors, counteract the high BMP activity derived from a ventral BMP signaling center. (D) Throughout gastrulation and elongation of the embryo, the organizer-emitted Wnt and BMP inhibitors create low level of Wnt and BMP activity anteriorly and dorsally respectively. Zygotically produced Wnt and BMP proteins give rise to high level of Wnt and BMP activity posteriorly and dorsally respectively. The perpendicular Wnt and BMP gradients play key roles in specifying the overall body plan, with Wnt specifying A-P axis and BMP specifying D-V axis.

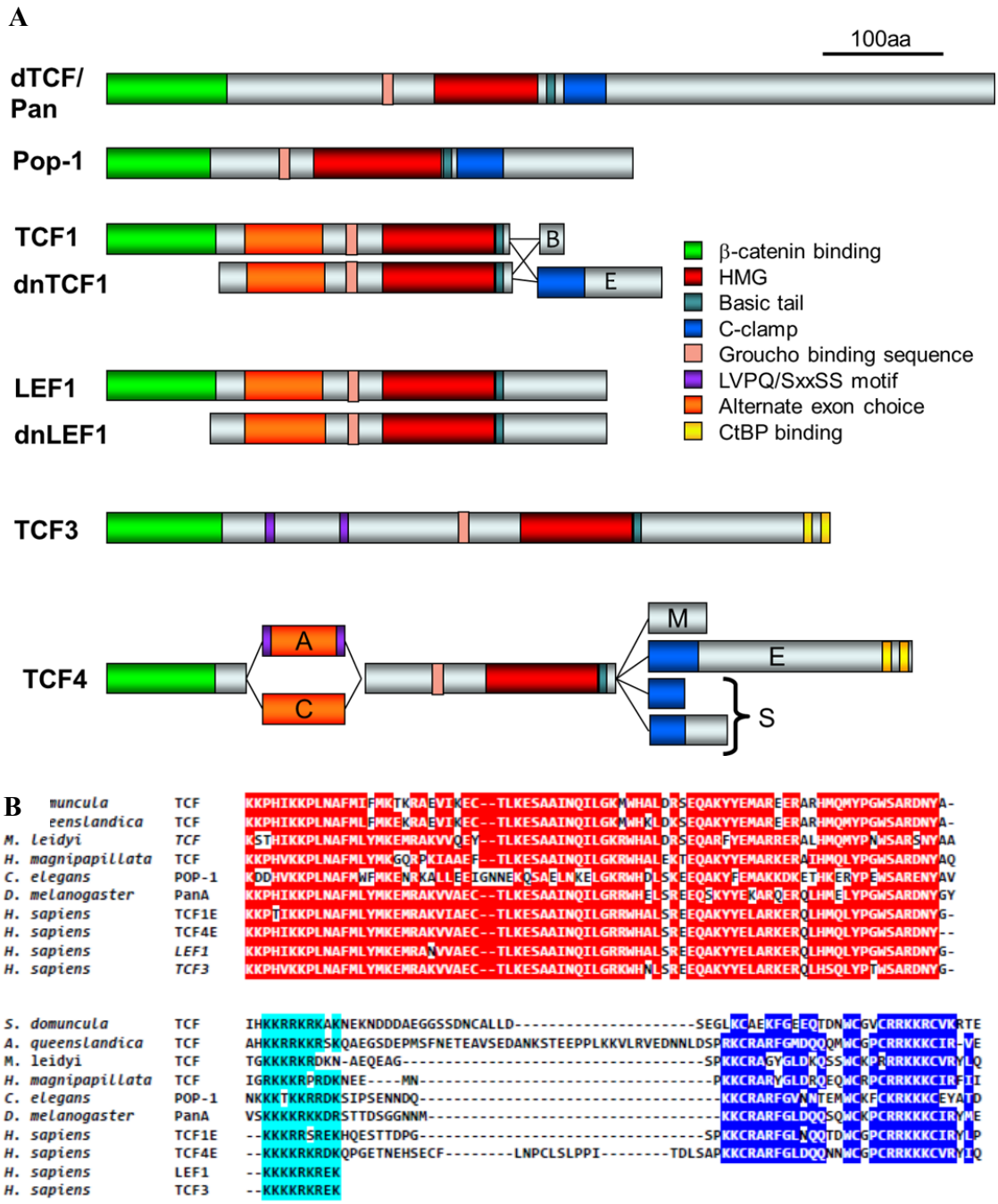

Figure 1.4 Diversity of TCF/LEFs and their conserved DNA binding domains.

(adapted from Archbold et al., 2012) (A) 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). Groucho binding sequence (salmon), which in part mediates direct interaction with co-repressor Gro/TLEs, is present in all TCFs. 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. B and 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.
(B) Alignment of the HMG domains, basic tails and C-clamps among metazoan TCFs. Non-conserved residues are not coloured in the alignment. 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 Suberities 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; HMG, high mobility group; LEF1, lymphoid enhancer-binding factor 1.

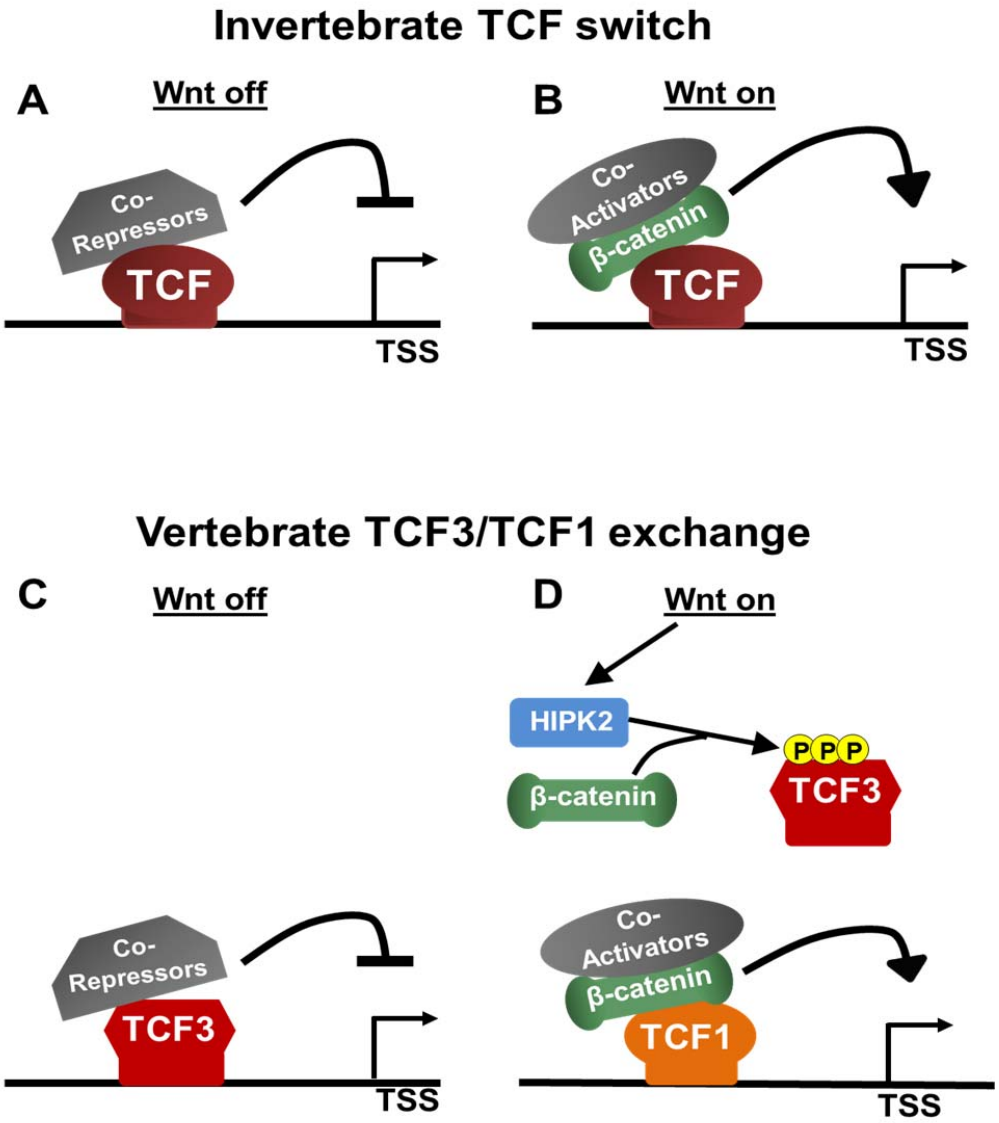

Figure 1.5 Models of TCF transcriptional switches in invertebrates and vertebrates.

Most invertebrates express a single TCF protein, which act as a docking station on W-CRM chromatin to switch co-regulators in response to Wnt signaling. (A) When no Wnts, TCF binds with co-repressors to suppress gene expression. (B) Upon signaling, TCF complexes with β-catenin and associated co-activators to activate gene expression. However, in vertebrates, multiple TCF proteins display specialized functions. (C) TCF3 almost exclusively functions in Wnt off conditions, to repress target gene with co-repressors. (D) Upon signaling, a TCF3/TCF1 exchange occurs, at least for some Xenopus Wnt target genes (i.e. *Siamois* and *Vent2*), Repressor TCF3 gets phosphorylated by Wnt-activated HIPK2 in a β-catenin dependent manner. This causes TCF3 to leave chromatin, allowing activating TCF1 and β-catenin to occupy the W-CRM and promoting transcriptional activation.

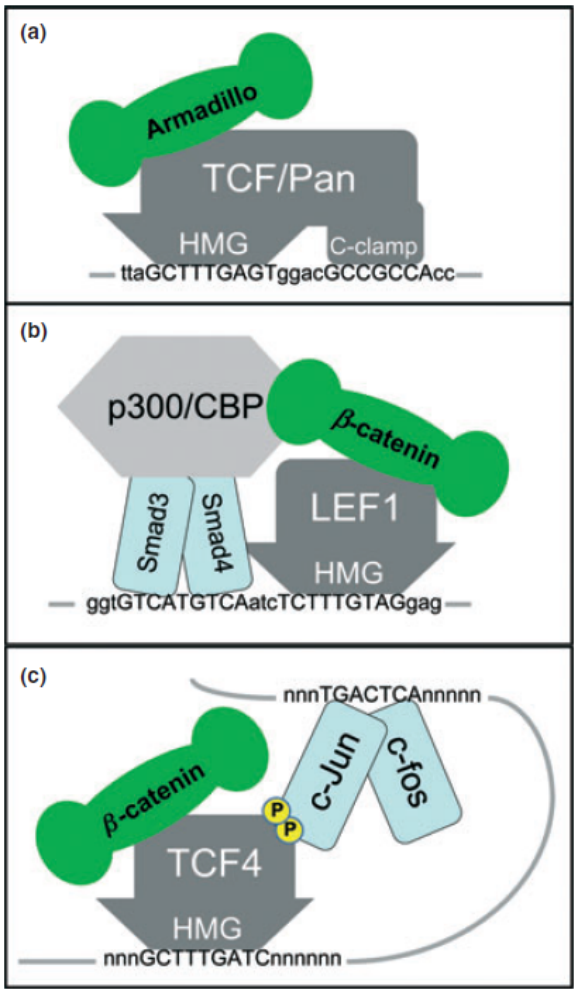

Figure 1.6 Three different mechanisms that contribute to TCF target selection in the nucleus.

Taken from Archbold et al., 2012. (a) Bipartite binding of dTCF/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 b-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.

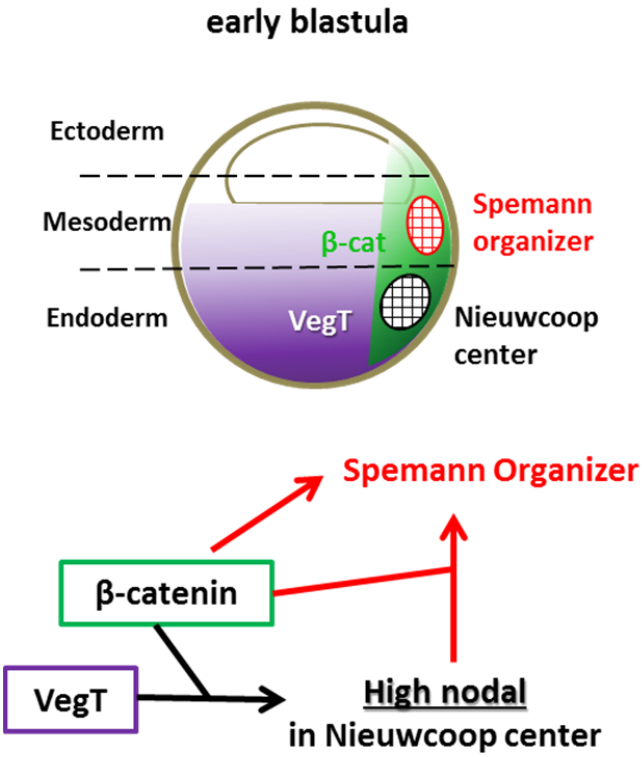

Figure 1.7 VegT is required for the organizer formation.

(A) Maternal VegT (purple shade) is localized in vegetal hemisphere of mature oocyte and cleaving blastula of Xenopus. Prior to MBT, VegT and dorsally enriched β-catenin (green shade) act together to establish Nieuwcoop Center (black meshed oval) in dorsal vegetal cells through inducing high level expression of nodal related genes (including xnr 1,2,4,5,6). The diffusible nodal activity induces overlying dorsal marginal cells to form Spemann organizer (red meshed oval) in combined action with maternal Wnt/β-catenin signaling (green). The black Dotted lines separate the early blastula into three sections roughly cooresponding to prospective endoderm, mesoderm,and ectoderm. The nieuwcoop arises in the endoderm region on dorsal side, and the spemann organizer subsequently forms in the dorsal mesoderm area.

CHAPTER 2

INVESTIGATING TARGET SELECTION BY NON-C-CLAMP VERTEBRATE TCFS

Abstract

Specific DNA recognition by transcription factors is essential for proper gene regulation. The TCF family members are the major transcription factors mediating Wnt/β-catenin signaling. However, how TCFs recognize specific targets in the information rich genome is not fully understood. All TCFs share a highly conserved DNA binding domain, the High Mobility Group (HMG) domain, which binds to specific sequences in Wnt-dependent *cis*-regulatory modules (W-CRMs). However, this DNA binding alone seems insufficient for recognizing specific targets in vivo. It has been suggested that most invertebrate TCFs and certain vertebrate TCF isoforms utilize a bipartite DNA binding mechanism to enhance recognition specificity, which involves HMG binding to TCF sites, and a secondary DNA binding domain, known as the C-clamp, binding to its cognate motif, known as the Helper sites. However, most vertebrate TCF isoforms have lost the C-clamp during evolution. How these non-C-clamp TCFs locate specific targets remains an unexplored question in the field. To examine this question, I tested two hypotheses in this chapter. First, the HMG domain of non-C-clamp TCFs has increased binding affinity and specificity to endogenous targets compared to the HMG domain of C-clamp-dependent TCFs, allowing non-C-clamp TCFs bypass the assistance from the C-clamp domain. Second, non-C-clamp TCFs cooperate with other TFs to locate and

coordinate regulation of Wnt targets. My findings do not support the first hypothesis by showing that LEF1, a vertebrate TCF lacking a C-clamp, could not activate fly W-CRMs, however, when fused with a C-clamp domain, LEF1-C-clamp chimeric protein was able to activate fly W-CRMs in vivo, suggesting the HMG domain of LEF1 is insufficient and still requires C-clamp to recognize Wnt targets in flies. To address the second hypothesis, I analyzed the *Siamois* W-CRM sequence by a mutagenesis screening, and found that additional DNA motifs besides TCF sites influence Wnt-dependent activation of *Siamois* reporter in HEK293T cells. Further studies need to be done to identify the TFs binding to the identified motifs and the mechanism of how they affect *Siamois* W-CRM activity (see Chapter 3).

Introduction

In all metazoans, Wnt/β-catenin signaling plays essential roles in embryonic development and adult tissue homeostasis (Archbold et al., 2012; Logan and Nusse, 2004b). TCF family members are the best studied transcription factors regulating the majority of Wnt target genes (Archbold et al., 2012). The classic transcriptional switch model, mainly based on the action of fly TCF, proposes that TCFs function as sequence - specific DNA anchors to recruit co-regulators to control transcription (Cadigan and Waterman, 2012). Thus, positioning TCFs at the right locations in the genome is key to Wnt signaling outputs. How TCFs select specific targets, however, is not fully understood.

The HMG domain is the major DNA binding domain highly conserved in all TCF proteins (Archbold et al., 2012). HMG binds to the consensus SCTTTGATS (S=G/C) with highest affinity in vitro (Atcha et al., 2007; van Beest et al., 2000; Giese et al., 1991;

Hallikas et al., 2006; van de Wetering et al., 1991, 1997). However, numerous in vivo functional TCF sites deviate from the consensus with varying degrees. The high degeneracy of TCF binding sites argues additional information is required to define the TCF binding code.

Most invertebrate TCFs and E-tail isoforms of vertebrate TCF1 and TCF4 possess a secondary DNA binding domain, known as the C-clamp (Archbold et al., 2012). It binds to a GC-rich DNA motif termed the Helper site (Atcha et al., 2007; Chang et al., 2008b). The bipartite DNA binding, involving the HMG-TCF site and the C-clamp-Helper site interactions, allows TCF to read an extended DNA sequence, increasing the target selection specificity (Chang et al., 2008b). Functionally, the bipartite regulation has been shown to be essential for regulation of many W-CRMs in various contexts (Atcha et al., 2007; Bhambhani et al., 2014; Chang et al., 2008b; Hoverter et al., 2012). *In silico* searches for clusters of TCF and Helper sites led to successful identification a handful of novel W-CRMs in flies and worms (Bhambhani et al., 2014, Archbold et al., 2014), demonstrating that understanding the targeting code of TCF improves our ability to learn new aspects of Wnt signaling.

However, bipartite recognition must not be the complete answer for TCF targeting, because most vertebrate TCF isoforms do not contain a C-clamp. Has the C-clamp been replaced by a separate protein in vertebrate systems? A related C-clamp domain has also been identified in other DNA binding transcription factors including HDBP1/GEF(GlutEF), HDBP2/PBF, and Gig1 (Tanaka et al., 2004). However, these C-clamp-containing TFs have not been implicated in the Wnt pathway. More importantly, Helper sites or similar sequences do not exist in the W-CRMs of many vertebrate Wnt

targets, e.g., *Siamois* and *c-myc* (Brannon et al., 1997; He et al., 1998; Yochum et al., 2008), suggesting these W-CRMs are regulated by TCF isoforms containing no C-clamp. How non-C-clamp TCFs accomplish specific targeting is largely uninvestigated.

To address this question, I tested two hypotheses. First, the HMG domain of non-C-clamp TCFs has increased binding affinity and specificity to endogenous targets compared to the HMG domain of C-clamp-dependent TCFs, allowing non-C-clamp TCFs bypass the assistance from the C-clamp domain. Second, non-C-clamp TCFs cooperate with other TFs to locate and coordinate regulation of the *Siamois* W-CRM. In the first half of this chapter, I showed that attaching the fly C-clamp domain to human LEF1 makes it capable to regulate endogenous Wingless (Wg, fly Wnt) targets in cells and transgenic flies. These results indicate the HMG domain of non-C-clamp vertebrate TCFs requires additional help to locate endogenous targets. In the latter half of this chapter, I focused on the previously described W-CRM of *Siamois* to search for additional *cis* motifs important for Wnt/TCF regulation. From a mutagenesis screen, sequence motifs enhancing or diminishing the *Siamois* reporter activation in HEK293T cells were identified. In particular, I described a set of three T-box sites collectively repressing *Siamois* reporter, and a set of five "CAGT" sites needed for full activation of the reporter. These functional motifs may positively or negatively influence the W-CRM activity via affecting TCF activities. Further studies characterizing the function of these motifs and identifying the associated *tran*s factors in *Xenopus* are needed to elucidate the mechanism (see Chapter 3).

Results and Discussion

LEF1 needs a C-clamp to activate *Drosophila* W-CRMs in Kc cells

Although the HMG domains of all TCFs bind to DNA with no detectable difference in vitro, this does not mean they display same DNA binding specificity in vivo. One possibility that has not been formally tested is whether the HMG domains of non-C-clamp TCFs are better at selecting endogenous targets than that of the invertebrate TCFs, which require aid by C-clamps. To test this hypothesis, we choose to characterize LEF1, which does not contain a C-clamp domain (Cadigan and Waterman, 2012). There are 6 amino acids differences in the HMG domains (about 80 amino acids) of fly TCF and LEF1 (Archbold et al., 2012). Could it be possible that the minor sequence variation underlies a difference in target selection in vivo?

To address this question, we assayed the ability of LEF1 to activate a known fly W-CRM, nkdIntE W-CRM, in Kc cells, an established *Drosophila* cell line (Chang et al., 2008b). This W-CRM was isolated from the first intron of the *naked cuticle* (*nkd*) gene. The *Drosophila* TCF (dTCF or Pangolin) binds to this region in vivo and mediates Wg activation of *nkd* (Chang et al., 2008a). The nkdIntE W-CRM contains three functional TCF sites and two Helper sites, and requires bipartite TCF binding for its regulation. Mutating the Helper sites or the C-clamp domain of fly TCF strongly reduces nkdIntE reporter activation in both Kc cells and in transgenic flies (Chang et al., 2008b; Ravindranath and Cadigan, 2014).

We first determined if LEF1 has activity in fly Kc cells. In LEF1-expressing transgenic flies, it has been shown that LEF1 activated the homeotic gene *Ultrabithorax* (*Ubx*) (Riese et al., 1997). This result suggests LEF1 is capable of coordinating with the

endogenous fly transcriptional machinery to turn on transcription. To validate that LEF1 retains similar activity in Kc cells, we first tested if LEF1 can induce a synthetic reporter driven by 6 consecutive copies of TCF binding sites (6xTCF reporter) in Kc cells. To rule out the contribution of endogenous fly TCF, RNAi targeting 5'UTR of fly TCF were introduced into Kc cells. The RNAi effectively depleted endogenous fly TCF, as indicated by that 6xTCF reporter no longer activated by constitutive active Armadillo, the fly β-catenin (Figure 2.1 B). Constructs expressing the fly TCF with a distinct 5'UTR sequence or LEF1 were transfected into RNAi treated cells to rescue loss of endogenous fly TCF activity. Fly TCF effectively restored reporter activity to a similar level as seen in control RNAi cells (Figure 2.1B). LEF1 also strongly restored reporter activity, suggesting LEF1 could recognize the synthetic reporter, and cooperate with Armadillo to activate transcription. Interestingly, LEF1 displayed much stronger rescue activity than fly TCF. This could be because LEF1 was expressed at higher level than fly TCF. Due to technical reasons, we were unable to compare the expression level of the two (the V5 epitope tag was not properly expressed in the fly TCF expression construct). Alternatively, it could be because LEF1 has a stronger ability to recruit Armadillo. This postulation agrees with previous observations implicating that LEF1 is better at recruiting β-catenin in vivo than other TCF members. For example, when injected in ventral cells of *Xenopus* embryos, LEF1 alone induces axis duplication, a hallmark of β-catenin-dependent Wnt signaling activation. However, TCF3 and TCF4 only do so when β-catenin was co-injected (Gradl et al., 2002; Huber et al., 1996; Molenaar et al., 1996 and data not shown).

Similar RNAi and rescue experiments were done with the *nkd*IntE reporter,

representing a natural W-CRM. As expected, dTCF rescued effectively. In contrast, LEF1 exhibited very minimal rescue activity, dramatically different from the situation seen with 6xTCF reporter (Figure 2.1C). This result indicates LEF1 is deficient at recognizing the endogenous *nkd*IntE which normally requires bipartite recognition, arguing the HMG domain of LEF1 is not intrinsically better than that of fly TCF at selecting endogenous targets, at least in Kc cells.

To test whether the inability of LEF1 to activate *nkd*IntE is specifically due to lack of target recognition, we constructed LEF1-C-clamp chimeric proteins and tested if they could rescue the *nkd*IntE reporter. As Figure 2.1A shows, two LEF1 chimeric proteins (LEF1-CC32, LEF1-CC44) were constructed by adding the dTCF C-clamp fragments at the C terminus of LEF1. When expressed at similar levels (Figure 2.1C), these chimeric LEF1s were able to rescue the 6xTCF reporter as well as LEF1. On the other hand, unlike LEF1, they robustly rescued *nkd*IntE reporter, suggesting the C-clamp domain markedly increased LEF1's capacity to recognize and associate with *nkd*IntE. Collectively, these results argue that the HMG domain of LEF1 is sufficient for recognizing an artificial W-CRM with a dense cluster of TCF sites, but insufficient for recognizing a natural W-CRM.

LEF1 needs a C-clamp to mediate Wg signaling in developing fly wing

In Kc cells, we demonstrated that the C-clamp is necessary for LEF1 to target a fly W-CRM. However, in the developing fly visceral mesoderm, LEF1 has been previously seen to activate a *Ubx* W-CRM reporter (Riese et al., 1997), which contains a Helper-like motif near the functional TCF binding site. Thus we wondered if LEF1 is capable of recognizing Wg targets in vivo without assistance by the C-clamp.

To directly address this question, a similar rescue assay as was performed in Kc cells was established in the developing wing. Wg signaling is required for specification of the wing margin and adjacent sensory bristles, with loss of signaling resulting in notches in the wing blade (Couso et al., 1994; Phillips and Whittle, 1993) and ectopic signaling causing ectopic sensory bristles (Blair, 1992; Cadigan et al., 1998). When dTCF/Pan was depleted in flies containing the wing margin specific Gal4 driver C96 (Krupp et al., 2005) and a UAS-TCF/Pan RNAi construct (Dietzl et al., 2007), notches along most of the distal margin were observed with 100% penetrance (Figure 2.2B; Table 2.1). In addition, a large number of ectopic wing margin bristles were seen (Figure 2.2B'; Table 2.1), likely due to derepression of the Wg targets specifying sensory bristles. The dTCF/Pan RNAi phenotypes were used to assay the ability of LEF1 or LEF1-C-clamp chimera (only the LEF1-CC32 was tested), to rescue the derepression and loss of activation phenotypes in C96::TCF/Pan RNAi wings.

Both the LEF1 and LEF1-C-clamp transgenes had biological activity in the fly wing, but with dramatically different specificities. LEF1 was unable to rescue the wing notch phenotype (Figure 2.2C; Table 2.1) but strongly suppressed the formation of ectopic bristles (Figure 2.2C'; Table 2.1). In contrast, the LEF1- C-clamp chimera (LEF1-CC32) was able to rescue both the notch and bristle phenotypes (Figure 2.2D, 2.2D'; Table 2.1). More than a dozen independent UAS-LEF1 and UAS-LEF1-C-clamp lines were generated, and the ones at the lower end of the expression spectrum were used in this rescue experiment, because higher expression of either transgene caused wing notches in an otherwise wild-type background (data not shown). We suspect that too much of either LEF1 protein inhibits Wg signaling by titrating out Armadillo in the

nucleus, causing dominant negative effects. Western blot analysis revealed that the LEF1 and LEF1-C-clamp transgenes used for the rescue were expressed at similar levels (Figure 2.2E). These data are largely in agreement with the results from Kc cell cultures, suggesting, for Wg-activated targets, LEF1 requires a C-clamp to constitute a bipartite DNA binding for specific target recognition. Additionally, the fact that extra bristles were rescued by both LEF1 and LEF1-C-clamp chimera indicated that C-clamp is dispensable for basal repression. This effect is consistent with findings in flies and *C.elegans*, where mutating Helper sites failed to derepress certain targets as mutating the TCF sites did in the domains where Wnt signaling is low (Bhambhani et al., 2014).

Mutagenesis screen uncovers novel *cis*-regulatory motifs controlling the Wnt responsiveness of the *Siamois* W-CRM in HEK293T cells

Based on the aforementioned results, we argue that LEF1, a vertebrate non-C-clamp TCF protein, is not superior to its fly counterpart in recognizing specific endogenous target genes. LEF1 still needs aid from C-clamp to locate targets in the fly genome. We reasoned that, in the human genome, which contains over 20 times more information than the fly genome, non-C-clamp TCF factors must utilize alternative approaches to improve their target selection.

When examining the DNA sequence of several previously characterized vertebrate W-CRMs, including W-CRMs from the *Axin2* (Jho et al., 2002), *c-myc* (Yochum et al., 2007) and *Siamois* loci (Brannon et al., 1997; Fan et al., 1998; Figure 2.3A), we found no Helper site-like motifs near the functional TCF sites in these W-CRMs, ruling out C-clamp/Helper site involvement in these W-CRM regulation.

In HEK293T cells, reporters driven by these three W-CRMs (previously

described in Brannon et al., 1997; Jho et al., 2002; Yochum et al., 2007) are only weakly activated by stabilized β-catenin S45F (about 2 to 3 fold activation, data not shown). p300, an acetyltransferase co-activator recruited by β-catenin, has been shown to potentiate W-CRM activation in synergistic action with β-catenin (Hecht et al., 2000; Li et al., 2007; Ma et al., 2005; Sun et al., 2000; Takemaru and Moon, 2000). We saw that p300 alone did not induce the *Siamois* W-CRM reporter, whereas p300 dramatically stimulated reporter activation when co-expressed with β-catenin (Figure 2.3B). This effect was also seen with the *Axin2* and *c-myc* reporters, but to a more modest extent (data not shown).

The *Siamois* W-CRM is composed of 804 bp upstream of the Transcription Start Site (TSS) of *Siamois*, including its endogenous promoter (Brannon et al., 1997). There are five putative TCF sites found in this region. Mutating all the TCF sites, abolished over 95% of the reporter activation (Figure 2.3C), confirming these sites are functional in mediating Wnt stimulation of the reporter in HEK293T cells. The residual activation of the mutant reporter may be the result of indirect regulation by Wnt signaling or more divergent TCF binding sites.

Siamois is known to be repressed by TCF3 in the absence of Wnt signaling in *Xenopus* embryos (Hikasa et al., 2010; Houston et al., 2002). To understand the TCF regulation of *Siamois* W-CRM in HEK293T cells, individual TCFs were knocked-down by shRNA. To control for off target effects, two separate shRNAs targeting different regions of each TCF were used. The *Siamois* reporter activity decreased modestly when LEF1 or TCF4 were depleted. In contrast, the reporter increased by over two-fold when TCF1 or TCF3 were knocked down (Figure 2.4). Similar results were observed with the

second set of shRNAs (Figure 2.4). These data suggested endogenous LEF1 and TCF4 in HEK293T cells activate *Siamois* W-CRM, consistent with the generic activating role of LEF1 and the previous findings suggesting *Xenopus* TCF4 as an activator of *Siamois* (Standley et al., 2006). On the other hand, TCF3 represses *Siamois* in HEK293T cells, indicating conserved regulation by TCF3 (Houston et al., 2002). However, it is surprising to see that TCF1 suppressed *Siamois*. We suspect the repressive effect of TCF1 was due to knockdown of dominant negative versions of TCF1 (dnTCF1). Interestingly, the predominant TCF1 isoform expressed in HEK293T cells was slightly smaller than the full length form (TCF1-B isoform, 48kD) (Weise et al., 2010), and close to the size of a dnTCF1 with a E-tail (dnTCF1-E, slightly smaller than 48kD) (Najdi et al., 2009)(Figure 2.4B). Thus, it is possible that the repressive effect is due to this dnTCF1-E in 293T cells. To formally test this, additional experiments, such as RT-PCR comparing abundance of dnTCF1-E and TCF1-B isoforms, need to be done.

Sequence mutagenesis is a useful approach to study the transcriptional regulation of a *cis*-regulatory DNA elements, e.g., Helper sites were originally identified from a mutagenesis screen on *nkd*IntE (Chang et al., 2008b). To search for additional *cis* information required for *Siamois* W-CRM regulation, we semi-systemically mutagenized the region surrounding the TCF site cluster (Figure 2.5). Mutant reporters were assessed in HEK293T cells. Relative activities of mutant reporters are summarized in Table 2.2.

This screen revealed several regions of interest for the regulation of the *Siamois* W-CRM. Besides TCF sites, multiple additional sequences contributed to the Wnt-dependent activation of the reporter (marked in green in Figure 2.5). Notably, five individual mutants (m4, m11, m24, m25 and m27), each containing a “CAGT” motif, all

showed reduction of activation (Figure 2.6, 40% to 60% loss of activation compared to WT). Two mutants with triple "CAGT" sites mutated had no further reduction compared to individual mutants (Figure 2.6). Interestingly, two mammalian W-CRMs that our lab is characterizing in mammalian cell cultures (a W-CRM 45kb downstream of the *Axin2* transcripiton unit and a W-CRM 335kb upstream of the *c-myc* gene also contain multiple "CAGT" sites (although some of them have a variable nucleotide at position "T"). The "CAGT" sites are required for the activation of the *Axin2* and *c-myc* W-CRMs (L. Chen and P. Burby, unpublished). The "CAGT" sites may be regulated by a common TF activating these W-CRMs. To determine the functional relevance in endogenous context, a reporter with all five CAGT sites mutated will be tested in *Xenopus* embryos.

It is not clear what transcription factors bind to the "CAGT" sites. It has been reported that a plant zinc finger protein ZPT2-2 binds to "CAGT" specifically through one of its two canonical TFIIIA-type zinc finger domains (Yoshioka et al., 2001). In amphibians and mammals, it is possible that transcription factors with conserved zinc finger domains act through these "CAGT" sites.

Another candidate is Odd-skipped related (Osr) proteins. *Odd-skipped* (*Odd*) in flies and *Osr-1, Osr-2* in mammals encode C2H2-type zing finger proteins, which are important regulators of multiple embryonic developmental processes, e.g., hindgut development in flies (Wang and Coulter, 1996), limb, heart and bone development in mammals (Verlinden et al., 2013). Intriguingly, these processes are also controlled by Wnt signaling (Archbold et al.,2012). *Odd* binds a DNA sequence, TA<u>CAGTAGC</u> with high affinity (Meng et al., 2005). The underlined nucleotides are critical for its in vitro

DNA binding (Meng et al., 2005), which contain the "CAGT" motif. However, the adjacent "AGC" sequence does not match with the flanking sequences of most functional "CAGT" sites in the *Siamois, c-myc* and *Axin2* W-CRMs. Consistent with this, recombinant Osr-1 failed to shift the DNA probe containing "CAGT" sites derived from the *Axin2* W-CRM (L. Chen and P. Burby, unpublished). Furthermore, Osr-1 and Osr-2 function as transcriptional repressors (Rankin et al., 2012), which is inconsistent with the function of the identified "CAGT" sites. Overexpressing Osr-1 or Osr-2 led to reduction of *Axin2* reporter (L. Chen and P. Burby, unpublished), suggesting negative regulation of *Axin2* W-CRM by Osr proteins. Thus, our data suggest Osr proteins may not directly act on these "CAGT" sites.

Besides mutants defective in activation, a number of mutants were also uncovered that had elevated Wnt activation, indicative of being bound by repressors. In particular, three mutants (m34, m7 and m10) sharing a "A/TGGTG" motif, which conforms to a T-box binding core site, all displayed over 2 fold higher activation than WT, indicating these T-box sites mediate repression of *Siamois* reporter (Table 2.2). A triple T-box site mutant was made and tested. An additive increase in activity was observed (Figure 2.7). The repressive function of these T-box sites were also confirmed in *Xenopus* embryos (Figure 3.1), suggestive of a conserved mechanism across species. These results will be discussed in detail in Chapter 3.

Materials and Methods

Drosophila cell culture, RNAi and transient transfection

Drosophila Kc167 cells were cultured and transient transfections were carried out as previously described (Fang et al., 2006). For RNAi treatment, cells were seeded at

1×10^6 cells/ml in growth media supplemented with 10 μg/ml dsRNA for 4 days, diluted to 1×10^6 cells/ml without additional dsRNA, and grown for 3 more days for luciferase assay. dsRNAs targeting the 3'UTR of dTCF (Chang et al., 2008b) or β-lactamase (negative control) were used (Blauwkamp et al., 2008). Transient transfections were carried out as previously described (Chang et al., 2008b). For the TCF rescue assays, 50 ng of luciferase reporters, 2 ng (for 6xTCF) or 10 ng (for *nkd*IntE) of pAcArm*, 20 ng of pAc-dTCF-V5 or pAc-LEF1-V5 or pAc-LEF1C32-V5 or pActin5.1 (negative control) and 10 ng of pAc-LacZ (β-galactosidase) were co-transfected. Each treatment was done in triplicates, containing 2.5×10^5 cells per well.

HEK293T cell culture, transient transfection and shRNA assays

HEK293T cells were cultured in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal bovine serum (BenchMark, 100-106) at 37°C, 5% CO_2. Transient transfection was carried out using LipofectamineTM 2000 (Invitrogen, 11668-027) as instructed by manual. Cells were seeded into a 48 well plate at a density of 0.25 million/ml one day prior to transfection. For regular reporter assays, cells were transfected with 50ng WT or mutant *Siamois* W-CRM luciferase reporter and 10ng pcDNA3-lacZ reporter plasmids and cultured for 24h before harvested for luciferase assay. For Wnt stimulation, 50ng p-cDNA3β-cateninS45F and 50ng pcDNA3-p300 were co-transfected with reporters. For basal expression, 100ng empty pcDNA3 vector were co-transfected to equalize total DNA amount. For all the shRNA experiments, 100ng of each indicated shRNA plasmid were co-transfected with 50ng reporter plasmid and 50ng p-cDNA3β-cateninS45F. Transfected cells were cultured for 48h before harvested for luciferase assay.

Luciferase reporter assay and statistical analysis

All luciferase assays (for both Kc cells and HEK293T cells) were carried out essentially as previously described (Chang et al., 2008b). In brief, cells were lysed in 100μl lysis buffer and assayed using the Tropix Luc-screen kit (Applied Biosciences). Luciferase and β-galactosidase activity were measured using the Promega Glamax system. The reporter activity was determined by the ratio of luciferase and β-galactosidase activity. To calculate relative fold activation (Y axis of the reporter assay bar graph), reporter activities, including basal activity of mutant reporters and Wnt-stimulated reporter activities of WT and mutant reporters were normalized to basal activity of WT reporter, which was set to 1. All experiments contained three technical replicates, and data shown are representative of at least two independent experiments.

Reporter activities were log transformed before statistical analysis with a two tailed Student's T-test conducted using GraphPad Prism software (GraphPad Prism, Inc., San Diego, CA). A P value <0.05 was considered statistically significant for all analyses.

***D. melanogaster* transgenics and genetics**

Transgenic *LEF1* and *LEF1 C-clamp* flies were generated by P-element transgenesis (performed by BestGene Inc.). *w1118* was obtained from Bloomington Stock Center. *C96::Gal4* was kindly provided by Dr. Rolf Bodmer. The *dTCF RNAi* line (#25940) was obtained from Vienna *Drosophila* RNAi Center. All fly crosses were performed at 25°C.

Plasmids

For luciferase reporter assays in Drosophila Kc167 cells, *nkd*IntE and 6xTCF luciferase reporters were described previously (Chang et al., 2008b). For expression plasmids, pAc-dTCF, pAc-Arm*, were described previously (Blauwkamp et al., 2008; Chang et al.,

2008b). pAc-LacZ (invitrogen) was used as a transfection control in reporter assays. pAc-LEF1-V5 was generated by subcloning a human *LEF1* fragment from an expression plasmid (kindly provided by Dr. Marian L. Waterman) into pActin5.1 (invitrogen) to introduce a V5 tag downstream LEF1 C terminus. PCR based amplification of *LEF1* was carried out using forward primer 5'CCCC*GGTACC*ATGCCCCAACTCTCCGGA3' and reverse primer 5'CAGT*GAATTC*TGCGATGTAGGCAGCTGTCATTCTTGG3' and inserted into the KpnI and EcoRI sites of the pActin5.1 vector, with the stop codon mutated (underlined). LEF1-C32 and LEF1-C44 were generated by inserting the C-clamp fragment PCR amplified from dTCF (LEF1-C32: KKCRARFGLDQQSQWCKPCRRKKKCIRYMEAL; LEF1-C44: KKCRARFGLDQQSQWCKPCRRKKKCIRYMEALNGNGPAEDGSCF) into EcorRI site of pAc-LEF1-V5.

For transgenic fly generation, pUAS LEF1V5 and pUAS LEF1C32(and C44)V5 were generated by subcloning LEF1V5 and LEF1C32 (and C44)V5 into pUAST vector. LEF1V5 and LEF1C32 (and C44)V5 were digested using the KpnI and PmeI sites and inserted into the KpnI and XbaI sites of pUAST. Prior to insertion, pUAST vector was restricted with XbaI and sticky ends filled in by Klenow to create blunt ends, followed by a restriction with KpnI.

For luciferase reporter assays, *Siamois* W-CRM luciferase reporter was previously described (Brannon et al., 1997). All the mutants were generated by site directed mutagenesis using Stratagene QuickChange kit (Agilent). The primers used for all mutagenesis contain the mutated sequences shown in Table 2.2 with at least 20nt flanking sequence on both sides. pcDNA3-β-cateninS45F and pcDNA3-p300 previously

described (Hecht et al., 2000; Yochum et al., 2007).

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I thank Nisa Penland for confirming LEF1-C-clamp rescue assay in Kc cell, and Aditi Ravindranath for cooperation in generating and analyzing the transgenic flies.

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Distinct DNA binding sites contribute to the TCF transcriptional switch in C. elegans and Drosophila.

Bhambhani C, Ravindranath AJ, Mentink RA, Chang MV, Betist MC, **Yang YX,** Koushika SP, Korswagen HC, Cadigan KM.

I generated Figure 2.2.

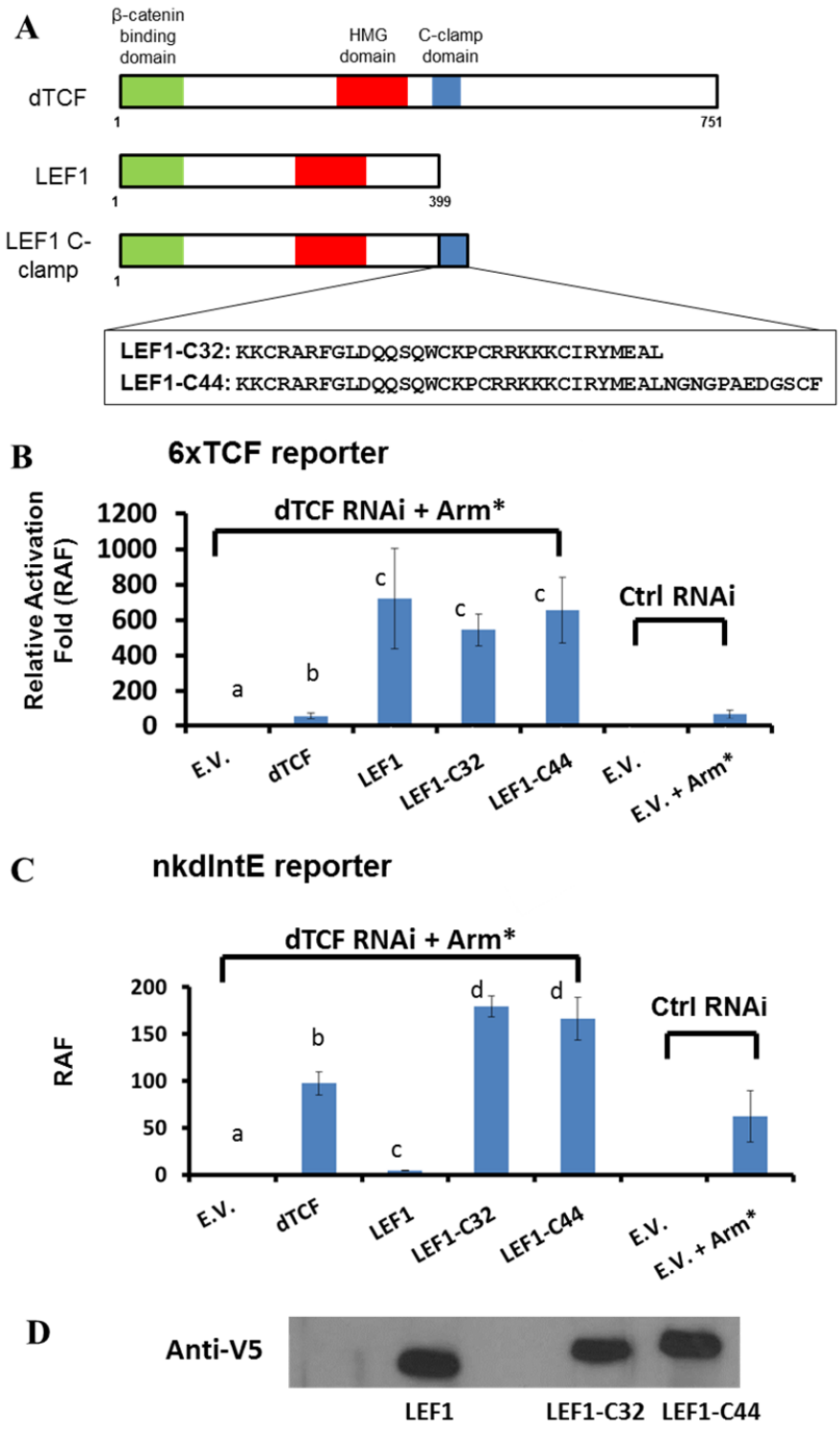

Figure 2.1 LEF1-C-clamp activates *nkd*IntE reporter in Kc cell, whereas LEF1 does not.

(A) Schematic depicting the *Drosophila* TCF (dTCF), LEF1, and LEF1-C-clamp

chimeras (LEF1-C32 and LEF1-C44), showing the location of the β-catenin binding domain (green), the HMG domain (red) and the C-clamp (blue). The amino acid sequence of C-clamp fragments (32aa and 44aa, respectively) in LEF1-C32 and LEF1-C44 is shown in the box. (B & C) TCF rescue assay in Kc cells. Endogenous dTCF was depleted with dsRNA targeting the 3' UTR region. 6xTCF reporter (B) or nkdintE reporter (C) was co-transfected with Arm* and indicated TCF expression plasmids. The 6xTCF reporter was rescued by all the TCFs but to different extents. All cells were co-transfected with pAC-lacZ for normalization of transfection efficiency. LEF1, LEF1-32 and LEF1-C44 displayed similar rescue activity, which were more than 10 times stronger than dTCF. The *nkd*intE reporter was efficiently rescued by dTCF, LEF1-C32, and LEF1-C44, but not by LEF1. Relative activation fold was calculated by normalizing each condition against mock rescue condition indicated by empty vector (E.V.), which was set as 1. In B and C, the data shown are representative of multiple independent experiments. Log transformed data were analyzed using One-way ANOVA with Tukey's post hoc test, $p < 0.05$, N=3 (technical repeats), bar = average $\pm$ SEM. Means with the same letter above are not significantly different from each other. Means with different letters above are significantly different. (D) Western-blot against V5 epitope showing comparative expression level of LEF1, LEF1-C32 and LEF1-C44 in the rescue assays. The dTCF expression construct does not express a V5 tag, thus the expression level of dTCF was not shown in this blot.

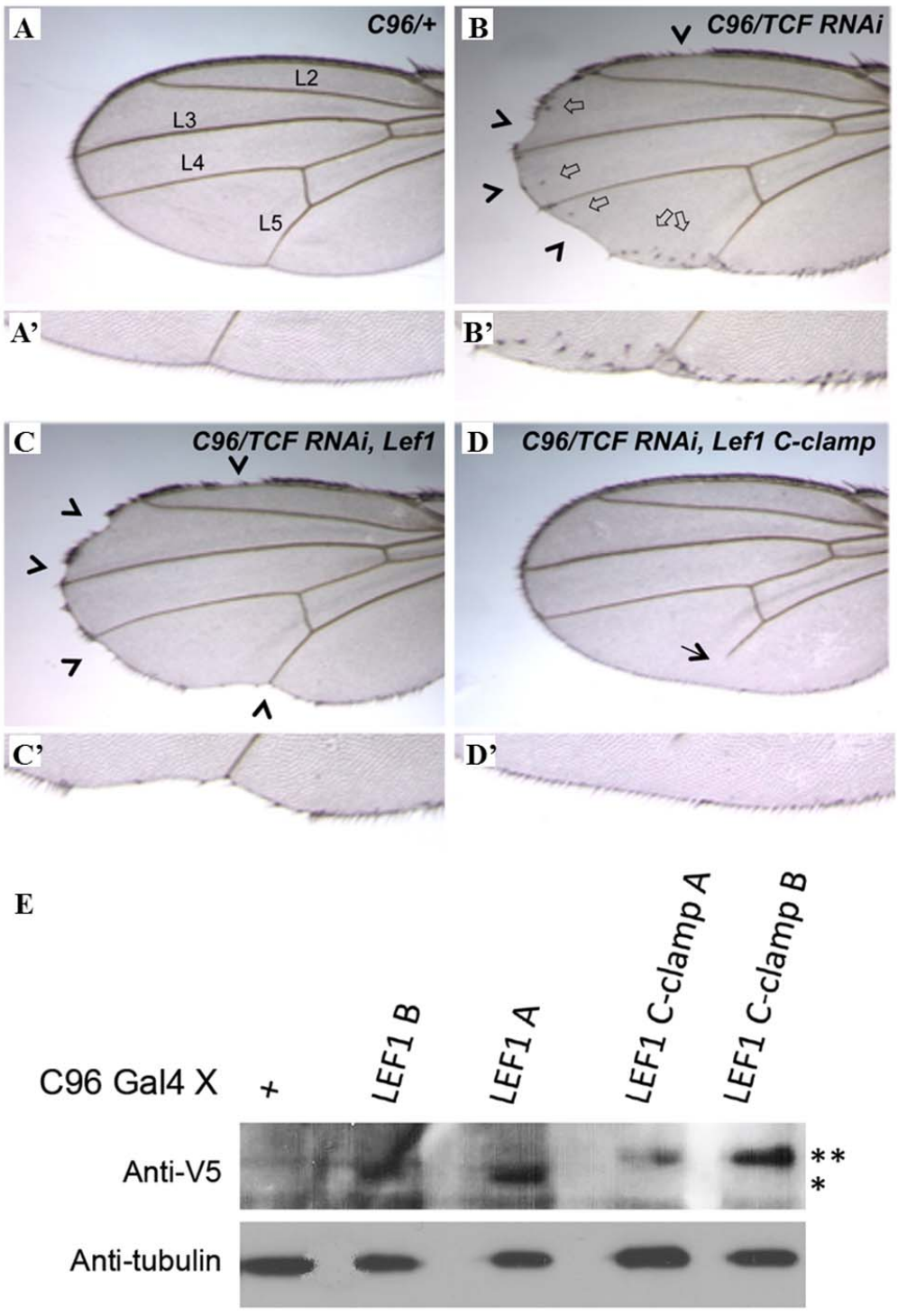

Figure 2.2 LEF1-C-clamp rescues Wing notches in dTCF RNAi transgenic flies, but LEF1 does not.

(A-D) Images of adult wings containing the wing driver C96-Gal4 crossed to WT (A,A'), UAS-dTCF/Pan RNAi (B, B') or UAS-TCF/Pan RNAi plus UAS-LEF1 (C, C') or UAS-

LEF1 plus the C-clamp of TCF/Pan (D, D'). Knockdown of dTCF leads to notches (arrowheads) and ectopic wing margin bristles (block arrows) along the periphery of the wing (where C96-Gal4 is active; B, B'). Expression of the human LEF1 transgene significantly rescues the ectopic bristle expression, but not the notches (C, C'). Expression of a LEF1-C-clamp chimera rescues the wing margin defects and prevents ectopic bristle formation, and causes a L5 vein defect (arrow). Details about the penetrance of these phenotypes are listed in Table 2.1. (E) Expression of human LEF1 and LEF1-C-clamp chimera in wing imaginal discs. Westernblot showing the expression levels in dissected wing discs from two lines (A and B) of V5 tagged LEF1 (*) or the LEF1-C-clamp chimera (**).

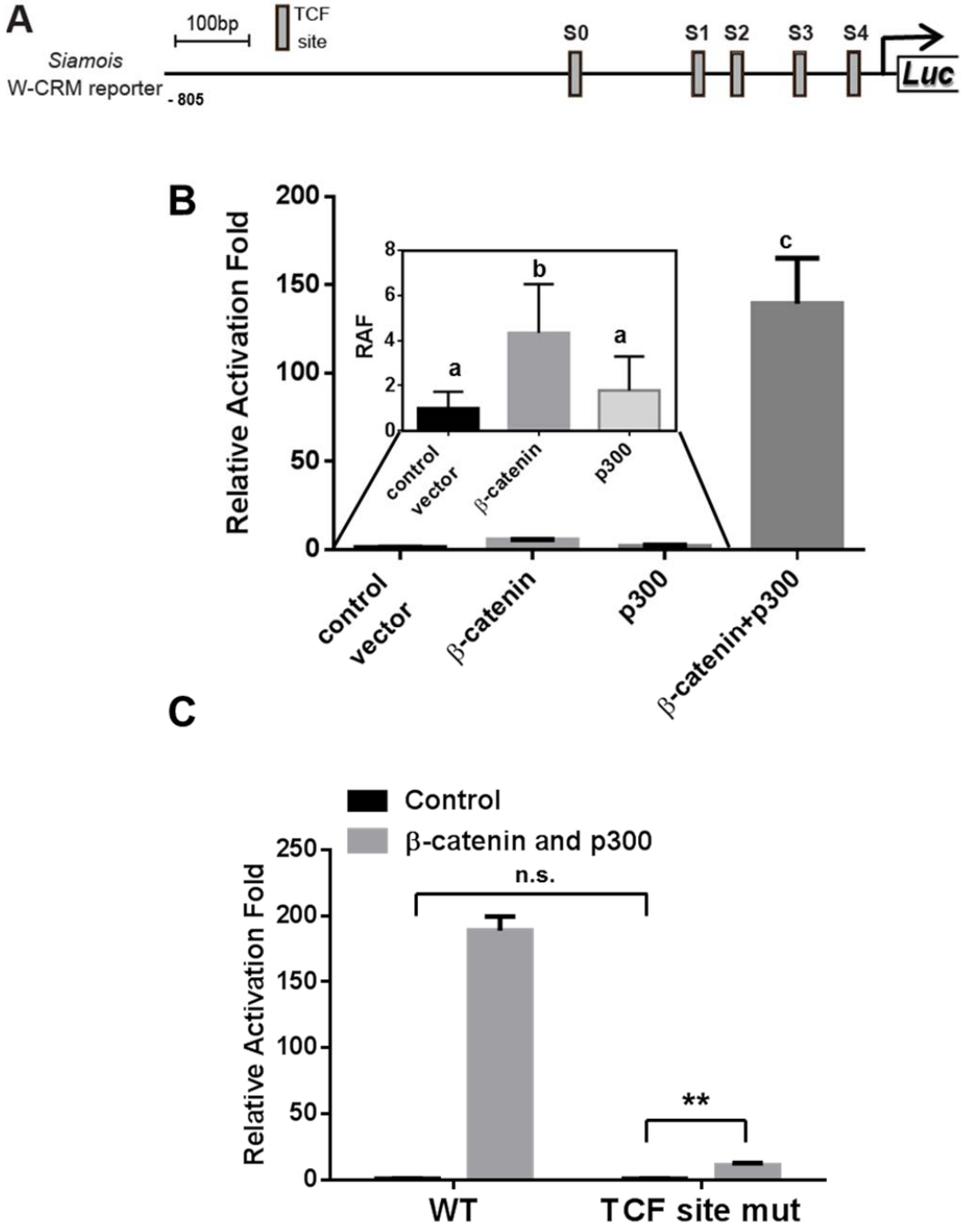

Figure 2.3 TCF sites are required for robust activation of the *Siamois* W-CRM reporter in HEK293T cells.

(A) Schematic depicting the *Siamois* promoter-proximal W-CRM luciferase reporter construct. The 804bp W-CRM contains five predicted TCF sites (grey rectangles). The bent arrow indicates the TSS (+1) of the firefly luciferase gene. (B) p300 potentiates β-catenin induced *Siamois* reporter activation in HEK293T cells. *Siamois* reporter (50ng)

was co-transfected with control vector, β-catenin (β-catS45F) (50ng), p300 (50ng), or both β-catenin and p300 as indicated. Total DNA was equalized by adding pCDNA3 empty vector. Relative Activation Fold (RAF) is all normalized against the control vector condition, which is set to 1. The inset showing enlarged RAF of the first three conditions. β-catenin alone induced ~ 4 fold activation of *Siamois* reporter, and p300 alone did not activate the reporter. Together, β-catenin and p300 synergistically induced dramatic activation of *Siamois* reporter. The data shown are representative of multiple independent experiments. Log transformed data were analyzed using One-way ANOVA with Tukey's post hoc test, $p < 0.05$, N=3 (technical repeats), bar = average $\pm$ SEM. Means with different letters above are significantly different. (C) TCF sites mediate most Wnt activation of *Siamois* reporter in HEK293T cells. TCF site mutant, with all five putative TCF sites mutated, lost most β-catenin/p300 induced activation. The data shown are representative of multiple independent experiments. Log transformed data were analyzed using a two tailed Student's T-test, $p < 0.05$. ** $p < 0.01$.

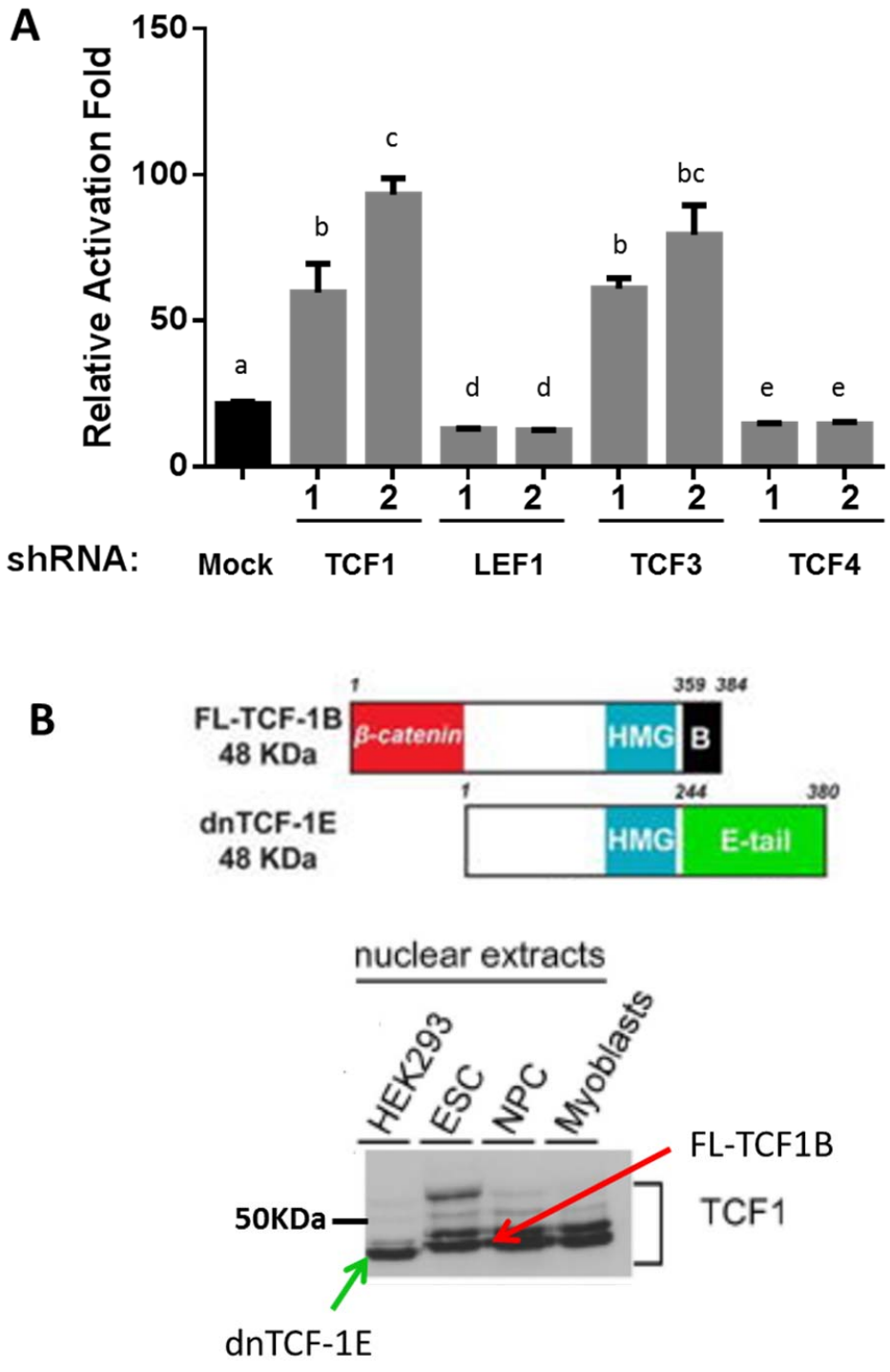

Figure 2.4 Endogenous TCFs in HEK293T cells display different regulatory activity of *Siamois* reporter.

TCF1 and TCF3 mediate repression of *Siamois* reporter whereas LEF1 and TCF4

mediate activation of the reporter. Plasmids expressing shRNA against each TCF were co-transfected with the reporter, β-catenin (β-catS45F) and p300 expression constructs. Baseline reporter activity is set as 1 (not shown in the graph), each treatment is normalized against baseline activity to calculate RAF. Knockdown of TCF1 or TCF3 resulted in large increase of reporter activity. In contrast, knockdown of LEF1 or TCF4 reduced reporter activity. Knockdown experiments were performed with two sets of different shRNAs indicated by "1" and "2" below X axis, and similar results were obtained from either shRNA treatment. The data shown are representative of multiple independent experiments. Log transformed data were analyzed using One-way ANOVA with Tukey's post hoc test, $p < 0.05$. N=3 (technical repeats), bar = average $\pm$ SEM. Significant difference is indicated by letters above the bars. Letters shared in common between or among the groups would indicate no significant difference. Different letters indicate significant difference. (B) TCF1 suppression of *Siamois* reporter may be mediated by dominant negative (dn) TCF1E. Upper cartoon illustrating dnTCF1E is slightly smaller than TCF1B (taken from Najdi et al.,2009). Lower western blott showing major TCF isoform in HEK293T cells is smaller than TCF1B in other cell lines, which corresponds to estimated size of dnTCF1E and TCF1B respectively (taken from Weise et al., 2010).

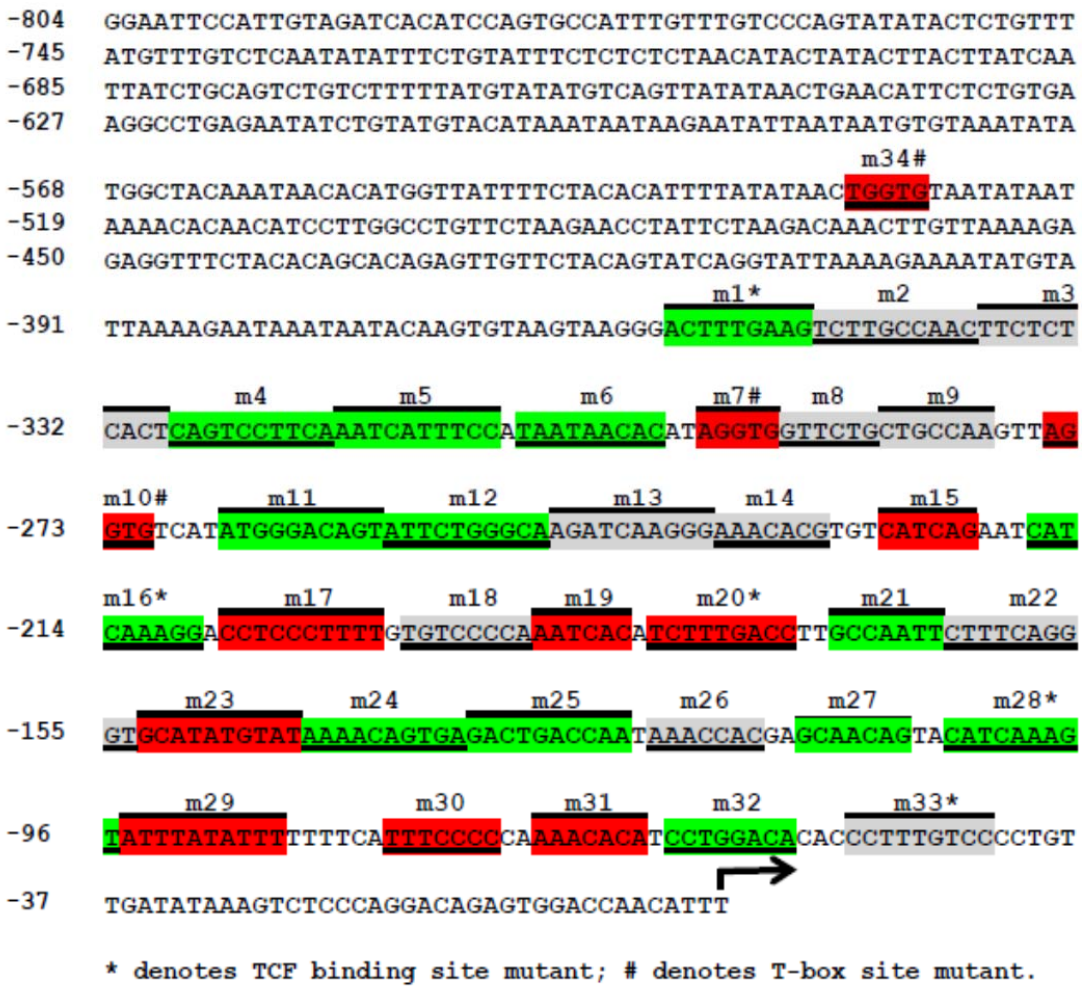

Figure 2.5 Mutagenesis screen uncovers novel *cis*-regulatory motifs controlling the Wnt responsiveness of the *Siamois* W-CRM in HEK293T cells.

Sequence of the *Siamois* W-CRM (GenBank: AF016226.1) is shown. Transcription start site is indicated with a bent arrow. Mutated motifs are alternately lined above and beneath, with names labeled above. Motifs contributing to activation, repression and no change are highlighted in green, red and grey, respectively. m1, m16, m20, m28, m33 correspond to five putative TCF binding sites labeled with *. m34, m7 and m10 correspond to three putative T-box binding sites labeled with #. See Table 2.2 for additional information.

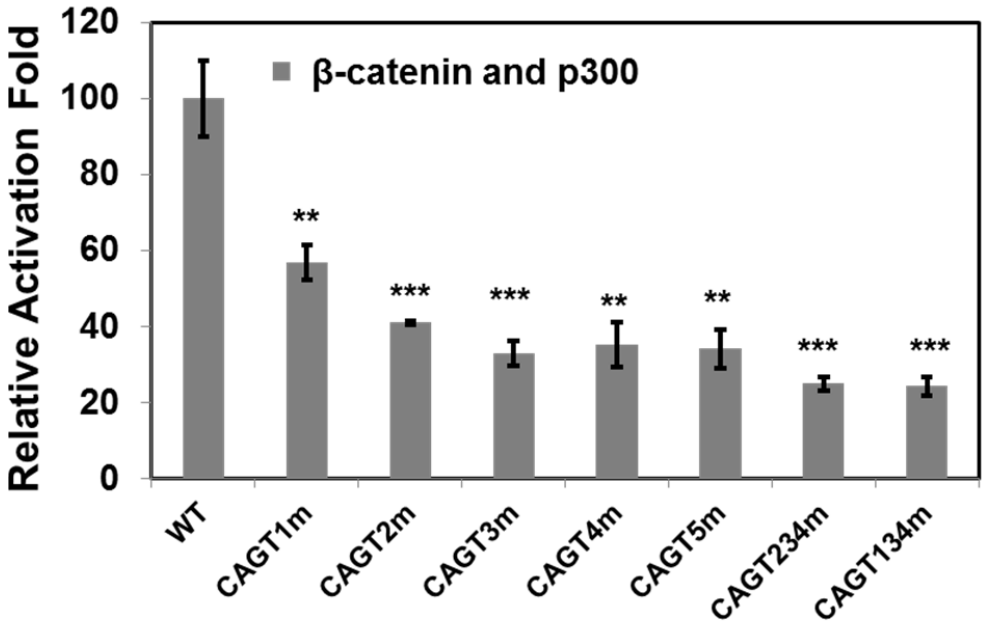

Figure 2.6 CAGT sites mediate activation of *Siamois* W-CRM in HEK293T cells.

HEK293T cells were transfected with WT or indicated CAGT site mutant reporter plasmids. For Wnt stimulation, β-cateninS45F and p300 expression constructs were co-transfected with the reporters. The CAGT site mutants all showed reduced activation. The data shown are representative of multiple independent experiments. Bar = average $\pm$ SEM, N=3 (technical repeats). Log transformed data were analyzed using a two tailed Student's T-test, $p < 0.05$. ** $p < 0.01$, *** $p < 0.001$.

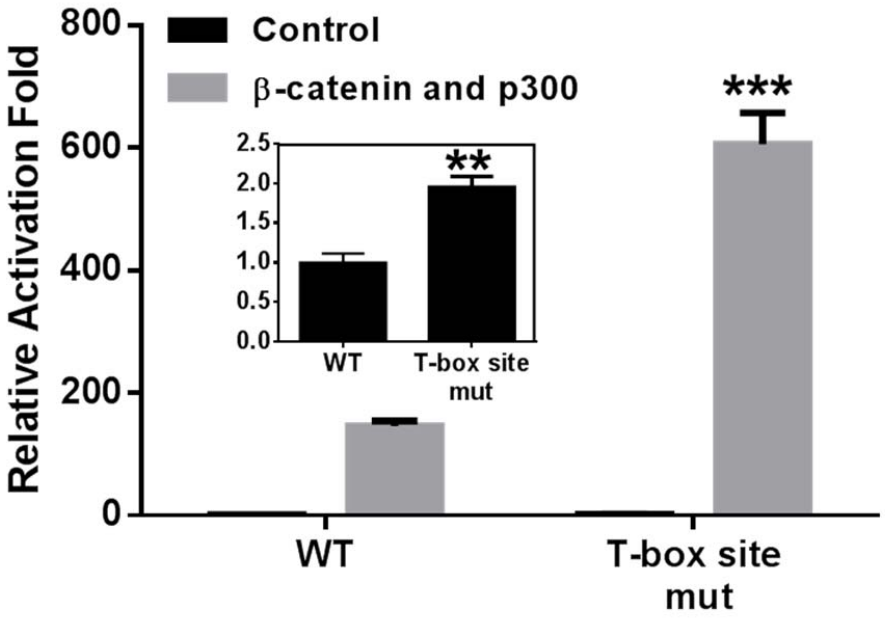

Figure 2.7 T-box sites mediate repression of *Siamois* W-CRM in HEK293T cells.

HEK293T cells were transfected with wild-type or triple T-box site mutant reporter plasmids and cultured for 24 h before harvesting for a luciferase assay. For Wnt stimulation, β-cateninS45F (a constitutive active mutant version) and p300 (to boost β-catenin-induced activation) expression constructs were co-transfected with the reporters. The T-box site mutant had increased activity with or without β-catenin/p300, shown by the grey bars and black bars (enlarged in the inset), respectively. Bars represent the mean of technical triplicates + SEM. RLUs were log10 transformed before statistical analysis with a two tailed Student's T-test. ** $p < 0.01$, *** $p < 0.001$.

Table 2.1					
C96-Gal4 crossed to: (n)	Notches (%)			Ectopic bristles/ wing (n)	L5 vein defect (%)*
	None	Small	Large		
+ (46)	100			0 (20)	0
Lef1 A (38)	100			0 (20)	5.2
Lef1 B (43)	95.3	4.7		0 (20)	4.7
Lef1-C-clamp A (38)	100			0 (20)	84.2
Lef-1-C-clamp B (39)	100			0 (20)	87.2
TCF/Pan-RNAi (46)		2.2	97.8	22.6 (20)	47.8
Lef1 A; TCF/Pan-RNAi (60)		8.3	91.7	3 (30)	3.3
Lef1 B; TCF/Pan-RNAi (52)		7.7	92.3	0.63 (30)	11.5
Lef1-C-clamp A; TCF/Pan-RNAi (46)	71.7	26.0	2.3	1.7 (20)	97.9
Lef-1-C-clamp B; TCF/Pan-RNAi (38)	100			0 (20)	55.3

Table 2.1 The C-clamp is required for Wg activation but not basal repression in a TCF/Pan rescue assay.

The C-clamp is required for Wg activation but not basal repression in a TCF/Pan rescue assay.
Two independent lines of UAS-Lef1 and UAS-Lef1-C-clamp with similar expression levels (see Figure 2.2E) were assayed. Expression of either transgene with the *C96-Gal4* driver had little or no effect on wing development in an otherwise wild-type background. Percentages tabulated for the wing phenotypes seen upon knock down of TCF. Depletion of TCF/Pan with a UAS-driven RNAi hairpin causes mostly large notches, and leads to more than 20 ectopic bristles per wing and a high penetrance of L5 vein defects. Expression of human Lef1 (Lef1) significantly rescues the ectopic bristles, but has little effect on the size and frequency of the wing notches. In contrast, expression of Lef1 with the C-clamp of TCF/Pan (Lef1-C-clamp) rescues both ectopic bristles and the wing notch phenotype. (n) represents the number of wings examined for each genotype. Depletion of TCF/Pan and expression of Lef1 and Lef1-C-clamp also resulted in a disruption of the L5 vein (see Figure 2.2). Since this phenotype has not been linked to Wg signaling, it is not considered in this chapter.

Name	Relative Basal Activity	S.D.	Relative Activation Fold	S.D.	Note	Original Motif Sequence	Mutated Motif Sequence
WT	1.0		100.0				
m1	1.4	0.2	72.1	6.7	TCF S0	ACTTTGAAG	ACTgTtcAG
m2	1.7	0.2	114.2	21.2		TCTTGCCAAC	gaggGCacca
m3	1.5	0.2	80.2	17.1		TTCTCTCACT	ggagCTacag
m4	1.7	0.5	56.5	3.1	CAGT 1	CAGTCCTTCA	actgCCggac
m5	1.5	0.2	69.0	9.9		AATCATTTCC	ccgacgggaa
m6	0.9	0.2	68.6	10.8		TAATAACAC	gccTAcaca
m7	3.9	0.2	229.2	21.0	TBS 2	AGGTG	ctcat
m8	2.2	0.3	102.5	23.1		GTTCTG	tggagt
m9	1.3	0.1	99.3	7.9		CTGCCAA	agtaacc
m10	1.4	0.1	216.3	16.2	TBS 3	AGGTG	ctcat
m11	0.9	0.0	41.0	5.8	CAGT 2	ATGGGACAGT	cgtttACctg
m12	1.0	0.1	72.0	3.8		ATTCTGGGCA	cggaTGttac
m13	1.6	0.1	90.8	9.8		AGATCAAGGG	ctcgCActtt
m14	0.7	0.2	98.0	4.3		AAACACG	cccacat
m15	1.2	0.2	385.8	12.4		CATCAG	acgact
m16	1.2	0.2	58.2	9.4	TCF S1	CATCAAAGG	CAgaAcAGG
m17	5.8	0.3	493.5	37.5		CCTCCCTTTT	aagCCaTggg
m18	1.2	0.1	122.9	5.5		TGTCCCCA	gtgaaaac
m19	1.1	0.1	197.9	16.4		AATCAC	gcagtg
m20	1.1	0.2	176.7	16.1	TCF S2	TCTTTGACC	TCTgTtcCC
m21	7.0	1.0	46.7	4.2		GCCAATT	taaccgg
m22	2.7	0.2	110.1	6.5		CTTTCAGGGT	aggTC
m23	4.3	0.7	296.7	8.9		GCATATGTAT	tacgATtgcg
m24	1.9	0.0	32.9	5.0	CAGT 3	AAAACAGTGA	cccacGTtc
m25	1.0	0.1	35.1	1.6	CAGT 4	GACTGACCAA	tcagGAaatg
m26	0.9	0.1	117.0	18.8		AAACCAC	cccCaca
m27	1.1	0.0	34.2	2.4	CAGT 5	GCAACAG	taccact
m28	1.0	0.3	17.6	1.1	TCF S3	CATCAAAGT	CAgaAcAGT
m29	1.0	0.1	192.2	43.8		ATTTATATTT	cgggcgcggg
m30	0.8	0.1	294.4	11.6		TTTCCCC	gggatat
m31	1.5	0.2	275.1	36.2		AAACACA	taccact
m32	0.4	0.2	43.2	6.7		CCTGGACA	aagttAac
m33	0.8	0.5	110	12.8	TCF S4	CCTTTGTCC	CCTgTtgCC
m34	1.7	0.6	307.7	2.5	TBS 1	TGGTG	gtcatg

Table 2.2 Summary of the *Siamois* W-CRM mutant reporter activity in HEK293T cells.

CHAPTER 3

VEGT PLAYS A DUAL ROLE IN THE TCF TRANSCRIPTIONAL SWITCH REGULATING *SIAMOIS* EXPRESSION IN *XENOPUS*

Abstract

The T-cell factor (TCF) family of transcription factors are major modulators of Wnt/β-catenin signaling in animals. TCFs regulate Wnt targets through a transcriptional switch, repressing transcription in the absence of signaling, while activating target gene expression when bound with β-catenin. Vertebrates contain several TCFs which are specialized for either basal repression (e.g., TCF3) or β-catenin-dependent activation (e.g, TCF1) of Wnt-dependent *cis*-regulatory modules (W-CRMs). Wnt/ β-catenin signaling is thought to promote an exchange of repressive and activating TCFs on W-CRMs, but how this is controlled is not well understood. In this study, we examined this question with TCF3, a Wnt target gene repressor, using a W-CRM controlling the dorsal organizer gene *Siamois* in *Xenopus* embryos. In addition to the TCF binding sites previously shown to mediate basal repression, we identified three T-box sites required for repression and provide evidence that the T-box protein VegT binds to these sites *in vitro* and *in vivo*. Knockdown of VegT causes vegetal-oriented expansion of *Siamois* and *Chordin* expression in *Xenopus* late blastula. VegT binds to TCF3, and this interaction is inhibited by the activity of homeodomain interacting protein kinase 2 (HIPK2), a kinase previously found to facilitate the TCF transcriptional switch in *Xenopus*. In addition to their role in basal

repression, the T-box sites also contributed to gene activation, and VegT remained associated with Wnt target gene chromatin after forced HIPK2 expression. These data support a model where VegT acts with TCF3 to repress *Siamois* in the absence of Wnt/β-catenin signaling, but VegT also contributes to *Siamois* activation. This adds a new level to our understanding of how the TCF transcriptional switch regulates Wnt targets.

Introduction

Wnt/β-catenin signaling plays essential roles in embryogenesis, tissue homeostasis and regeneration among all metazoans that have been studied (Clevers and Nusse, 2012; Logan and Nusse, 2004a; Nusse, 2012). Dysregulation of Wnt/β-catenin signaling has been linked to many human diseases, including various cancers (Polakis, 2012). The Wnt pathway is active in many tissues, where it regulates different target genes in a context-dependent manner (Archbold et al.,2012). How this signaling pathway regulates such a large diversity of target genes is incompletely understood.

Wnt/β-catenin target genes are regulated through a conserved signaling cascade that is still being elucidated (Cadigan and Peifer, 2009; MacDonald et al., 2009; Valenta et al., 2012). The hallmark event of the activated pathway is stabilization and nuclear accumulation of the transcriptional co-regulator β-catenin. Nuclear β-catenin is recruited to target gene chromatin by binding to transcription factors (Archbold et al., 2012; Valenta et al., 2012), the best characterized of which are the T-cell factor (TCF) family (Cadigan and Waterman, 2012). TCFs are thought to act as transcriptional switches, where in the absence of signaling, TCFs complexed with co-

repressors inhibit target gene expression. Upon Wnt signaling, β-catenin binds to TCFs and converts them into transcriptional activators (Cadigan, 2012).

The transcriptional switch model can explain several aspects of Wnt target gene regulation, but the situation has an added complication in vertebrates. Organisms like *Drosophila* and *C. elegans* have a single TCF with little isoform diversity (Archbold et al., 2012; Cadigan and Waterman, 2012), and it is clear that these transcription factors perform both sides of the switch, i.e., basal repression and Wnt-dependent activation (Cadigan, 2012). Amphibians and mammals have four TCFs, TCF1 (also called TCF7L), LEF1, TCF3 (also called TCF7L1) and TCF4 (TCF7L2). Vertebrate TCFs have become more specialized in regulating the transcriptional switch (Archbold et al., 2012; Hoppler and Kavanagh, 2007). TCF3 primarily functions as a transcriptional repressor in regulating Wnt targets (Houston et al., 2002; Kim et al., 2000; Liu et al., 2005; Merrill et al., 2004), and many of its functions do not require its β-catenin binding domain (Liu et al., 2005; Wu et al., 2012). LEF1 and TCF1, on the other hand, are primarily involved in activation of Wnt targets (Galceran et al., 1999; Hoverter et al., 2012; Kratochwil et al., 2002; Liu et al., 2005), and this functions requires binding to β-catenin (Galceran et al.,2004). The isoforms of LEF1 and TCF1 lacking the β-catenin binding domain function as dominant negatives to suppress Wnt targets (Hovanes et al., 2001; Roose et al., 1999; Tiemessen et al., 2012). TCF4 can act as both an activator or a repressor of Wnt signaling, depending on the context (Lien et al., 2014; Liu et al., 2005; Nguyen et al., 2009; Standley et al., 2006; Tang et al., 2008; Van de Wetering et al., 2002). How these distinct TCFs act together to regulate Wnt targets is not well understood.

TCFs bind DNA through a highly conserved High Mobility Group (HMG) domain (Cadigan and Waterman, 2012). The DNA binding specificity of vertebrate TCFs is very similar (Badis et al., 2009; van Beest et al., 2000), suggesting that they compete for the same binding sites at Wnt target genes. This implies that a "TCF exchange" might occur when Wnt targets are induced by the pathway (Cadigan, 2012). For example, in mouse embryonic stem cells (ESCs), Wnt signaling promotes an undifferentiated, pluripotent state (Clevers and Nusse, 2012), with TCF3 antagonizing and TCF1 promoting self-renewal, respectively, consistent with the TCF transcriptional switch model (Yi et al., 2011). Wnt/β-catenin signaling has been shown to reduce TCF3 expression in ESCs, either by inhibiting its transcription (Atlasi et al., 2013) or by removing it from Wnt target gene chromatin, resulting in TCF3 degradation (Shy et al., 2013). Thus, in this context, the Wnt pathway appears to effect a TCF exchange by repressing TCF3 levels, allowing activating TCFs (e.g., TCF1) to take its place.

Another well characterized example of a Wnt-induced TCF exchange occurs during early embryogenesis in *Xenopus*. Wnt/β-catenin signaling is required for dorsoventral patterning in this organism, through specification of a dorsal organizer after fertilization (Hikasa and Sokol, 2013). The organizer depends on β-catenin induction of the homeobox genes *Siamois* and *twin* (Bae et al., 2011; Fan and Sokol, 1997; Laurent et al., 1997; Lemaire et al., 1995). In addition, *Xenopus* Wnt8 is zygotically expressed in ventral cells during gastrulation, where it activates the homeobox gene *Vent2* (Karaulanov et al., 2004; Ramel and Lekven, 2004). TCF3 is a repressor of these Wnt target genes (Hikasa et al., 2010; Houston et al., 2002; Liu et al., 2005) and acts to limit the expression domain of Wnt targets (Houston et al.,

2002). Wnt/β-catenin signaling promotes phosphorylation of TCF3, via the homeodomain interacting protein kinase 2 (HIPK2) (Hikasa et al., 2010). This phosphorylation results in removal of TCF3 from the Wnt-dependent *cis*-regulatory modules (W-CRMs) that control *Vent2* and *Siamois* expression (Hikasa and Sokol, 2011; Hikasa et al., 2010). The HIPK2 sites in TCF3 that mediate this regulation are not present in TCF1, which can explain why Wnt stimulation promotes an exchange of TCF3 for TCF1 on *Vent2* and *Siamois* W-CRM chromatin (Hikasa and Sokol, 2011).

In this report, we examined the *Siamois* W-CRM in detail to understand how the TCF transcriptional switch operates. In our initial screen we identified three predicted T-box binding sites that contribute to repression of the W-CRM. These sites are bound by the T-box factor VegT, which physically interacts with TCF3. This interaction is disrupted by HIPK2, which supports a model where Wnt/β-catenin signaling disrupts the TCF3-VegT complex, resulting in removal of TCF3 from the W-CRM chromatin. We also found that Wnt/HIPK2 activation does not remove VegT from W-CRMs, and that VegT and the T-box sites are required for full activation of *Siamois*. Taken together, these data support a dual role for VegT in regulating *Siamois* expression, working with TCF3 to repress expression in the absence of Wnt signaling, but also contributing to *Siamois* activation. Our findings reveal an additional component of the TCF transcriptional switch in an important vertebrate Wnt target.

Results

T-box and TCF sites in the *Siamois* W-CRM contribute to its repression in ventral blastomeres

The *Siamois* W-CRM has been described previously (Brannon et al., 1997; Fan et al., 1998). For our analysis, we used a reporter constructed by Brannon and coworkers, composed of the 804 bp upstream of the Transcription Start Site (TSS) of *Siamois*, including its endogenous promoter (Brannon et al., 1997). This stretch of DNA contains five predicted TCF binding sites (Figure 3.1A), three of which (S0, S1 and S3) were reported to mediate Wnt/β-catenin activation (Brannon et al., 1997; Fan et al., 1998). In our assays, mutation of these three sites resulted in a modest decrease in response to pathway activation (data not shown), suggesting that the other two sites may also contribute to activation. Therefore, we characterized the regulation of a *Siamois* reporter with all five TCF sites mutated by base substitution.

The initial assay for Wnt regulation involved injection of the reporters into the animal side of one-cell stage *Xenopus* embryos, with or without β-catenin mRNA. Injected embryos were harvested for luciferase analysis at stage 10.5, when endogenous *Siamois* expression reach peak levels (Brannon et al., 1997; Lemaire et al., 1995). Consistent with previous reports (Brannon et al., 1997; Fan et al., 1998), we observed a large activation of the wild-type reporter activity by β- catenin (Figure 3.1B). Mutation of all five TCF sites caused a dramatic derepression of the reporter, and no further activation by β-catenin (Figure 3.1B). The level of baseline activity in the TCF site mutant reporter was similar as that seen with the wild type reporter with β-catenin activation (Figure 3.1B), highlighting the importance of TCF mediated repression in regulating this W-CRM.

To identify additional *cis*-regulatory elements that contribute to the regulation of the *Siamois* W-CRM reporter, we systematically mutated the regions surrounding the

five TCF sites and tested for Wnt responsiveness in HEK293T cells, where the *Siamois* reporter is known to be active (Fan et al., 1998; Hecht et al., 2000). This screen identified several regions that dampened or heightened reporter activation when mutated (Figure 2.5; Table 2.2). In particular, two mutants destroying predicted T-box sites (core consensus: AGGTG) (Conlon et al., 2001; Garnett et al., 2009; Howard et al., 2007) drew our attention. When these two sites, plus another T-box site further upstream were simultaneously mutated (Figure 3.1A; Figure 2.5), the reporter was activated three fold higher than wild-type by β-catenin in HEK293T cells (Figure 2.7). When assayed in *Xenopus* embryos, the T-box sites were also found to repress reporter expression. Without β- catenin co-injection, mutation of the T-box sites resulted in a small but significant derepression of the reporter (Figure 3.1C). Consistent with the T-box sites antagonizing Wnt signaling, the mutant reporter was activated more than five-fold higher than wild-type by exogenous β-catenin (Figure 3.1C). These data support that the T-box sites contribute to repression of the *Siamois* W-CRM.

After fertilization, the embryo undergoes cortical rotation, which results in β-catenin accumulation on the dorsal side as early as the two cell stage (Houston, 2012; Larabell et al., 1997; Schneider et al., 1996). This dorsally localized Wnt signaling activity induces *Siamois* expression in dorsal marginal cells during the midblastula transition (MBT) (Brannon and Kimelman, 1996; Carnac et al., 1996; Kuroda et al., 2004; Lemaire et al., 1995; Sudou et al., 2012).

To take advantage of this ventral/dorsal asymmetry in endogenous Wnt/β-catenin signaling, the wild-type and binding site mutant reporters were injected into the equatorial region of the ventral or dorsal blastomeres of four-cell stage

embryos. As previously reported (Brannon et al., 1997; Fan et al., 1998), the wild-type reporter is much more active on the dorsal side (Figure 3.1D, 3.1E). Mutation of the five TCF sites resulted in high reporter activity both ventrally and dorsally, indicating derepression (Figure 3.1C). When the three T-box sites were mutated, ventral and dorsal expression were higher than in wild-type controls (Figure 3.1D). These results confirm that with endogenous levels of Wnt/β-catenin signaling, the TCF and T-box sites contribute to repression of the *Siamois* W-CRM *in vivo*.

VegT regulates *Siamois* transcription by directly binding to the *Siamois* W-CRM

Although amphibians have many T-box genes in their genomes (Ryan et al., 1996; Smith et al., 1991; Stennard et al., 1999; Uchiyama et al., 2001), VegT stands out as a candidate for a direct regulator of *Siamois* transcription. Several T-box proteins are involved in *Xenopus* embryogenesis, but they are zygotically expressed (Stennard et al., 1999), perhaps too late to regulate *Siamois*, one of the earliest zygotic genes transcribed at the onset of MBT (Blythe et al., 2010; Lemaire et al., 1995). VegT, on the other hand, is maternally expressed and localized to the vegetal hemisphere of ooctyes and cleavage stage embryos, where it is required for mesendoderm specification (Horb and Thomsen, 1997; Showell et al., 2004; Zhang and King, 1996). In addition, VegT has previously been shown to regulate *Siamois* expression, though with conflicting results. Two groups reported that VegT is an activator of *Siamois* (Cao et al., 2007; Houston et al., 2002; Xanthos et al., 2002), while another provided evidence that VegT represses *Siamois* expression (Ishibashi et al., 2007). These studies used different approaches to address the relationship between VegT and *Siamois* expression, and they do not rule out the

possibility of multiple levels of regulation (see Discussion for further comments).

To examine the role of VegT in *Siamois* regulation, we knocked down expression of VegT using a morpholino approach. The vegetal pole of each blastomere was injected with a morpholino targeting VegT at the four cell stage, as previously described (Heasman et al., 2001; Ishibashi et al., 2007). Overall, VegT morphants gastrulated normally, except that involution and blastopore closure was slightly slower than in embryos injected with control morpholino (data not shown). At the tadpole stage, VegT morphants exhibited several defects, including a bent tail toward the ventral side, a less distinct somite muscle pattern, a larger head and expanded cement gland (Figure 3.2A and B). These phenotypes are reminiscent of mild LiCl treatment of early embryos (Kao and Elinson, 1988), which activates Wnt/β-catenin signaling (Klein and Melton, 1996).

To investigate how VegT regulates *Siamois* transcription *in vivo*, we conducted whole mount in situ hybridization. Knockdown of VegT resulted in the expansion of *Siamois* transcripts towards the vegetal pole (Figure 3.2C and 3.2D). These data indicate that VegT represses *Siamois* in the dorsal-vegetal region, which is consistent with VegT being localized to the vegetal hemisphere of the embryo (Horb and Thomsen, 1997; Zhang and King, 1996). In addition to the expanded expression domain, the mRNA level of *Siamois* was lower in VegT morphants compared to controls (Figure 3.2C and 3.2D). We also extended our analysis to another hallmark organizer gene, *Chordin*, whose expression requires *Siamois* (Collart et al., 2005; Ishibashi et al., 2007). *Chordin* transcripts also expanded vegetally in VegT morphants (Figure 3.2 E-G). These data are in

agreement with a previous report (Ishibashi et al., 2007) which proposed that VegT restricts *Siamois* expression and the dorsal organizer to the dorsal/animal region of the mid/late blastula.

The T-box sites in the *Siamois* W-CRM are similar to previously defined VegT consensus sites (Conlon et al., 2001) (Table 3.1). They also resemble the functional T-box sites identified in CRMs of several VegT direct target genes, such as *derrière, Sox17α, Xnr1, Xnr5* and *Bix4* (Casey et al., 1999; Hilton et al., 2003; Howard et al., 2007; Hyde and Old, 2000; White et al., 2002) (Table 3.1). To test whether VegT could bind to the predicted T-box sites from the *Siamois* W-CRM, we conducted electromobility shift assays (EMSAs) using recombinant GST- tagged VegT protein. The T-box site probe sequence corresponded to a 38 bp region upstream of the *Siamois* TSS, which contains two T-box sites (Figure 3.3A). GST-VegT bound to the T-box site probe, and binding was abolished by mutating the first five nucleotides of both T-box sites (Figure 3.3B). This demonstrates GST-VegT can specifically recognize these T-box sites *in vitro*.

To examine if VegT associates with *Siamois* W-CRM *in vivo*, chromatin immuno-precipitation (ChIP) was performed. mRNA encoding a myc-tagged VegT was injected animally into one-cell stage embryos and processed for ChIP at stage 10.5. We observed significant association of VegT with *Siamois* W-CRM chromatin over controls, which was not observed at the control *EF1α* locus (Figure 3.3C). In addition, we tested the W-CRM upstream of the *Vent2* gene for VegT binding, since it is known to be regulated by TCF3 (Hikasa et al., 2010) and has a predicted T-box site near the functional TCF site (Figure 3.3C). TCF3 binding was observed at this locus (Figure

3.3C). These data support a model where VegT associates with the *Siamois* and *Vent2* W-CRMs, likely via directly binding to T-box sites.

HIPK2 disrupts VegT binding to TCF3

Because TCF3 and VegT both contribute to repression of the *Siamois* W-CRM, we hypothesized that these TFs might physically interact. Indeed TCF3 binding to VegT has been previously reported (Cao et al., 2007). To investigate whether VegT associates with different TCFs, we coexpressed myc-tagged VegT with *Xenopus* TCF1, TCF3, TCF4 or mouse LEF1 (which were Flag tagged) in HEK293T cells. Interestingly, when expressed alone, VegT was not be detected by Western blot (Figure 3.4A, lane 5). Strikingly, co-expression with TCF3 or TCF4 resulted in a large increase in VegT levels (Figure 3.4A, lanes 3 & 4). In contrast, co-expression with TCF1 or LEF1 had little effect on VegT expression (Figure 3.4A, lanes 1 & 2). These data suggested that TCF3 and TCF4 can stabilize VegT, allowing it to accumulate to detectable levels. Consistent with this, treatment of VegT transfected cells with the proteasome inhibitor MG132 for 15 h prior to harvesting resulted in a dose-dependent increase in VegT levels, similar to that obtained with TCF3 co-expression (Figure 3.4B). Together, these results suggest TCF3 and TCF4 can stabilize VegT, likely by preventing its degradation.

To test if TCF3 and TCF4 can physically interact with VegT, a co-immunoprecipitation (co-IP) assay was performed. Anti-Flag epitope antibodies precipitated myc-VegT from extracts where it was co-expressed with Flag tagged TCF3 or TCF4 (Figure 3.4C). To examine if VegT specifically interacts with TCF3, a similar

co-IP was conducted using lysates from *Xenopus* embryos injected with myc-VegT and Flag-TCF1 or Flag TCF3 mRNA (Figure 3.4D). As expected, VegT specifically co-immunoprecipated with TCF3 but not with TCF1, suggesting VegT specifically binds to TCF3 but not TCF1.

The physical interaction between TCF3 and VegT supports a model where both TFs cooperate to repress *Siamois* transcription in the absence of Wnt/β-catenin signaling. As HIPK2 phosphorylation of TCF3 promotes its removal from the *Siamois* W-CRM (Hikasa and Sokol, 2011; Hikasa et al., 2010), we examined whether it regulated the interaction between TCF3 and VegT. We found that expression of HIPK2 in HEK293T cells blocked TCF3-dependent stabilization of VegT (Figure 3.5A, lanes 5 and 6). This destabilization of VegT was not observed when a kinase-dead version of HIPK2 was used (Figure 3.5A, lane 7). HIPK2 has been shown to promote cell apoptosis (D'Orazi et al., 2002, 2012), thus it is possible that loss of VegT signal is due to death of the transfected cells. Therefore, experiments were repeated with HIPK2ΔP, a kinase active mutant that is unable to induce apoptosis (Hikasa et al., 2010). This HIPK2 variant was still able to reduce VegT levels in the presence of TCF3 (Figure 5C). These results suggest HIPK2 kinase activity disrupts the TCF3 and VegT interaction.

To test the ability of HIPK2 phosphorylation of TCF3 to stabilize VegT, we took advantage of a TCF3 mutant where three serine residues were substituted with alanines. This TCF3 mutant was shown to be resistant to HIPK2 regulation in *Xenopus* (Hikasa et al., 2010). Like the wild- type TCF3, the mutant was able to stabilize VegT (Figure 3.5A, lane 5). Surprisingly, HIPK2 was able to disrupt the mutant TCF3's stabilization

of VegT similar to wild-type TCF3 (Figure 3.5A, lane 8). This suggests that HIPK2 may be able to regulate VegT independently of TCF3. Interestingly, the kinase dead HIPK2 stabilized VegT (Figure 3.5B), suggesting that the two proteins interact. Consistent with this, expression of HIPK2 causes a small but reproducible mobility shift in VegT (e.g., Figure 3.5C, compare lanes 2 and 3).

To test if VegT may be a substrate of HIPK2, we mutagenized the potential HIPK2 phosphorylation sites on VegT. There are ten sites with the consensus S/T-P (S30, S135, S188, S247, S260, T300, S303, T350 and S401), which are similar to the sites found in TCF3 (Hikasa and Sokol, 2011; Hikasa et al., 2010). These, residues were all converted to alanine in the same mutant construct. When this mutant VegT was expressed with TCF3 or the TCF3 phosphorylation site mutant in HEK293T cells, it was resistant to destabilization by HIPK2ΔP (Figure 3.5C, lanes 5-7). In addition, the HIPK2-dependent mobility shift was not observed with the VegT mutant (Figure 3.5C, lanes 5-7). These data suggest that HIPK2 promotes phosphorylation of VegT to disrupt its interaction with TCF3.

VegT also contributes to *Siamois* activation

Sokol and coworkers have previously showed that Wnt-activated HIPK2 phosphorylates TCF3, causing TCF3 to dissociate from W-CRM chromatin (Hikasa and Sokol, 2011; Hikasa et al.,2010). Thus, we investigated if HIPK2 also affects VegT W-CRM occupancy by expressing VegT-V5 in *Xenopus* embryos in the presence of HIPK2ΔP or the HIPK2 kinase dead mutant. We used the *Vent2* W-CRM because the VegT ChIP signal is reproducibly higher at this locus (Figure 3.3C).

VegT associated with this chromatin at similar levels with either HIPK2ΔP or the kinase dead HIPK2 KD (Figure 3.6A). As a positive control, we also assayed Flag tagged TCF3. A small but significant decrease in TCF3 binding to the *Vent2* W-CRM was observed when coexpressed with HIPK2ΔP (Figure 3.6B). These data suggest that unlike TCF3, VegT binding to W-CRM chromatin is not affected by Wnt/HIPK2 signaling. The observation that VegT remained associated with the W-CRM under conditions that favor target gene activation suggests a possible role for VegT in this process. Several results support this hypothesis. First, in the context of a *Siamois* W-CRM reporter lacking TCF sites, the three T-box sites are required for expression (Figure 3.6C). This effect was observed when the reporter was injected in both ventral and dorsal blastomeres (Figure 3.6C). Second, *Siamois* transcript levels were reduced in VegT morphants compared to controls (Figure 3.6D). While this result seems contradictory to the vegetal expansion of *Siamois* in VegT morphants, it is consistent with the overall lower staining intensity in the morphants compared to controls (Figure 3.2D). These data support a dual role for VegT in regulating *Siamois* W-CRM activity, with the protein contributing to repression in regions of low Wnt/β-catenin signaling, but also being required for activation in the presence of signaling.

Discussion

Given the specialization of vertebrate TCFs for repression or activation of Wnt targets, mechanisms must exist that regulate their ability to associate with W-CRMs in a signal-dependent manner. In this report, we describe how the T-box protein VegT contributes to this regulation in early *Xenopus* development. A model summarizing our findings on

VegT in *Siamois* regulation is shown in Figure 3.7. In cells in which Wnt/β-catenin signaling is low, TCF3 and VegT cooperate to directly repress *Siamois* transcription. Mutation of either the TCF or T- box binding sites results in higher expression of a *Siamois* W-CRM reporter (Figure 3.1). VegT specifically binds the T-box sites and is found associated with the *Siamois* W-CRM (Figure 3.3), similar to TCF3 (Hikasa and Sokol, 2011; Hikasa et al., 2010). VegT prevents expression of dorsal organizer genes in the vegetal hemisphere, which is required for patterning the body plan (Figure 3.2; Ishibashi et al., 2007).

The TCF3 repression of *Siamois* is relieved by Wnt/β-catenin signaling through HIPK2, which results in phosphorylation of TCF3 and its release from W-CRM chromatin (Hikasa and Sokol, 2011; Hikasa et al., 2010). We found that VegT physically associates with TCF3 (Figure 3.4) and this association is disrupted by HIPK2 (Figure 3.5). Mutation of putative HIPK2 phosphorylation sites on TCF3 and VegT prevents HIPK2-mediated dissociation (Figure 3.5C). This dissociation may contribute to the reduction of TCF3 binding to W-CRM chromatin by Wnt/HIPK2 signaling (Figure 3.7), though further studies are required to address this.

In addition to TCF3, HIPK2 also promotes the phosphorylation of LEF1 and TCF4, but not TCF1 (Hikasa and Sokol, 2011). This suggests a model where TCF1 replaces TCF3 on W- CRMs in the presence of Wnt/HIPK2 signaling, leading to β-catenin recruitment and transcriptional activation (Figure 3.7; Hikasa and Sokol, 2011; Hikasa et al., 2010). We found that TCF3 and TCF4 interacted with VegT in HEK293T cells (Figure 3.4A). In *Xenopus*, we confirmed that VegT specifically binds to TCF3 but not to TCF1 (Figure 3.4D). The selective binding of VegT to TCF family members

and its modification by HIPK2 could explain how Wnt signaling facilitates the exchange of TCFs on W-CRM chromatin in the early *Xenopus* embryo (Figure 3.7).

In addition to its role in repressing *Siamois*, we also found evidence that VegT contributes to activation of this Wnt target. We note that this activating function of T-box sites was not observed in our initial reporter assays (Figure 3.1), indicating that they are not absolutely required for expression, at least in the context of an injected reporter. However, mutation of the T-box sites in a *Siamois* W-CRM reporter lacking TCF sites resulted in a dramatic reduction in reporter activity (Figure 3.6C). In addition, VegT morphants showed a reduction in *Siamois* transcripts (Figure 3.2C, 3.6D). Consistent with a role in Wnt target gene activation, VegT remained bound to W-CRM chromatin in the presence of HIPK2 (Figure 3.6A). We propose that VegT provides some transcriptional activation of *Siamois* expression in a TCF-independent manner (Figure 3.7).

Our findings that VegT has a dual role in regulating *Siamois* provide a resolution for the controversy in the literature. While depletion of VegT with morpholinos injected into 4-cell embryos largely resulted in expansion of the *Siamois* expression domain (Figure 3.2; Ishibashi et al., 2007), depletion of VegT via antisense oligonucleotide-mediated mRNA knockdown in *Xenopus* oocytes using the host transfer technique resulted in a reduction in overall *Siamois* transcript abundance (Houston et al., 2002; Xanthos et al., 2002). While some of this effect could be due to loss of VegT activation of *Siamois*, this early depletion of VegT also disrupted vegetal accumulation of *xWnt11* mRNA prior to cortical rotation (Heasman et al., 2001), which is required for *Siamois* expression (Tao et al., 2005). Stronger evidence for a

direct activating role for VegT comes from animal cap studies, where injection of *VegT* and β-*catenin* mRNA lead to a large increase in *Siamois* reporter activity (Cao et al., 2007). This is further supported by our findings that T- box sites contribute to *Saimois* W-CRM reporter activation (Figure 3.6C) and the reduction in *Siamois* transcripts in VegT morphants (Figure 3.6D), where VegT was depleted after cortical rotation and *XWnt11* mRNA dorsal accumulation has already occurred.

Regulation of other Wnt targets by VegT or other Tbx proteins

It is interesting to note that VegT has also been linked to regulation of nodal-regulated genes in *Xenopus* embryos. β-catenin and VegT cooperatively activating *Xnr5* and *Xnr6* expression in dorsal vegetal cells (Agius et al., 2000; Rex et al., 2002; Takahashi et al., 2000), while VegT antagonizes Wnt/β-catenin activation of *Xnr3* within the dorsal organizer (Rex et al.,2002). In the case of a *Xnr5* W-CRM reporter, mutation of the T-box sites results in loss of expression (Hilton et al., 2003) without the derepression we observed when similar sites were altered in the *Siamois* reporter (Figure 3.1). These data suggest the action of VegT is context and target gene specific. In the case of the *Siamois* W-CRM, our data support a role for both repression and activation by VegT through T-box sites (Figure 3.7).

In addition to the *Siamois* W-CRM, TCF3 and HIPK2 also regulate *Vent2* expression, a Wnt target expressed in ventral blastomeres (Hikasa et al., 2010). We found that VegT is associated with the *Vent2* W-CRM in chromatin (Figure 3.3C). There is a predicted T-box site near the functional TCF site in this W-CRM (Figure 3.3C). Mutation of the TCF site results in derepression of the reporter (Hikasa et al.,

2010). Further studies are needed to determine whether and how the T-box site contributes to this W-CRM's regulation.

Do other T-box proteins (Tbxs) work with TCFs to regulate Wnt target gene transcription? In the mouse, Tbx6 and Wnt/β-catenin signaling act together to activate *Hes7*, *Delta 1* and *mesogenin 1* expression, which all contribute to patterning the presomitic mesoderm. CRMs for each of these genes were found to contain functional TCF and T-box binding sites (González et al., 2013; Hofmann et al., 2004; Wittler et al., 2007). At the genomic level, a ChIP-seq analysis in *Drosophila* embryos demonstrated that TCF/Pangolin (the sole fly TCF) and Dorsocross (a fly Tbx) co-localize to many cardiogenic CRMs (Junion et al., 2012). More evidence linking TCFs with other TFs is accumulating, largely driven by genome-wide surveys of TF binding (Archbold et al., 2012; Blahnik et al., 2010; Bottomly et al., 2010; Cole et al., 2008; Junion et al., 2012; Marson et al., 2008; Tam et al., 2008; Trompouki et al., 2011; Verzi et al., 2010) and it seems likely that some W-CRMs use a strategy where TCF and Tbx proteins act in concert. Further studies are required to determine whether the dual regulation of a Tbx protein (i.e., VegT) on the *Siamois* W-CRM is a common feature of Wnt target gene regulation.

Materials and Methods

Plasmids

The *Siamois* W-CRM reporter was described previously (Brannon et al., 1997; GenBank AF016226.1). All mutations in this reporter were generated by site-directed mutagenesis (QuickChange II kit, Strategene) and subcloned into the original reporter

backbone using StuI and SacI sites. See Table SII for mutagenesis primer sequences. pRenilla-luc (Promega) was used as an injection control for luciferase reporter assays. pCS2-myc-VegT and pCS2-VegT-V5 were derived from pCS2-VegT (kindly provided by Walter Knöchel) by inserting 2 myc epitopes or the V5 epitope at the N or C terminus, respectively. Mutations of predicted HIPK2 sites on VegT were introduced by Assembly PCR as previously described (Swanson et al., 2010) using the primers listed in Table SII. The coding fragment containing the mutated HIPK2 sites was subcloned into pCS2-VegT-V5 using PstI and EcoRI sites. To generate pGEX-GST-VegT, the VegT ORF was PCR amplified and inserted into pGEX-6P-1 vector using XmaI and XhoI sites. Constructs of *Xenopus* TCF1, TCF3 (WT and P2/3/4 mutant), TCF4, HIPK2 (WT, ΔP and KD mutant) and mouse LEF1 were described previously (Hikasa et al., 2010). pcDNA3-β- cateninS45F and pcDNA3-p300 are previously described (Hecht et al., 2000; Yochum et al.,2007).

Embryo Microinjections and Luciferase Reporter Assays

In vitro fertilization, staging and culture in 0.01x Marc's modified Ringer's solution were carried out as previously described (Nieuwkoop and Faber, 1967; Peng, 1991). Capped synthetic RNAs were generated by *in vitro* transcription using the mMessage mMachine kit (Ambion) with the following linearized plasmid templates: pSP36T-β-catenin, pCS2-2xmyc-VegT, pCS2-VegT-V5, pCS2-flag-TCF3HA, pCS2-6xmyc-HIPK2(WT/KD/ΔP),. VegT morpholino (Ishibashi et al.,2007) and control morpholino (Hikasa et al., 2010) were previously described (sequences are found in Table SII). For microinjections, embryos were injected at one cell or four cell stage with 5~10 nl

mRNA, DNA, or morpholino solution.

For dual luciferase assays, 100 pg of the indicated *Siamois* reporter, 20 pg pRLRenilla-luc, with or without 200 pg β-catenin mRNA were injected into one-cell stage or four-cell stage embryos. Lysates of injected embryos were prepared at stage 10.5. Luciferase activity was measured as described by manufacturer (Dual-Luciferase® Reporter Assay System, Promega). For one-cell injections, a group of four embryos was mixed and lysed as one sample for measurement, and five replicates were analyzed for each condition. For dorsal/ventral assays, each individual embryo was lysed and measured as one sample, and eight replicates were analyzed for each condition. Due to variations in the batch of embryos and microinjections, direct comparisons between data were only made for embryos injected on the same day. Therefore, each figure panel only contains data collected on the same day.

Whole-Mount In Situ Hybridization

Whole-mount in situ hybridization was carried out as previously described (Harland, 1991). Digoxygenin-labeled antisense RNA probes were synthesized from pBluescript II SK+-Siamois and pCS2+-Chordin using DIG labeling mixture (Roche). 1-Step NBT/BCIP (Thermo Scientific) was used for chromogenic reactions. For detection of *Siamois* transcripts, after fixation, embryos were bisected along the left-right axis to expose interior. The bisected embryos were re-fixed for 0.5h, and dehydrated directly in 100% ethanol before proceeding with the in situ hybridization.

Electrophoretic Mobility Shift Assay

Electrophoretic Mobility Shift Assays (EMSAs) were carried out as previously described (Blauwkamp et al., 2008). GST-tagged VegT protein was purified from *E.coli* as previously described (Chang et al., 2008b). For binding reactions, 400 fmoles biotinylated probes (IDT, Coraville, IA) and 0, 2, 4, 6 or 8 pmoles of protein were incubated in 20 µl total volume.

Chromatin Immunoprecipitaiton Assays and quantitative RT-PCR

Chromatin immunoprecipitation (ChIP) assays were carried out with *Xenopus* embryos as previously described (Blythe et al., 2009), except that freshly fixed embryos were used, because freezing/thawing of fixed embryos decreased the ChIP signal. DNA was fragmented using a Fisher Model 100 sonicator to an average size of 200-600 bp (determined by agarose gel electrophoresis). Polyclonal anti-myc antibody (Millipore, 06-549), polyclonal anti-V5 antibody (Abcam, ab15828) and anti-flag M2 antibody (Sigma, F3165) were used to precipitate myc- VegT, VegT-V5 and flag-TCF3-HA, respectively. Precipitated DNA was analyzed with qPCR (ABI 7500 Fast System) using the primers listed in Table SII. For quantitative RT-PCR, total RNA was extracted from injected embryos using RNeasy Mini Kit (QIAGEN). cDNA was synthesized using SuperScript® III RT (Life Technologies) with oligo dT primers. For quantification, *Siamois* level among different samples was normalized to *ornithine decarboxylase* (*ODC*). See Table 3.2 for primers used in qPCR.

Cell Culture, Transfection, Co-Immunoprecipitation and Western blot analysis

HEK293T cells were cultured in Dulbecco's modified Eagle's medium (GIBCO)

supplemented with 10% fetal bovine serum at 37˚C, 5% CO_2. Cells were seeded at 2.5 x 10^5 per ml in 12-well plates for expression assays, in 60 mm plates for CoIP assays and in 48-well plates for luciferase reporter assays. At about 60% confluency, cells were transfected with the following plasmids. For expression assays, 400 ng myc-VegT or VegT-V5 (WT or mutant), 300 ng flag-TCFs/LEF1 and 300 ng myc-HIPK2. For co-IP assays, 4 µg myc-VegT and 4 µg flag-TCF3 or flag-TCF4 were transfected. For luciferase reporter assays, 50 ng reporter plasmids, 50 ng β-cateninS45F and 50 ng p300 plasmids. All DNA were transfected using Lipofectamine 2000 (Life Technologies) and total DNA transfected was normalized with empty backbone plasmid. Cells were harvested 48 h post transfection and lysed in 1x SDS sample buffer for western analysis. For proteasome inhibitor treatments, MG132 (Sigma) was dissolved in DMSO and added to media at the indicated concentrations 15 h before harvesting, with control cultures receiving the same amount of DMSO. For co-IP assays, transfected cells were lysed in 400 µl of buffer containing 10mM HEPES pH 7.9, 1.5mM $MgCl_2$, 10mM KCl, 0.5% NP40, 0.5mM DTT and 1x protease inhibitor cocktail (Roche). 40 injected *Xenopus* embryos were lysed in buffer containing 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.5% Triton X-100, 1 mM EDTA, and 1x protease inhibitor cocktail (Roche). Cell or embryo lysates were pre-cleared with protein G agarose, fast flow (Millipore) followed by overnight incubation with anti-flag M2 antibody (Sigma, F3165) and one hour protein G agarose binding. The bound beads were washed four times with lysis buffer supplemented with 150 mM NaCl and boiled in SDS sample buffer for western blot analysis. Monoclonal 9E10, M2 (Sigma) and anti-V5 (Invitrogen) were used for detection of myc-, flag-, V5-tagged proteins, respectively.

Acknowledgements

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The data presented in this chapter is a major portion of the manuscript**: "VegT plays a dual role in the TCF transcriptional switch regulating Siamois expression in *Xenopus*"**

Yaxuan X. Yang, Ann L. Miller, Robert J. Denver and Ken M. Cadigan

This manuscript has been submitted to Developmental Biology, and is currently in revision.

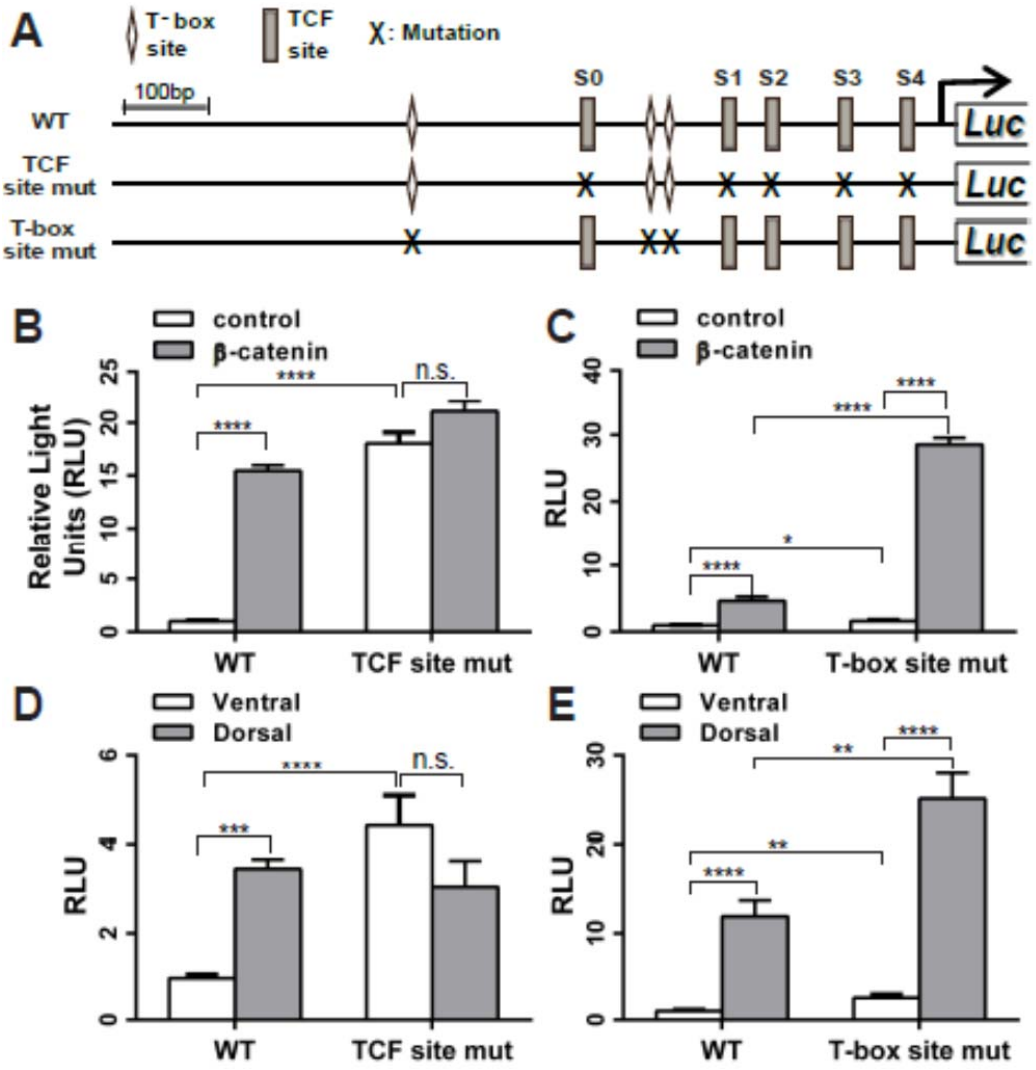

Figure 3.1 TCF sites and T-box sites both mediate repression of the *Siamois* W-CRM.

(A) Diagram of *Siamois* promoter-proximal W-CRM luciferase reporter constructs. The 804bp W-CRM contains five predicted TCF sites (grey rectangles) and three predicted T-box sites (white diamonds). Mutations of the sites are denoted by Xs. The bent arrow indicates the TSS (+1) of the firefly luciferase gene. (B, C) Injection of *Siamois* reporter constructs into one-cell stage embryos with or without β-catenin mRNA. The wild-type reporter was activated by β- catenin. Mutation of the TCF sites resulted in a large derepression, which was not further activated by β-catenin. The T-box site mutant reporter had increased activity over the wild-type control with and without β-catenin. (D, E) Four-cell stage embryos were injected with *Siamois* reporters into the equatorial region of ventral or dorsal blastomeres. As previously reported (Brannon et al., 1997; Fan et al., 1998), wild-type reporter activity was higher in the dorsally injected embryos. Expression was equally high for the TCF site mutant reporter injected ventrally and dorsally, while the T-box site mutant reporter was higher than wild-type in both cases,

but still retained enhanced expression dorsally. All injections also contained a renilla luciferase plasmid for normalization of firefly luciferase activity and injected embryos were harvested at stage 10.5 for dual luciferase assays. Relative light units (RLUs) are defined by the ratio of firefly luciferase counts to renilla luciferase counts and normalized to WT control (B,C) or WT ventral (D,E). Bars represent the mean + SEM of at least five biological replicates. RLUs were log10 transformed before statistical analysis using a two tailed Student's T-test. * $P< 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, n.s., not significant.

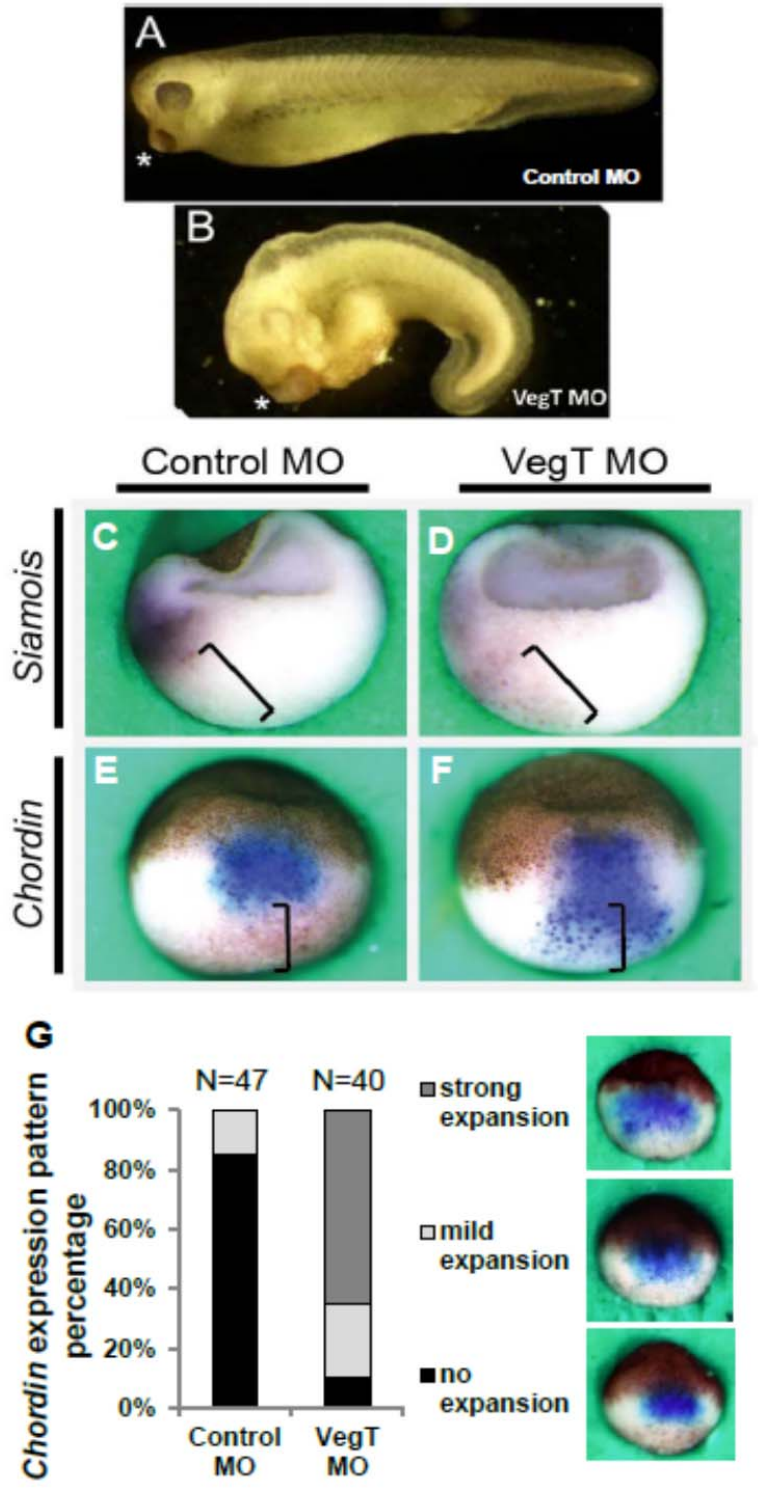

Figure 3.2 VegT represses organizer gene expression.

Approximately 5 pmol control or VegT morpholino was injected into the vegetal pole of each blastomere of four cell-stage embryos. (A- B) Stage 40 VegT morphants showed

bent tail and enlarged cement gland (white asterisks). (C, D) In situ hybridization of *Siamois* transcripts in control and VegT morphants. Stage 9 embryos were fixed and bisected along right-left axis prior to in situ hybridization and positioned with animal pole upward and the dorsal organizer on the left. *Siamois* transcripts are reduced in the Organizer region and expanded toward the vegetal pole (brackets). (E, F) In situ hybridization of *Chordin* transcripts in control and VegT morphants at stage 10. Dorsal is towards the reader. *Chordin* expression is expanded vegatally in VegT morphants (brackets). (G) Bar graph summarizing the penetrance of the VegT effect on *Chordin* expression, with examples of embryos displaying normal *Chordin* expression or mild and strong vegetal expansion.

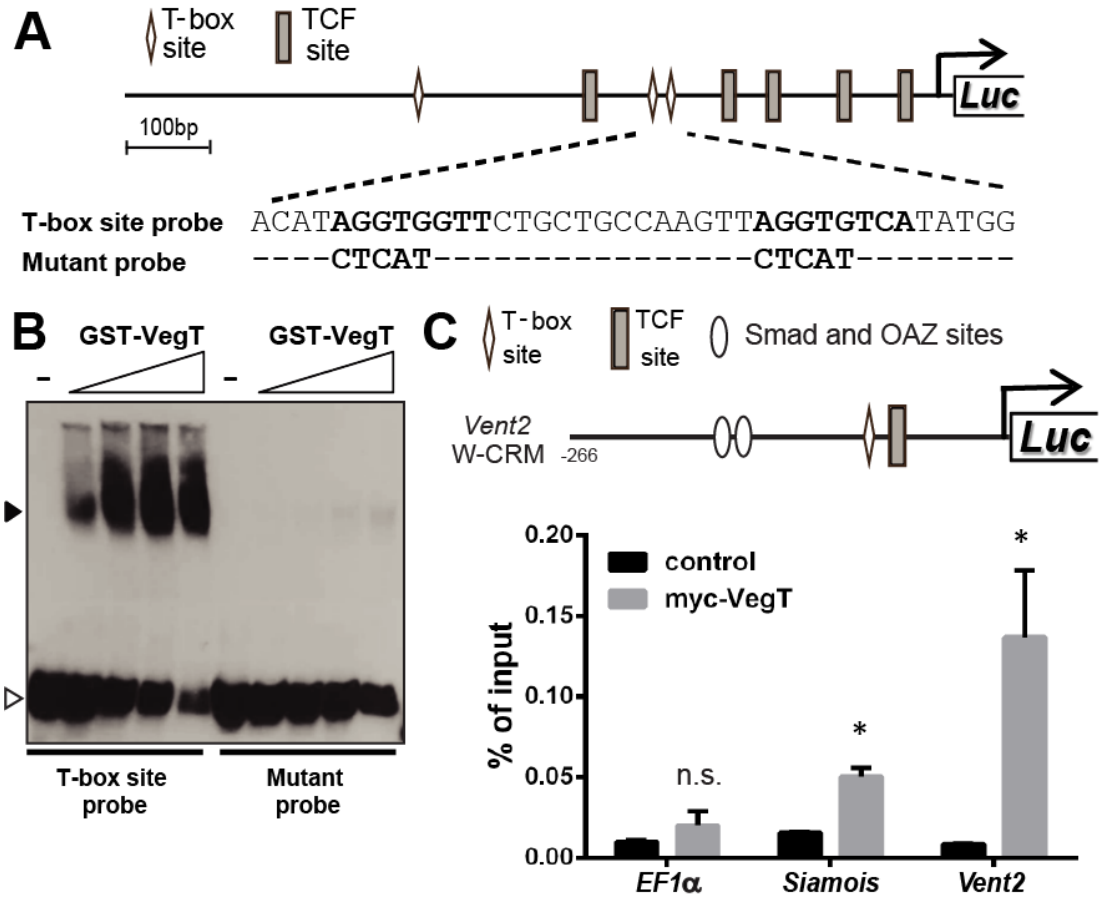

Figure 3.3 VegT binds to *Siamois* and *Vent2* W-CRM chromatin.

(A) Cartoon showing the T-box site probe from the *Siamois* W-CRM (-300 to -263) used for the EMSA experiments. The alterations in the mutant probe are indicated below. (B) EMSA showing GST-VegT specifically binds to the T-box site probe but not the mutant probe. Black and white triangles indicate bound and free probes, respectively. (C) ChIP assay showing that injected myc-tagged VegT associates with *Siamois* and *Vent2* W-CRM chromatin. One-cell embryos were injected with 300 pg myc- VegT mRNA at the animal pole. Uninjected embryos served as the negative control. Embryos were fixed at stage 10.5 and processed for ChIP as described in Materials and Methods. The amplicons of *Siamois* and *Vent2* are located at -360 to -240 and -170 to -40 from the TSS of each gene, respectively. Primers for the *EF1α* locus served as a negative control. Bars represent the mean + SEM (n = 3, each biological replicate contains 50 embryos). The data shown are representative of three independent experiments. ChIP data (the ratio of ChIP signal to input) were log10 transformed before statistical analysis. * $P < 0.05$. Sequences of the PCR primers used are provided in Table 3.2.

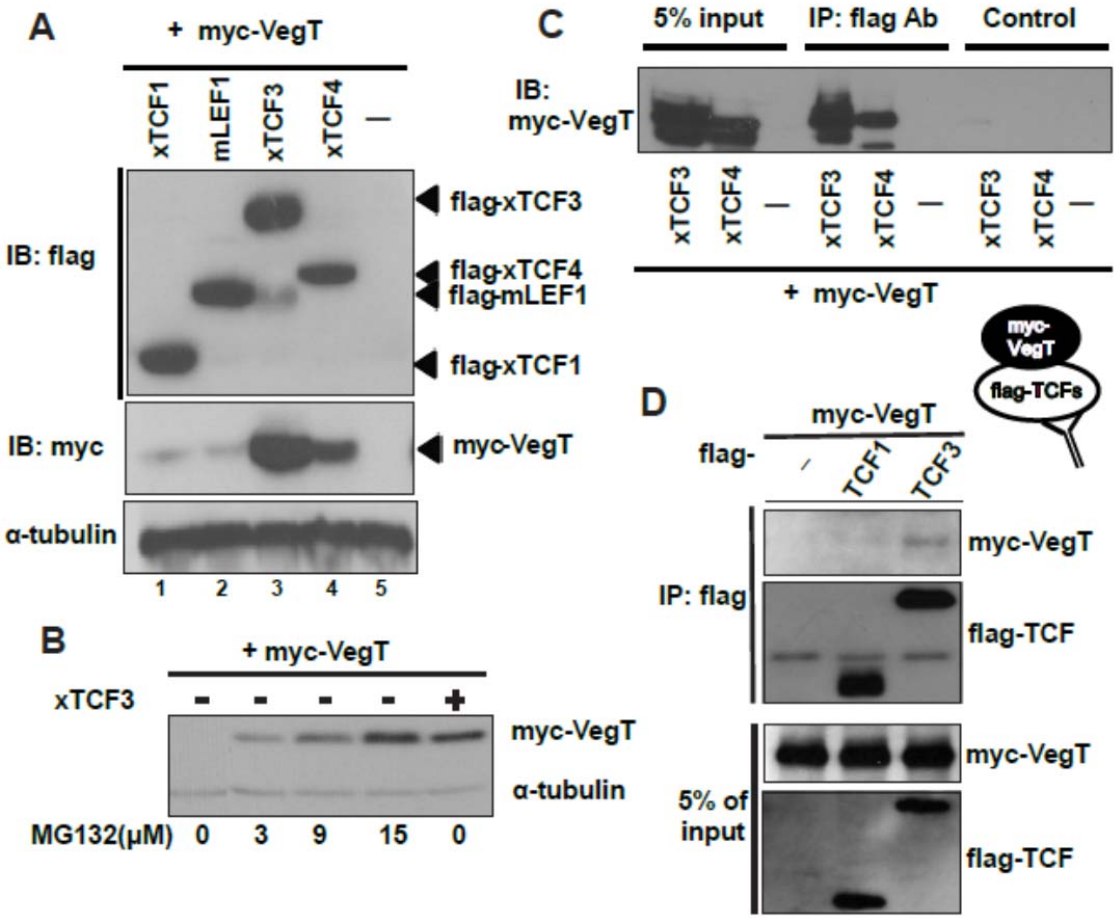

Figure 3.4 VegT interacts with TCF3 in HEK239T cells and *Xenopus* embryos.

(A) Western blot showing that VegT protein is stabilized by co-expression of xTCF3 and xTCF4 but not xTCF1 or mLEF1 in HEK293T cells. (B) Western blot showing that xTCF3 prevents VegT degradation. HEK293T cells were transfected with the myc-VegT expression vector and were treated with proteasome inhibitor MG132 at 3, 9, 15 μM for 15 h. VegT levels in MG132 (15μM) treated cells were similar to those seen in xTCF3 co-transfected cells. (C) Co-IP assay showing that VegT interacts with xTCF3 and xTCF4 in HEK293T cells. (D) Co-IP of VegT and xTCF1 or xTCF3 in *Xenopus* embryos injected with the respective mRNAs at the one cell stage and harvested at stage 10.5. VegT is specifically co-IPed with xTCF3. All blots are representative of three independent experiments.

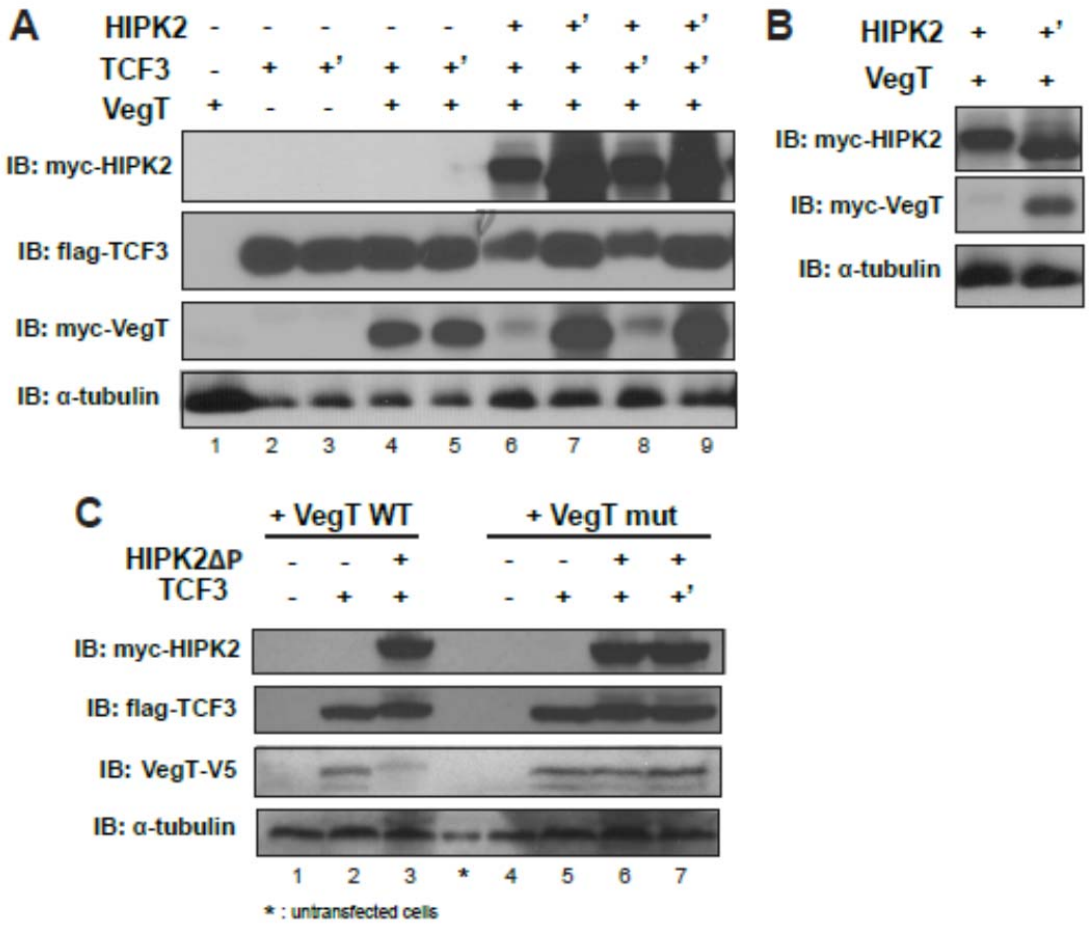

Figure 3.5 VegT is destabilized by HIPK2 in HEK293T cells.

(A) Western blots showing that catalytically active HIPK2 blocks the stabilizing effect of *Xenopus* TCF3 on VegT protein levels in HEK293T cells. Cells were transfected with VegT, TCF3 and HIPK2 as indicated by the plus signs. Apostrophes indicate where a kinase dead version of HIPK2 or TCF3 lacking three HIPK phosphorylation sites (i.e., p2/3/4) was used (Hikasa et al. 2010). Wild-type HIPK2 but not HIPK2 kinase dead (KD) mutant greatly reduced VegT protein levels. (B) Western blots of HEK293T cells transfected with VegT and HIPK2 or the kinase dead mutant (without exogenous TCF3). The kinase dead HIPK2 mutant stabilized VegT protein, whereas wild-type HIPK2 did not. (C) Western blots showing HIPK2ΔP, a kinase active version unable to induce apoptosis, also inhibits the stabilizing effect of TCF3 on VegT in HEK293T cells. However, a VegT mutant with its predicted HIPK2 phosphorylation sites mutated remained stabilized by TCF3 in the presence of HIPK2ΔP, and did not display the HIPK2-dependent mobility shift observed with wild-type VegT. All blots are representative of three independent experiments.

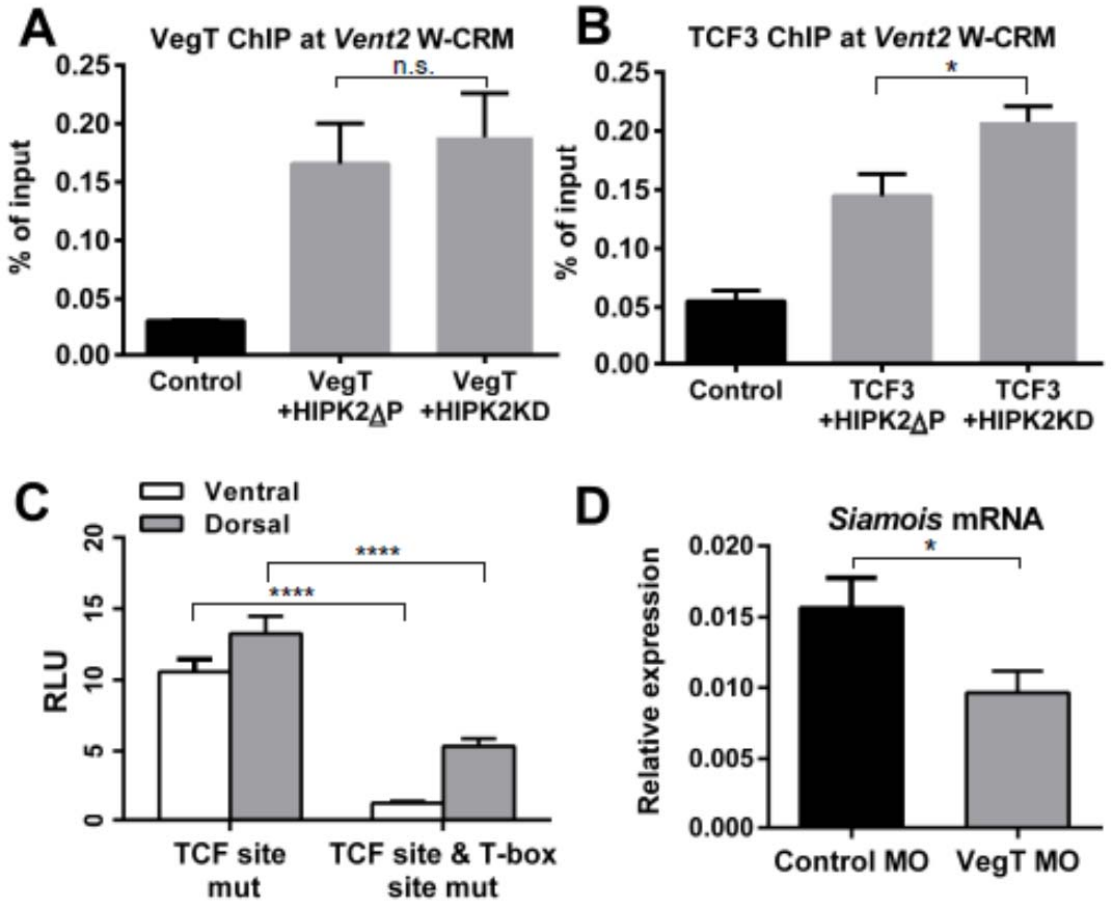

Figure 3.6 VegT also plays a role in *Siamois* activation.

(A) A ChIP assay showing that injected V5 tagged VegT remained bound to Vent2 W-CRM chromatin at similar levels in the presence of HIPK2ΔP (lacking apoptosis promoting activity) or HIPK2 KD mutant. (B) ChIP assay showing that injected flag tagged TCF3 binds less at *Vent2* W-CRM in the presence of HIPK2ΔP than in the presence of HIPK2 KD mutant. RNAs encoding VegT (300 pg), TCF3 (300 pg) and HIPK2 (150 pg) were injected into animal pole of embryos at the one cell stage. Embryos were fixed at stage 10.5. Bars represent the mean of biological triplicates ± SEM. The data shown are representative of three independent experiments. ChIP data were log10 transformed before statistical analysis. * $P < 0.05$. (C) Four-cell stage embryos were injected with the indicated *Siamois* reporters into the equatorial region of both ventral or dorsal blastomeres. The expression of TCF site & T-box site double mutant reporters were significantly lower than the TCF site mutant in ventral and dorsal cells. The double mutant is more active when in dorsal than ventral cells. Bars represent the mean of six replicates ± SEM. RLUs were log10 transformed before statistical analysis with a two tailed Student's T-test. **** $P < 0.0001$. (D) Quantitative RT-PCR assay showing that *Siamois* transcript levels are reduced in VegT morphants. Morpholinos were injected as previously described for in situ experiments in Figure 3.2. RNA were extracted from embryos at stage 10.5. *Siamois* expression was normalized to the housekeeping gene

ODC. Bars represent the mean of biological triplicates (each replicate contained four embryos) +SEM. The data shown were representative of three independent experiments. Data were analyzed with a two tailed Student's T-test. * $P < 0.05$.

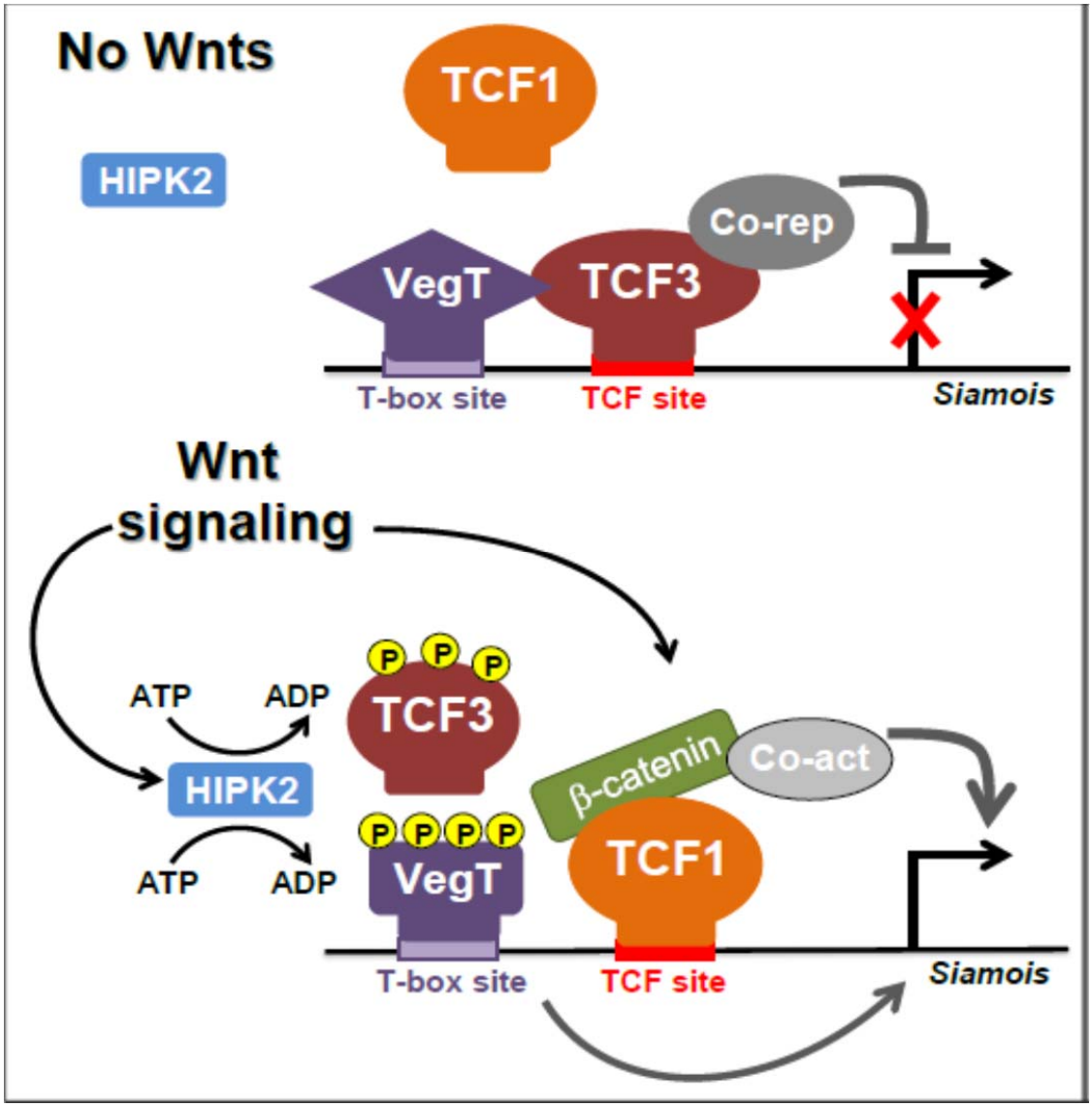

Figure 3.7 Incorporating VegT into the *Xenopus* TCF transcriptional switch model.

This model is a modification of the one proposed by Hikasa and Sokol (Hikasa and Sokol, 2011; Hikasa et al., 2010). (upper cartoon) In the absence of Wnt signaling, VegT binds to T-box sites and forms a complex with TCF3 to suppress *Siamois* expression. (lower cartoon) Upon Wnt signaling, TCF3-VegT interaction is disrupted by activated HIPK2. TCF3 dissociates from *Siamois* W-CRM chromatin, allowing activating TCFs such as TCF1 to bind to W-CRM and activate transcription with β-catenin and co-activators. VegT stays bound to *Siamois* W-CRM and contributes to *Siamois* activation as well. See Discussion for further explanation.

	sequence	Reference
VegT highest-affinity site *in vitro*	AGGTGTGA	Conlon et al, 2001
functional VegT sites *in vivo*		
derrière	AGGTGTCA	White et al., 2002
	AGGTGTCA	
sox17a1	TGGTGTGA	Howard et al., 2007
	AGGTGTCA	
Xnr1	AGGTGTGA	Hyde and Old, 2000
	AGGAGTGA	
Xnr5	AGGTGACA	Hilton et al., 2003
	AGGTCTCT	
	AGGTGACA	
	AGGTGCCA	
Bix4	AGGTGTCA	Casey et al, 1999
	AGGTGTGA	
	ACGTGTGA	
VegT sites in this report		
Siamois	TGGTGTAA	
	AGGTGGTT	
	AGGTGTCA	
Vent2 (putative)	AGGTGGTA	

Table 3.1 Comparison of the optimal in vitro VegT binding site and verified functional VegT sites.

Purpose	Primer name	Primer sequence	Reference
Mutagenesis of TCF sites	S0_f	CAAGTGTAAGTAAGGGACTGTTCAGTCTTGCCAACTTCTCTCACTCAGTCC	
	S0_r	GGACTGAGTGAGAGAAGTTGGCAAGACTGAACAGTCCCTTACTTACACTTG	
	S1_f	ATCAAGGGAAACACGTGTCATCAGAATCAGAACAGGACCTCCCTTTTG	
	S1_r	CAAAAGGGAGGTCCTGTTCTGATTCTGATGACACGTGTTTCCCTTGAT	
	S2_f	TCCCTTTTGTGTCCCCAAATCACATCTGTTCCCTTGCCAATTCTTTCAG	
	S2_r	CTGAAAGAATTGGCAAGGGAACAGATGTGATTTGGGGACACAAAAGGGA	
	S3_f	CCAATAAACCACGAGCAACAGTACAGAACAGTATTTATATTTTTTTCATTT CCC	
	S3_r	GGGAAATGAAAAAAATATAAATACTGTTCTGTACTGTTGCTCGTGGTTTAT TGG	
	S4_f	CCCCAAAACACATCCTGGACACACCCTGTTCCCCCTGTTGATATA	
	S4_r	TATATCAACAGGGGGAACAGGGTGTGTCCAGGATGTGTTTTGGGG	
Mutagenesis of T-box sites	T1_f	CTACACATTTTATATAACgtcatgAATATAATAAAACACAACATCCTTGGCC	
	T1_r	GGCCAAGGATGTTGTGTTTTATTATATTcatgacGTTATATAAAATGTGTAG	
	T2_f	CAAATCATTTCCATAATAACACATctcatGTTCTGCTGCCAAGTTAGG	
	T2_r	CCTAACTTGGCAGCAGAACatgagATGTGTTATTATGGAAATGATTTG	
	T3_f	GGTTCTGCTGCCAAGTTcttTGgacgATGGGACAGTATTCTGGGC	
	T3_r	GCCCAGAATACTGTCCCATcgtcCAaagAACTTGGCAGCAGAACC	
ChIP	Sia-f	GGGACTTTGAAGTCTTGCCA	Hikasa et al 2010
	Sia-r	TCTGATGACACGTGTTTCCC	
	Vent2-f	GGCAGACATGGTGGAGCCAG	Hikasa et al 2010
	Vent2-r	GTATGCAAATGCAGCCACTA	
	EF1α-f	ACAAAAGAGCTGGGAGCT	Blythe et al 2010
	EF1α-r	TTCCTTTCCCATTGTGGA	
Morpholino	VegT MO	CCCGACAGCAGTTTCTCATTCCAGC	Ishibashi et al 2008
	Control MO	AGAGACTTGATACAGATTCGAGAAT	Hikasa et al 2010
EMSA probe	T-box site probe	ACATAGGTGGTTCTGCTGCCAAGTTAGGTGTCATATGG	
	mutant probe	ACATCTCATGTTCTGCTGCCAAGTTCTCATTCATATGG	
qRT-PCR	siamois_f	AACTTTCTCCAGAACC	Yang et al 2002
	siamois_r	GTCAGTGTGGTGATTC	Yang et al 2002
	ODC _f	AATGGATTTCAGAGACCA	Yang et al 2002
	ODC _r	CCAAGGCTAAAGTTGCAG	Yang et al 2002
Assembly PCR of VegT mutant	flanking_f	caccctgcaggacgtatgtccacccagat	
	assembly 1	gacgtatgtccacccagatGCACCTgctcctggtgcccactggatgaaggatccgatctg	
	assembly 2	ttgatccaatgtgttgttggtgagtttgagcttttgaaagcagatcggatccttcatcca	
	assembly 3	accaacaacacattggatcaacaaggccatattatcttgcattcaatgcatcgctacaag	
	assembly 4	attgtacatgtcatcagactgaactacatggaacctgggcttgtagcgatgcattgaatg	
	assembly 5	tcagtctgatgacatgtacaatGCTCCAtggggattggtacaagtgtttagcttcccaga	
	assembly 6	cttttcattctggtaggcagtcactgaagtaaactctgtctctgggaagctaaacacttg	
	assembly 7	actgcctaccagaatgaaaagattactaaactgaaaattaatcacaacccatttgctaaa	
	assembly 8	catccctcttgtgactcctttcctgctcccggaatcctttagcaaatgggttgtgattaa	
	assembly 9	aggagtcacaagagggatgatgttttaaagattctacaacaaGCTCCTagtaaaaggcag	
	assembly 10	aaatatcagcctcAGGAGCgtcctcccacttcttcctcttctgccttttactAGGAGCtt	
	assembly 11	GCTCCTgaggctgatatttcagatttccccaaggctatatgtgtgaaggaggaatccatt	
	assembly 12	taaactcctgctgggtccataatggattcctccttcacac	
	assembly 13	tggacccagcaggagtttatcagaactgggtttcagatcacgaggctaaccaaggcttg	
	assembly 14	tgctcctgattggcaccctcagactcAGGGGCgtgGGGTGCcaagccttggttagcctcg	
	assembly 15	agggtgccaatcaggagcagcaagtccccacatcttcctctaacttctacaacaagagcc	
	assembly 16	ggagagatgttgggaactccttcgataatggctcttgttgtagaagttagag	
	assembly 17	ggagttcccaacatctctccGCGCCAtttgaattgggagagccctctagcagacgtctt	
	assembly 18	gaatctggatcggaatccggcactgtagcaatgtcAGGGGCaagacgtctgctagagggc	
	flanking_r	agcagaattctgtgttggaatgacatgaaacactgctaaagaatctggatcggaatccgg	

Table 3.2 List of primers used in this study.

CHAPTER 4

FUTURE DIRECTIONS

HIPK2 regulation of VegT and its functional relevance in the TCF transcriptional switch

Our model (Figure 3. 7) proposes HIPK2 promotes VegT phosphorylation. This is supported by the size shift of VegT in the presence of HIPK2 (Figure 3.5). This size shift also correlates with VegT destabilization, even in the presence of TCF3 (Figure 3.5), suggesting that HIPK2 disrupts the TCF3-VegT complex. These data suggest HIPK2 regulation of VegT might play an important role in promoting the TCF transcriptional switch (Figure 3.7). To further investigate this model, I propose the following experiments and discuss possible results below.

The VegT mutant with ten putative HIPK2 phosphorylation sites substituted was no longer shifted and destabilized by HIPK2 (Figure 3.5C). Based on this, it is reasonable to assume HIPK2 directly phosphorylates VegT. To formally test this, an in vitro phosphatase assay could be conducted using immuno-precipitated VegT from cells co-expressing HIPK2WT or HIPK2 kinase dead mutant.

HIPK2 is a promiscuous kinase with a poorly-defined phosphorylation site sequence. However, examination of previously identified phosphorylation sites of HIPK2 substrates indicates that serine or threonine residues followed by a proline tend to be phosphorylated (D'Orazi et al., 2002; Hikasa and Sokol, 2011; Hofmann et al., 2002;

Kim et al., 2006). The aforementioned VegT mutant was generated by using these loose criteria to identify potential VegT phosphorylation sites. The VegT mutant resists HIPK2 regulation (Figure 3.5C), implying a subset of the 10 sites might be target sites of HIPK2. To identify the *bona fide* phosphorylation sites, tandem mass spectrometry analysis could be conducted with purified phosphorylated VegT (Dephoure et al., 2013; Gingras et al., 2007). False positive or non-specific results remain as a key concern of MS/MS (Dephoure et al., 2013), thus it would be important to verify the MS/MS identified sites with functional experiments.

My data suggests HIPK2 phosphorylation does not affect VegT chromatin binding (Figure 3.6A) and VegT participates in target gene activation after TCF3 is replaced by TCF1 (Figure 3.6 C and D). It has been reported that overexpressing VegT and β-catenin substantially increases *Siamois* reporter activation in animal caps, where endogenous VegT or Wnt/β-catenin are absent (Cao et al., 2007). This data indicates that the activating role of VegT is more evident with this assay. My working model predicts that unphosphorylated VegT interacts with TCF3 on chromatin to repress *Siamois*. Thus, a mutant VegT resistant to HIPK2 phosphorylation is predicted to activate *Siamois* reporter less well than WT VegT. If true, this suggests that HIPK2 phosphorylation switches VegT from repressing to activating *Siamois.*

Mapping interaction domains on TCF3 and TCF4 responsible for VegT stabilization and interaction

In HEK293T cells, VegT is stabilized by TCF3 and TCF4, but not TCF1 or LEF1 (Figure 3.4A). My data suggest this is due to specific interactions between VegT and TCF3/TCF4 (Figure 3.4B&C). Consistent with this, CoIP experiment demonstrated that

VegT interacted with TCF3 but not TCF1 in *Xenopus* embryos (Figure 3.4D). These data indicate VegT may bind to certain unique regions in TCF3 and TCF4.

Sequence alignment of TCF1, LEF1, TCF3 and TCF4 uncovers three common regions shared by TCF3 and TCF4, but absent in TCF1 and LEF1 (Figure 4.1). Among the three candidate domains, region A has not been explored before. It partially overlaps with the HIPK2 phosphorylation sites on TCF3 and TCF4, raising the possibility that phosphorylation may affect the conformation of region A, thereby influencing their interaction with VegT. LVPQ and SXXSS motifs (Figure 4.1; region B) have been implicated in the repressive function of TCF3 and TCF4A isoform (Liu et al., 2005), which corresponds to the TCF4 expressed in the stabilization and CoIP experiments (Figure 3.4 A and C). Region C, corresponding to C terminal end, has not been characterized before either. A truncated fragment of TCF3 containing the entire C-terminal region downstream of HMG has been reported to bind with T-box domain of VegT in vitro (Cao et al., 2007). In agreement with this, preliminary data indicates a TCF3 with truncated C terminus (TCF3ΔC; Liu et al., 2005) exhibited slightly less VegT-stabilizing effect than WT TCF3 (Figure 4.2). Although modest, this effect has been observed twice. On the other hand, a TCF3 with region B altered (TCF3ΔL-SA with LVPQ motif deleted and SXXSS motif mutated) (Liu et al., 2005) was able to stabilize VegT to normal levels (Figure 4.2). The expression of two mutants (TCF3ΔC and TCF3ΔL-SA) was driven by identical heterologous promoter, indicating their different VegT-stabilizing activities are due to the difference of translation products. This preliminary data implies that the C terminus or region C is more linked to VegT interaction, or region B inhibits TCF3-VegT interaction somehow. Expression levels of

these TCF3 constructs have to be checked to better interpret these results. A region A-deleted TCF3 mutant could also be tested in this assay. Double or triple mutant could be considered if mutating individual region was not sufficient to observed a dramatic effect. Then, the mutants with least VegT stabilizing ability will be tested in CoIP experiments in *Xenopus* embryos to check if they lose binding to VegT in vivo.

Functional relevance of TCF3 and VegT interaction

Because of the dual role of VegT, it is difficult to tear apart the specific effect of each role using simple loss of function experiments. Once the interaction domain is mapped out, the interaction mutant of TCF3 will be useful to specifically address if TCF3 and VegT interaction is critical for *Siamois* repression and whether the VegT binding domain is needed for TCF3 repressive function. *Siamois* expression is elevated in TCF3 depleted embryos due to derepression (Houston et al., 2002) A TCF3 rescue experiment using WT TCF3 and VegT-interacting mutant TCF3 will be conducted to compare which form has stronger ability to rescue derepressed *Siamois.* If the mutant is defective in suppressing *Siamois,* it suggests interacting with VegT is necessary for TCF3 repression of *Siamois*.

Does VegT facilitate TCF3 recruitment on chromatin

My thesis began with the goal of understanding vertebrate TCF target selection mechanism; however this question is not directly addressed in my work. My findings suggest functionally VegT interacts with TCF3 to cooperatively suppress *Siamois*. At the mechanistic level, it could be because that VegT and TCF3 bind to DNA in a cooperative fashion.

It is well established that Hox proteins require cooperative DNA binding cofactors to achieve specific gene regulation (Mann et al., 2009). Using an electrophoretic mobility shift assay (EMSA), Gebelein and colleagues nicely demonstrated Ultrabithorax (Ubx), Extradenticle (Exd) and Homothorax (Hth) trimers synergistically enhanced binding to a probe containing recognition sites of each factor, whereas Ubx alone or Exd/Hth heterodimer binding to the same probe is barely detectable (Gebelein et al., 2002). To test if VegT and TCF3 bind DNA cooperatively, I tried similar EMSAs using recombinant TCF3 and VegT proteins and synthesized DNA probes containing a TCF binding site and a T-box site linked by a 12bp spacer. Unfortunately, cooperative DNA binding was not seen in vitro. In the case of Ubx/Exd/Hht, the binding sites of each factor are closely linked, with 1bp between Ubx and Exd sites and 7bp between Exd and Hth (Gebelein et al., 2002). In contrast, in the case of TCF and T-box sites in *Siamois* W-CRM, the closest pair is 72bp apart, possibly indicating that an unknown mechanism is needed to facilitate the cooperative binding of TCF3 and VegT in vivo.

To study if VegT and TCF3 bind DNA cooperatively in vivo, ChIP assays combined with morpholino depletion could be employed. This experiment requires a ChIP quality antibody against endogenous TCF3, which is currently unavailable. If such an antisera were available, the *Vent2* and *Siamois* W-CRMs could be tested. TCF3 chromatin binding is expected to be less in VegT morphants than controls. This would support that VegT is needed for full recruitment of TCF3.

A more exquisite but more difficult experiment to further elucidate the mechanism would be reporter ChIP. Reporter plasmid undergoes chromatinization after

injected into *Xenopus* embryos (Roche et al., 2006). This offers a system with editable DNA sequences. WT, T-box site mutant and TCF site mutant reporter plasmids will be compared for TCF3 binding level. If TCF3 binding is reduced in T-box site mutant, that would argue VegT positively influences TCF3 recruitment. To obtain specific signal in reporter ChIP, non-target region within the reporter construct has to be included as a negative control. In addition, injected DNA amount has to be optimized to ensure balanced signal to noise ratio.

Are TCF and Tbx site clusters a general paradigm or unique to early embryogenesis of *Xenopus*

A T-box site is also seen near the functional TCF site in the *Vent2* W-CRM (Figure 3.3C and Table 3.1; Hikasa et al., 2010). Also, VegT binds to the W-CRM locus of *Vent2* in vivo (Figure 3.3C). To determine the function of the T-box site, WT and T-box site mutant *Vent2* reporter will be assessed in *Xenopus* embryos.

Cdx4/Xcad3 plays important roles in posterior development of *Xenopus* (Isaacs et al., 1998). It is directly repressed by TCF3 (Haremaki et al., 2003; Ro and Dawid, 2011), and regulated by Wnt/HIPK2 as well (Hikasa et al., 2010). An intronic *cis*-regulatory element controlling posterior neural specific expression of *cdx4* contains multiple functional TCF binding sites as well as three T-box sites (Haremaki et al., 2003). It would be interesting to learn if the T-box sites influence the *cdx4* W-CRM regulation. Note that the maternal pool of VegT quickly degrades after MBT, whereas the expression level of zygotic T-box proteins, e.g. xBrachyury, Eomesodermin and zygotic VegT isoform, increases fast (Yanai et al., 2011). Furthermore, the expression pattern of these zygotic T-box proteins largely overlaps with cdx4 (Keenan et al., 2006; Tada and Smith,

2001), raising the possibility that these T-box proteins are involved in *cdx4* W-CRM regulation instead of maternal VegT.

Beyond body patterning during *Xenopus* embryogenesis, TCFs and T-box (Tbx) family proteins are both essential for cardiogenesis in mammals (Archbold et al., 2012; Greulich et al., 2011; Naiche et al., 2005). Wnt signaling is required for early cardiac specification of precursor cells and plays important roles in establishing second heart field (SHF) as well as subsequent differentiation of heart tissue (Archbold et al., 2012). Evidence indicates a number of essential genes involved in heart development are directly controlled by Wnt/TCF, including *tinmann* (fly)/*Nkx2.5* (Klaus et al., 2012; Archbold et al., 2012), *Pitx2* (Ai et al., 2007), *Islet1* (Klaus et al., 2012; Lu et al., 2014) In developing mammalian heart, 6 of the 17 family members (Tbx1, Tbx2, Tbx3, Tbx5, Tbx18 and Tbx20) are expressed and required in a combinatorial fashion in different cardiac progenitor pools as well as in different differentiated compartments (Greulich et al., 2011). Accumulating evidence implicates interplay between Wnt signaling and Tbx activity in heart development. For example, a recent report demonstrated Tbx20 acts upstream of Wnt signaling to regulate endocardial cushion formation and valve remodeling during mouse cardiogenesis (Cai et al., 2013).

In addition to their hierarchal relationship in some contexts, combinatory regulation by TCF and Tbx has also been implicated before. For instance, *Pitx2* has been suggested as a common target regulated by TCF and Tbx1 (Ai et al., 2007; Nowotschin et al., 2006). Furthermore, *Connexin43* (*Cx43)* encodes an important gap junctional protein essential for heart function. It has been shown Tbx2, Tbx3 and Tbx18 suppress *Cx43* promoter activity in a rat heart derived cell line (Boogerd et al., 2008) and guinea pig

heart (Kapoor et al., 2011). Interestingly, *Cx43* has also been suggested to be a direct target activated by TCF/β-cat (van der Heyden et al., 1998). Both T-box sites and TCF binding sites were identified in the *Cx43* promoter region (van der Heyden et al., 1998; Boogerd et al., 2008), serving as a good candidate to investigate cooperative regulation by TCF and Tbx in heart development.

A common feature of these TCF and Tbx co-regulated CRMs is that there lacks discernable organization of TCF binding sites and T-box sites. This actually fits with the Transcription Factor collective model, where the combinatory binding by multiple TFs operates with very flexible arrangement of binding sites and does not require specific binding motif orientation or spacing. A TF collective involving TCF and a Tbx factor has been demonstrated in cardiac specification in fly embryos (Junion et al., 2012).

My work has investigated the dual role of VegT in regulating *Siamois* transcription in *Xenopus,* and provided mechanistic insights on how VegT facilitates operation of the vertebrate TCF transcriptional switch. It further illustrates the complexities of Wnt/β-catenin-dependent gene regulation and sheds light on the general requirement of cooperation with context-specific TFs by TCFs. As a complementary approach to genomic studies, our strategy offers a thorough understanding of the action of TCF-involved TF collectives and improves our understanding how TCFs and other TFs "read" the genome to identify WREs.

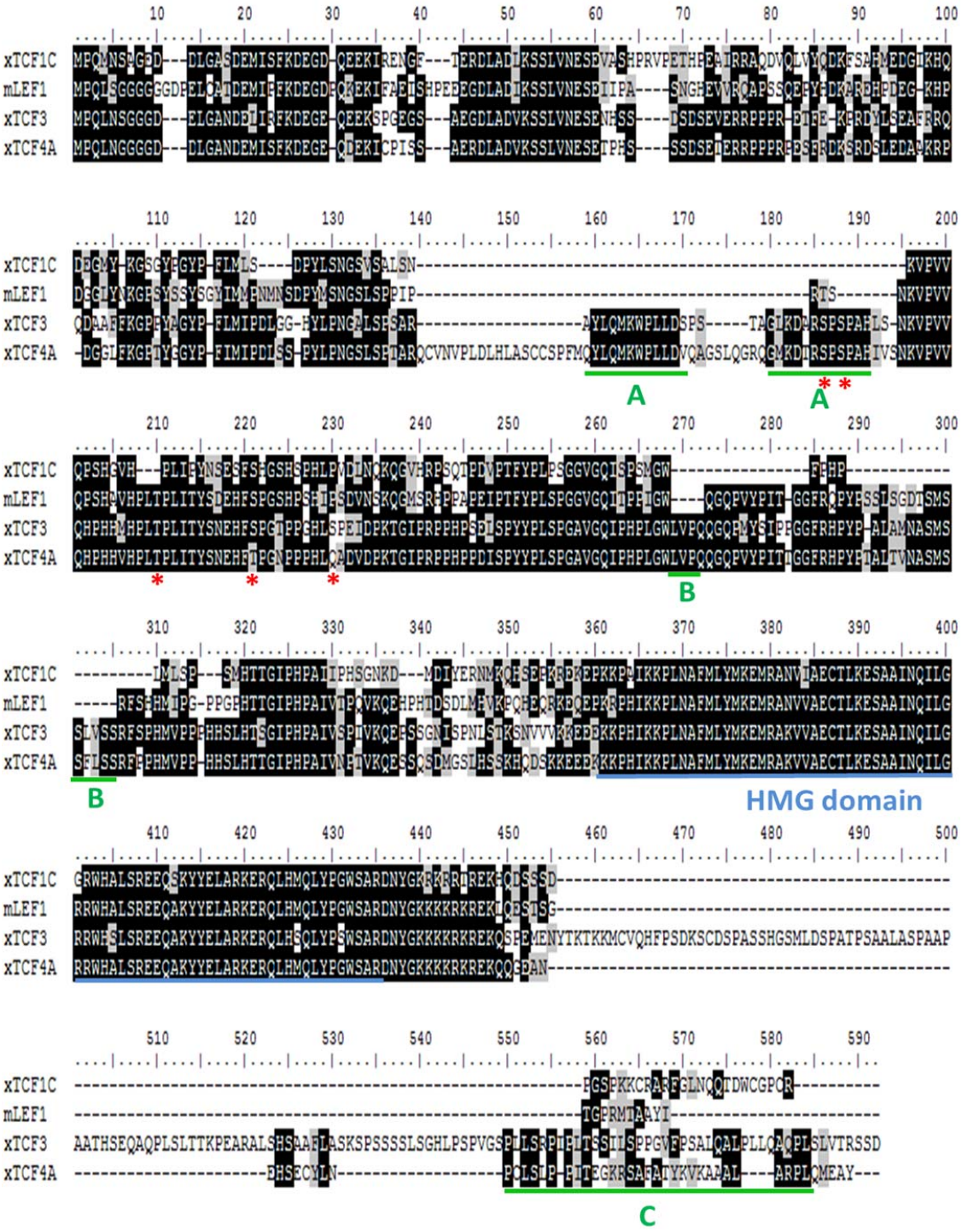

Figure 4.1 Candidate VegT-interacting domains of TCF3 and TCF4.

Sequence alignment of xTCF1C (AAO23663.1), mLEF1 (NP_034833.2), xTCF3 (NP_001080938.1), and xTCF4A (NP_001083866.1). Identical and similar residues are shaded in black and grey respectively. Three unique domains/motifs only present in

TCF3 and TCF4A, marked with green lines and letters, are candidate VegT interaction domains. HIPK2 phosphorylation sites shared by TCF3, TCF4 and LEF1 are indicated by red asterisk (Hikasa et al., 2010).

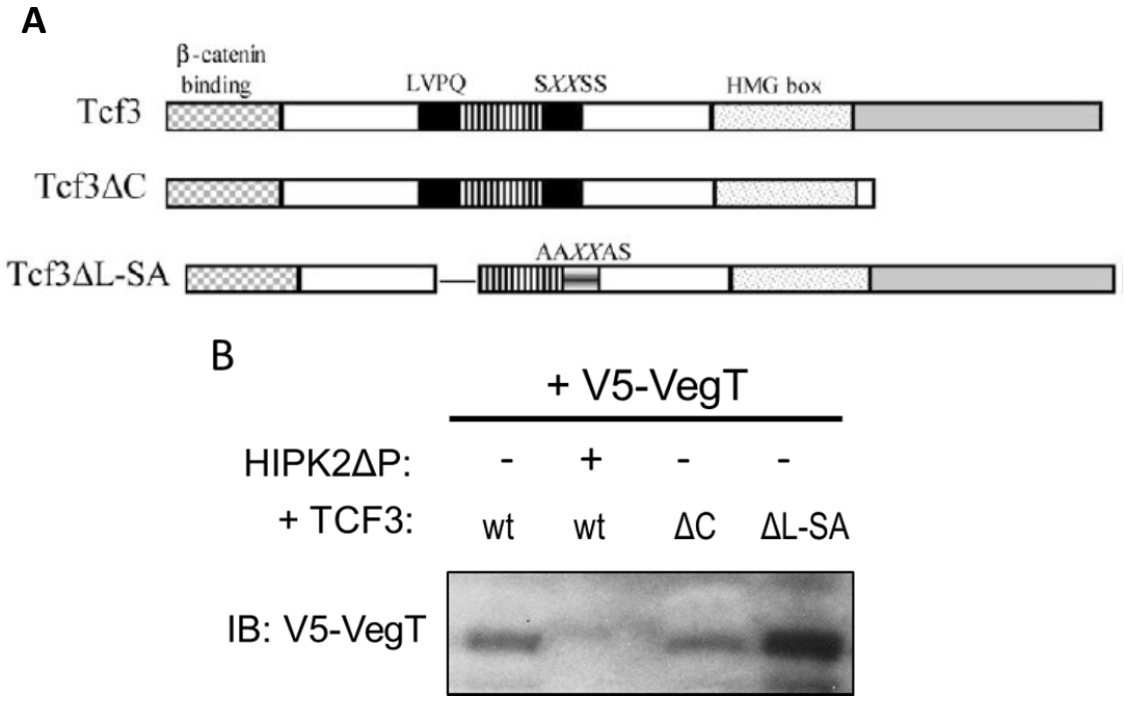

Figure 4.2 VegT-stabilizing effects of different TCF3 mutants in HEK293T cells.

(A) Cartoon depicting TCF3ΔC and TCF3ΔL-SA (adapted from Liu et al.,2005).
(B) Western blot showing VegT level in the presence of TCF3 WT or TCFΔC or TCF3ΔL-SA. HEK293T cells are transfected with constructs expressing V5-VegT, WT or indicated mutant forms of TCF3. TCF3ΔC displays slightly less VegT-stabilizing activity than WT, whereas TCF3ΔL-SA shows stronger VegT-stabilizing activity. Expression level of TCF3 is lacking in this experiment.

REFERENCES

Agius, E., Oelgeschläger, M., Wessely, O., Kemp, C., and De Robertis, E.M. (2000). Endodermal Nodal-related signals and mesoderm induction in Xenopus. Development *127*, 1173–1183.

Ai, D., Wang, J., Amen, M., Lu, M.-F., Amendt, B.A., and Martin, J.F. (2007). Nuclear factor 1 and T-cell factor/LEF recognition elements regulate Pitx2 transcription in pituitary development. Mol. Cell. Biol. *27*, 5765–5775.

Van Amerongen, R., and Nusse, R. (2009). Towards an integrated view of Wnt signaling in development. Development *136*, 3205–3214.

Van Amerongen, R., Nawijn, M., Franca-Koh, J., Zevenhoven, J., van der Gulden, H., Jonkers, J., and Berns, A. (2005). Frat is dispensable for canonical Wnt signaling in mammals. Genes Dev. *19*, 425–430.

Angus-Hill, M.L., Elbert, K.M., Hidalgo, J., and Capecchi, M.R. (2011). T-cell factor 4 functions as a tumor suppressor whose disruption modulates colon cell proliferation and tumorigenesis. Proc. Natl. Acad. Sci. U. S. A. *108*, 4914–4919.

Arce, L., Yokoyama, N.N., and Waterman, M.L. (2006). Diversity of LEF/TCF action in development and disease. Oncogene *25*, 7492–7504.

Arce, L., Pate, K.T., and Waterman, M.L. (2009). Groucho binds two conserved regions of LEF-1 for HDAC-dependent repression. BMC Cancer *9*, 159.

Archbold, H.C., Yang, Y.X., Chen, L., and Cadigan, K.M. (2012). How do they do Wnt they do?: regulation of transcription by the Wnt/β-catenin pathway. Acta Physiol. (Oxf). *204*, 74–109.

Archbold, H.C., Broussard C., Chang M.V., and Cadigan, K.M. (2014). Bipartite Recognition of DNA by TCF/Pangolin is Remarkably Flexible and Contributes to Transcriptional Responsiveness and Tissue Specificity of Wingless Signaling. PloS Genetics, in revision.

Atcha, F.A., Syed, A., Wu, B., Hoverter, N.P., Yokoyama, N.N., Ting, J.-H.T., Munguia, J.E., Mangalam, H.J., Marsh, J.L., and Waterman, M.L. (2007). A unique DNA binding domain converts T-cell factors into strong Wnt effectors. Mol. Cell. Biol. *27*, 8352–8363.

Atlasi, Y., Noori, R., Gaspar, C., Franken, P., Sacchetti, A., Rafati, H., Mahmoudi, T., Decraene, C., Calin, G.A., Merrill, B.J., et al. (2013). Wnt Signaling Regulates the

Lineage Differentiation Potential of Mouse Embryonic Stem Cells through Tcf3 Down-Regulation. PLoS Genet. *9*.

Badis, G., Berger, M.F., Philippakis, A.A., Talukder, S., Gehrke, A.R., Jaeger, S.A., Chan, E.T., Metzler, G., Vedenko, A., Chen, X., et al. (2009). Diversity and complexity in DNA recognition by transcription factors. Science *324*, 1720–1723.

Bae, S., Reid, C.D., and Kessler, D.S. (2011). Siamois and Twin are redundant and essential in formation of the Spemann organizer. Dev. Biol. *352*, 367–381.

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

Barolo, S. (2006). Transgenic Wnt/TCF pathway reporters: all you need is Lef? Oncogene *25*, 7505–7511.

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

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

Béland, M., Pilon, N., Houle, M., Oh, K., Sylvestre, J.-R., Prinos, P., and Lohnes, D. (2004). Cdx1 autoregulation is governed by a novel Cdx1-LEF1 transcription complex. Mol. Cell. Biol. *24*, 5028–5038.

Bhambhani, C., Ravindranath, A.J., Mentink, R.A., Chang, M. V, Betist, M.C., Yang, Y.X., Koushika, S.P., Korswagen, H.C., and Cadigan, K.M. (2014). Distinct DNA Binding Sites Contribute to the TCF Transcriptional Switch in C. elegans and Drosophila. PLoS Genet. *10*, e1004133.

Bhambhani, C. and Cadigan, K.M. (2014). Finding a needle in a genomic haystack: genome-wide approaches to identify Wnt/TCF transcriptional targets. In Wnt Signaling in Development and Disease: Molecular Mechanisms and Biological Functions, S.P. Hoppler and R.T. Moon, ed. (USA: Wiley)

Blahnik, K.R., Dou, L., O'Geen, H., McPhillips, T., Xu, X., Cao, A.R., Iyengar, S., Nicolet, C.M., Ludäscher, B., Korf, I., et al. (2010). Sole-Search: an integrated analysis program for peak detection and functional annotation using ChIP-seq data. Nucleic Acids Res. *38*, e13.

Blair, S.S. (1992). shaggy (zeste-white 3) and the formation of supernumerary bristle precursors in the developing wing blade of Drosophila. Dev. Biol. *152*, 263–278.

Blauwkamp, T.A., Chang, M. V, and Cadigan, K.M. (2008). Novel TCF-binding sites specify transcriptional repression by Wnt signalling. EMBO J. *27*, 1436–1446.

Blythe, S. a, Cha, S.-W., Tadjuidje, E., Heasman, J., and Klein, P.S. (2010). beta-Catenin primes organizer gene expression by recruiting a histone H3 arginine 8 methyltransferase, Prmt2. Dev. Cell *19*, 220–231.

Blythe, S.A., Reid, C.D., Kessler, D.S., and Klein, P.S. (2009). Chromatin immunoprecipitation in early Xenopus laevis embryos. Dev Dyn *238*, 1422–1432.

Boogerd, K.J., Wong, L.Y.E., Christoffels, V.M., Klarenbeek, M., Ruijter, J.M., Moorman, A.F.M., and Barnett, P. (2008). Msx1 and Msx2 are functional interacting partners of T-box factors in the regulation of Connexin43. Cardiovasc. Res. *78*, 485–493.

Bottomly, D., Kyler, S.L., McWeeney, S.K., and Yochum, G.S. (2010). Identification of {beta}-catenin binding regions in colon cancer cells using ChIP-Seq. Nucleic Acids Res. *38*, 5735–5745.

Brandt, T., Petrovich, M., Joerger, A.C., and Veprintsev, D.B. (2009). Conservation of DNA-binding specificity and oligomerisation properties within the p53 family. BMC Genomics *10*, 628.

Brannon, M., and Kimelman, D. (1996). Activation of Siamois by the Wnt pathway. Dev. Biol. *180*, 344–347.

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

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

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

Brunner, E., Peter, O., Schweizer, L., and Basler, K. (1997). pangolin encodes a Lef-1 homologue that acts downstream of Armadillo to transduce the Wingless signal in Drosophila. Nature *385*, 829–833.

Cadigan, K.M. (2012). TCFs and Wnt/β-catenin signaling: more than one way to throw the switch. (Elsevier Inc.).

Cadigan, K.M., and Peifer, M. (2009). Wnt signaling from development to disease: insights from model systems. Cold Spring Harb. Perspect. Biol. *1*, a002881.

Cadigan, K.M., and Waterman, M.L. (2012a). TCF/LEFs and Wnt signaling in the nucleus. Cold Spring Harb. Perspect. Biol. *4*, 1–22.

Cadigan, K.M., and Waterman, M.L. (2012b). TCF/LEFs and Wnt Signaling in the Nucleus. Cold Spring Harb. Perspect. Biol. 1–22.

Cadigan, K.M., Fish, M.P., Rulifson, E.J., and Nusse, R. (1998). Wingless repression of Drosophila frizzled 2 expression SHAPES THE wingless morphogen gradient in the wing. Cell *93*, 767–777.

Cai, X., Zhang, W., Hu, J., Zhang, L., Sultana, N., Wu, B., Cai, W., Zhou, B., and Cai, C.-L. (2013). Tbx20 acts upstream of Wnt signaling to regulate endocardial cushion formation and valve remodeling during mouse cardiogenesis. Development *140*, 3176–3187.

Cao, Y., Siegel, D., Donow, C., Knöchel, S., Yuan, L., and Knöchel, W. (2007). POU-V factors antagonize maternal VegT activity and beta-Catenin signaling in Xenopus embryos. EMBO J. *26*, 2942–2954.

Carnac, G., Kodjabachian, L., Gurdon, J.B., and Lemaire, P. (1996). The homeobox gene Siamois is a target of the Wnt dorsalisation pathway and triggers organiser activity in the absence of mesoderm. Development *122*, 3055–3065.

Carrera, I., Janody, F., Leeds, N., Duveau, F., and Treisman, J.E. (2008). Pygopus activates Wingless target gene transcription through the mediator complex subunits Med12 and Med13. Proc. Natl. Acad. Sci. U. S. A. *105*, 6644–6649.

Casey, E.S., Tada, M., Fairclough, L., Wylie, C.C., Heasman, J., and Smith, J.C. (1999). Bix4 is activated directly by VegT and mediates endoderm formation in Xenopus development. Development *126*, 4193–4200.

Cavallo, R.A., Cox, R.T., Moline, M.M., Roose, J., Polevoy, G.A., Clevers, H., Peifer, M., and Bejsovec, A. (1998). Drosophila Tcf and Groucho interact to repress Wingless signalling activity. Nature *395*, 604–608.

Cha, S.-W., Tadjuidje, E., Tao, Q., Wylie, C., and Heasman, J. (2008). Wnt5a and Wnt11 interact in a maternal Dkk1-regulated fashion to activate both canonical and non-canonical signaling in Xenopus axis formation. Development *135*, 3719–3729.

Chang, J.L., Chang, M. V., Barolo, S., and Cadigan, K.M. (2008a). Regulation of the feedback antagonist naked cuticle by Wingless signaling. Dev. Biol. *321*, 446–454.

Chang, M. V, Chang, J.L., Gangopadhyay, A., Shearer, A., and Cadigan, K.M. (2008b). Activation of wingless targets requires bipartite recognition of DNA by TCF. Curr. Biol. *18*, 1877–1881.

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

Cho, K.W., Blumberg, B., Steinbeisser, H., and De Robertis, E.M. (1991). Molecular nature of Spemann's organizer: the role of the Xenopus homeobox gene goosecoid. Cell *67*, 1111–1120.

Christian, J.L., and Moon, R.T. (1993). Interactions between Xwnt-8 and Spemann organizer signaling pathways generate dorsoventral pattern in the embryonic mesoderm of Xenopus. Genes Dev. *7*, 13–28.

Clevers, H. (2006). Wnt/beta-catenin signaling in development and disease. Cell *127*, 469–480.

Clevers, H., and Nusse, R. (2012). Wnt/β-Catenin Signaling and Disease. Cell *149*, 1192–1205.

Cole, M.F., Johnstone, S.E., Newman, J.J., Kagey, M.H., and Young, R.A. (2008). Tcf3 is an integral component of the core regulatory circuitry of embryonic stem cells. Genes Dev. *22*, 746–755.

Collart, C., Verschueren, K., Rana, A., Smith, J.C., and Huylebroeck, D. (2005). The novel Smad-interacting protein Smicl regulates Chordin expression in the Xenopus embryo. Development *132*, 4575–4586.

Conlon, F.L., Fairclough, L., Price, B.M., Casey, E.S., and Smith, J.C. (2001). Determinants of T box protein specificity. Development *128*, 3749–3758.

Couso, J.P., Bishop, S.A., and Martinez Arias, A. (1994). The wingless signalling pathway and the patterning of the wing margin in Drosophila. Development *120*, 621–636.

D'Orazi, G., Cecchinelli, B., Bruno, T., Manni, I., Higashimoto, Y., Saito, S., Gostissa, M., Coen, S., Marchetti, A., Del Sal, G., et al. (2002). Homeodomain-interacting protein kinase-2 phosphorylates p53 at Ser 46 and mediates apoptosis. Nat. Cell Biol. *4*, 11–19.

D'Orazi, G., Rinaldo, C., and Soddu, S. (2012). Updates on HIPK2: a resourceful oncosuppressor for clearing cancer. J. Exp. Clin. Cancer Res. *31*, 63.

Daniels, D.L., and Weis, W.I. (2005). Beta-catenin directly displaces Groucho/TLE repressors from Tcf/Lef in Wnt-mediated transcription activation. Nat. Struct. Mol. Biol. *12*, 364–371.

Dephoure, N., Gould, K.L., Gygi, S.P., and Kellogg, D.R. (2013). Mapping and analysis of phosphorylation sites: a quick guide for cell biologists. Mol. Biol. Cell *24*, 535–542.

Dietzl, G., Chen, D., Schnorrer, F., Su, K.-C., Barinova, Y., Fellner, M., Gasser, B., Kinsey, K., Oppel, S., Scheiblauer, S., et al. (2007). A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. Nature *448*, 151–156.

Dominguez, I., Itoh, K., and Sokol, S.Y. (1995). Role of glycogen synthase kinase 3 beta as a negative regulator of dorsoventral axis formation in Xenopus embryos. Proc. Natl. Acad. Sci. U. S. A. *92*, 8498–8502.

Dorsky, R.I., Itoh, M., Moon, R.T., and Chitnis, A. (2003). Two tcf3 genes cooperate to pattern the zebrafish brain. Development *130*, 1937–1947.

Elkouby, Y.M., Elias, S., Casey, E.S., Blythe, S.A., Tsabar, N., Klein, P.S., Root, H., Liu, K.J., and Frank, D. (2010). Mesodermal Wnt signaling organizes the neural plate via Meis3. Development *137*, 1531–1541.

Fan, M.J., and Sokol, S.Y. (1997). A role for Siamois in Spemann organizer formation. Development *124*, 2581–2589.

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

Fang, M., Li, J., Blauwkamp, T., Bhambhani, C., Campbell, N., and Cadigan, K.M. (2006). C-terminal-binding protein directly activates and represses Wnt transcriptional targets in Drosophila. EMBO J. *25*, 2735–2745.

Frietze, S., Wang, R., Yao, L., Tak, Y., Ye, Z., Gaddis, M., Witt, H., Farnham, P.J., and Jin, V.X. (2012). Cell type-specific binding patterns reveal that TCF7L2 can be tethered to the genome by association with GATA3. Genome Biol. *13*, R52.

Fukuda, M., Takahashi, S., Haramoto, Y., Onuma, Y., Kim, Y.-J., Yeo, C.-Y., Ishiura, S., and Asashima, M. (2010). Zygotic VegT is required for Xenopus paraxial mesoderm formation and is regulated by Nodal signaling and Eomesodermin. Int. J. Dev. Biol. *54*, 81–92.

Galceran, J., Fariñas, I., Depew, M.J., Clevers, H., and Grosschedl, R. (1999). Wnt3a-/--like phenotype and limb deficiency in Lef1(-/-)Tcf1(-/-) mice. Genes Dev. *13*, 709–717.

Garnett, A.T., Han, T.M., Gilchrist, M.J., Smith, J.C., Eisen, M.B., Wardle, F.C., and Amacher, S.L. (2009). Identification of direct T-box target genes in the developing zebrafish mesoderm. Development *136*, 749–760.

Gebelein, B., Culi, J., Ryoo, H.D., Zhang, W., and Mann, R.S. (2002). Specificity of Distalless repression and limb primordia development by abdominal Hox proteins. Dev. Cell *3*, 487–498.

Gentsch, G.E., Owens, N.D.L., Martin, S.R., Piccinelli, P., Faial, T., Trotter, M.W.B., Gilchrist, M.J., and Smith, J.C. (2013). In Vivo T-Box Transcription Factor Profiling Reveals Joint Regulation of Embryonic Neuromesodermal Bipotency. Cell Rep. *4*, 1185–1196.

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

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

Gingras, A.-C., Gstaiger, M., Raught, B., and Aebersold, R. (2007). Analysis of protein complexes using mass spectrometry. Nat. Rev. Mol. Cell Biol. *8*, 645–654.

Glinka, A., Wu, W., Onichtchouk, D., Blumenstock, C., and Niehrs, C. (1997). Head induction by simultaneous repression of Bmp and Wnt signalling in Xenopus. Nature *389*, 517–519.

Glinka, A., Wu, W., Delius, H., Monaghan, A.P., Blumenstock, C., and Niehrs, C. (1998). Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. Nature *391*, 357–362.

González, A., Manosalva, I., Liu, T., and Kageyama, R. (2013). Control of Hes7 expression by Tbx6, the Wnt pathway and the chemical Gsk3 inhibitor LiCl in the mouse segmentation clock. PLoS One *8*, e53323.

Gradl, D., König, A., and Wedlich, D. (2002). Functional diversity of Xenopus lymphoid enhancer factor/T-cell factor transcription factors relies on combinations of activating and repressing elements. J. Biol. Chem. *277*, 14159–14171.

Greulich, F., Rudat, C., and Kispert, A. (2011). Mechanisms of T-box gene function in the developing heart. Cardiovasc. Res. *91*, 212–222.

Grewal, S.I., and Moazed, D. (2003). Heterochromatin and epigenetic control of gene expression. Science (80-.). *301*, 798.

Guger, K.A., and Gumbiner, B.M. (1995). beta-Catenin has Wnt-like activity and mimics the Nieuwkoop signaling center in Xenopus dorsal-ventral patterning. Dev. Biol. *172*, 115–125.

Hallikas, O., Palin, K., Sinjushina, N., Rautiainen, R., Partanen, J., Ukkonen, E., and Taipale, J. (2006). Genome-wide prediction of mammalian enhancers based on analysis of transcription-factor binding affinity. Cell *124*, 47–59.

Haremaki, T., Tanaka, Y., Hongo, I., Yuge, M., and Okamoto, H. (2003). Integration of multiple signal transducing pathways on Fgf response elements of the Xenopus caudal homologue Xcad3. Development *130*, 4907–4917.

Harland, R., and Gerhart, J. (1997). Formation and function of Spemann's organizer. Annu. Rev. Cell Dev. Biol. *13*, 611–667.

Hatzis, P., van der Flier, L.G., van Driel, M.A., Guryev, V., Nielsen, F., Denissov, S., Nijman, I.J., Koster, J., Santo, E.E., Welboren, W., et al. (2008). Genome-wide pattern of TCF7L2/TCF4 chromatin occupancy in colorectal cancer cells. Mol. Cell. Biol. *28*, 2732–2744.

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

Heasman, J., Crawford, A., Goldstone, K., Garner-Hamrick, P., Gumbiner, B., McCrea, P., Kintner, C., Noro, C.Y., and Wylie, C. (1994). Overexpression of cadherins and underexpression of β-catenin inhibit dorsal mesoderm induction in early xenopus embryos. Cell *79*, 791–803.

Heasman, J., Wessely, O., Langland, R., Craig, E.J., and Kessler, D.S. (2001). Vegetal localization of maternal mRNAs is disrupted by VegT depletion. Dev. Biol. *240*, 377–386.

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

Van der Heyden, M.A., Rook, M.B., Hermans, M.M., Rijksen, G., Boonstra, J., Defize, L.H., and Destrée, O.H. (1998). Identification of connexin43 as a functional target for Wnt signalling. J. Cell Sci. *111 (Pt 1*, 1741–1749.

Hikasa, H., and Sokol, S.Y. (2011). Phosphorylation of TCF proteins by homeodomain-interacting protein kinase 2. J. Biol. Chem. *286*, 12093–12100.

Hikasa, H., and Sokol, S.Y. (2013). Wnt signaling in vertebrate axis specification. Cold Spring Harb. Perspect. Biol. *5*, a007955.

Hikasa, H., Ezan, J., Itoh, K., Li, X., Klymkowsky, M.W., and Sokol, S.Y. (2010). Regulation of TCF3 by Wnt-dependent phosphorylation during vertebrate axis specification. Dev. Cell *19*, 521–532.

Hilton, E., Rex, M., and Old, R. (2003). VegT activation of the early zygotic gene Xnr5 requires lifting of Tcf-mediated repression in the Xenopus blastula. Mech. Dev. *120*, 1127–1138.

Hofmann, M., Schuster-Gossler, K., Watabe-Rudolph, M., Aulehla, A., Herrmann, B.G., and Gossler, A. (2004). WNT signaling, in synergy with T/TBX6, controls Notch signaling by regulating Dll1 expression in the presomitic mesoderm of mouse embryos. Genes Dev. *18*, 2712–2717.

Hofmann, T.G., Möller, A., Sirma, H., Zentgraf, H., Taya, Y., Dröge, W., Will, H., and Schmitz, M.L. (2002). Regulation of p53 activity by its interaction with homeodomain-interacting protein kinase-2. Nat. Cell Biol. *4*, 1–10.

Hoppler, S., and Kavanagh, C.L. (2007). Wnt signalling: variety at the core. J. Cell Sci. *120*, 385–393.

Horb, M.E., and Thomsen, G.H. (1997). A vegetally localized T-box transcription factor in Xenopus eggs specifies mesoderm and endoderm and is essential for embryonic mesoderm formation. Development *124*, 1689–1698.

Houliston, E., and Elinson, R.P. (1991). Evidence for the involvement of microtubules, ER, and kinesin in the cortical rotation of fertilized frog eggs. J. Cell Biol. *114*, 1017–1028.

Houston, D.W. (2012). Cortical rotation and messenger RNA localization in Xenopus axis formation. Wiley Interdiscip. Rev. Dev. Biol. *1*, 371–388.

Houston, D.W., Kofron, M., Resnik, E., Langland, R., Destree, O., Wylie, C., and Heasman, J. (2002). Repression of organizer genes in dorsal and ventral Xenopus cells mediated by maternal XTcf3. Development *129*, 4015–4025.

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

Hoverter, N.P., Ting, J.-H., Sundaresh, S., Waterman, M.L., and Baldi, P. (2012). A WNT/p21 Circuit Directed by the C-Clamp, a Sequence-Specific DNA Binding Domain in TCFs. Mol. Cell. Biol. *32*, 3648–3662.

Howard, L., Rex, M., Clements, D., and Woodland, H.R. (2007). Regulation of the Xenopus Xsox17alpha(1) promoter by co-operating VegT and Sox17 sites. Dev. Biol. *310*, 402–415.

Hu, M.C., and Rosenblum, N.D. (2005). Smad1, beta-catenin and Tcf4 associate in a molecular complex with the Myc promoter in dysplastic renal tissue and cooperate to control Myc transcription. Development *132*, 215–225.

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

Hussein, S.M., Duff, E.K., and Sirard, C. (2003). Smad4 and beta-catenin co-activators functionally interact with lymphoid-enhancing factor to regulate graded expression of Msx2. J. Biol. Chem. *278*, 48805–48814.

Hyde, C.E., and Old, R.W. (2000). Regulation of the early expression of the Xenopus nodal-related 1 gene, Xnr1. Development *127*, 1221–1229.

Isaacs, H. V., Pownall, M.E., and Slack, J.M.W. (1998). Regulation of Hox gene expression and posterior development by the Xenopus caudal homologue Xcad3. EMBO J. *17*, 3413–3427.

Ishibashi, H., Matsumura, N., Hanafusa, H., Matsumoto, K., De Robertis, E.M., and Kuroda, H. (2007). Expression of Siamois and Twin in the blastula Chordin/Noggin signaling center is required for brain formation in Xenopus laevis embryos. Mech. Dev. *125*, 58–66.

Jessen, S., Gu, B., and Dai, X. (2008). Pygopus and the Wnt signaling pathway: A diverse set of connections. BioEssays *30*, 448–456.

Jesuthasan, S., and Stähle, U. (1997). Dynamic microtubules and specification of the zebrafish embryonic axis. Curr. Biol. *7*, 31–42.

Jho, E., Zhang, T., Domon, C., Joo, C.-K., Freund, J.-N., and Costantini, F. (2002). Wnt/beta-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway. Mol. Cell. Biol. *22*, 1172–1183.

Joubin, K., and Stern, C.D. (2001). Formation and maintenance of the organizer among the vertebrates. Int. J. Dev. Biol. *45*, 165–175.

Junion, G., Spivakov, M., Girardot, C., Braun, M., Gustafson, E.H., Birney, E., and Furlong, E.E.M. (2012). A transcription factor collective defines cardiac cell fate and reflects lineage history. Cell *148*, 473–486.

Kao, K.R., and Elinson, R.P. (1988). The entire mesodermal mantle behaves as Spemann's organizer in dorsoanterior enhanced Xenopus laevis embryos. Dev. Biol. *127*, 64–77.

Kapoor, N., Galang, G., Marbán, E., and Cho, H.C. (2011). Transcriptional suppression of connexin43 by TBX18 undermines cell-cell electrical coupling in postnatal cardiomyocytes. J. Biol. Chem. *286*, 14073–14079.

Karaulanov, E., Knöchel, W., and Niehrs, C. (2004). Transcriptional regulation of BMP4 synexpression in transgenic Xenopus. EMBO J. *23*, 844–856.

Keenan, I.D., Sharrard, R.M., and Isaacs, H. V. (2006). FGF signal transduction and the regulation of Cdx gene expression. Dev. Biol. *299*, 478–488.

Khokha, M.K., Yeh, J., Grammer, T.C., and Harland, R.M. (2005). Depletion of three BMP antagonists from Spemann's organizer leads to a catastrophic loss of dorsal structures. Dev. Cell *8*, 401–411.

Kiecker, C., and Niehrs, C. (2001). A morphogen gradient of Wnt/beta-catenin signalling regulates anteroposterior neural patterning in Xenopus. Development *128*, 4189–4201.

Kim, C.H., Oda, T., Itoh, M., Jiang, D., Artinger, K.B., Chandrasekharappa, S.C., Driever, W., and Chitnis, A.B. (2000). Repressor activity of Headless/Tcf3 is essential for vertebrate head formation. Nature *407*, 913–916.

Kim, E.A., Noh, Y.T., Ryu, M.-J., Kim, H.-T., Lee, S.-E., Kim, C.-H., Lee, C., Kim, Y.H., and Choi, C.Y. (2006). Phosphorylation and transactivation of Pax6 by homeodomain-interacting protein kinase 2. J. Biol. Chem. *281*, 7489–7497.

Kim, S.-E., Huang, H., Zhao, M., Zhang, X., Zhang, A., Semonov, M. V, MacDonald, B.T., Zhang, X., Garcia Abreu, J., Peng, L., et al. (2013). Wnt stabilization of β-catenin reveals principles for morphogen receptor-scaffold assemblies. Science *340*, 867–870.

Klaus, A., Muller, M., Schulz, H., Saga, Y., Martin, J.F., and Birchmeier, W. (2012). Wnt/ -catenin and Bmp signals control distinct sets of transcription factors in cardiac progenitor cells. Proc. Natl. Acad. Sci. *109*, 10921–10926.

Klein, P.S., and Melton, D.A. (1996). A molecular mechanism for the effect of lithium on development. Proc. Natl. Acad. Sci. U. S. A. *93*, 8455–8459.

Kofron, M., Demel, T., Xanthos, J., Lohr, J., Sun, B., Sive, H., Osada, S., Wright, C., Wylie, C., and Heasman, J. (1999). Mesoderm induction in Xenopus is a zygotic event regulated by maternal VegT via TGFbeta growth factors. Development *126*, 5759–5770.

Kofron, M., Birsoy, B., Houston, D., Tao, Q., Wylie, C., and Heasman, J. (2007). Wnt11/beta-catenin signaling in both oocytes and early embryos acts through LRP6-mediated regulation of axin. Development *134*, 503–513.

Kratochwil, K., Galceran, J., Tontsch, S., Roth, W., and Grosschedl, R. (2002). FGF4, a direct target of LEF1 and Wnt signaling, can rescue the arrest of tooth organogenesis in Lef1(-/-) mice. Genes Dev. *16*, 3173–3185.

Krupp, J.J., Yaich, L.E., Wessells, R.J., and Bodmer, R. (2005). Identification of genetic loci that interact with cut during Drosophila wing-margin development. Genetics *170*, 1775–1795.

Ku, M., and Melton, D.A. (1993). Xwnt-11: a maternally expressed Xenopus wnt gene. Development *119*, 1161–1173.

Kuroda, H., Wessely, O., and De Robertis, E.M. (2004). Neural induction in Xenopus: requirement for ectodermal and endomesodermal signals via Chordin, Noggin, beta-Catenin, and Cerberus. PLoS Biol. *2*, E92.

Labbé, E., Letamendia, A., and Attisano, L. (2000). Association of Smads with lymphoid enhancer binding factor 1/T cell-specific factor mediates cooperative signaling by the transforming growth factor-beta and wnt pathways. Proc. Natl. Acad. Sci. U. S. A. *97*, 8358–8363.

Lam, N., Chesney, M.A., and Kimble, J. (2006). Wnt signaling and CEH-22/tinman/Nkx2.5 specify a stem cell niche in C. elegans. Curr. Biol. *16*, 287–295.

Larabell, C.A., Torres, M., Rowning, B.A., Yost, C., Miller, J.R., Wu, M., Kimelman, D., and Moon, R.T. (1997). Establishment of the dorso-ventral axis in Xenopus embryos is presaged by early asymmetries in beta-catenin that are modulated by the Wnt signaling pathway. J. Cell Biol. *136*, 1123–1136.

Laurent, M.N., Blitz, I.L., Hashimoto, C., Rothbächer, U., and Cho, K.W. (1997). The Xenopus homeobox gene Twin mediates Wnt induction of Goosecoid in establishment of Spemann ' s organizer. *4916*, 4905–4916.

Lemaire, P., Garrett, N., and Gurdon, J.B. (1995). Expression cloning of Siamois, a Xenopus homeobox gene expressed in dorsal-vegetal cells of blastulae and able to induce a complete secondary axis. Cell *81*, 85–94.

Levine, M. (2010). Transcriptional enhancers in animal development and evolution. Curr. Biol. *20*, R754–63.

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

Li, B., Kuriyama, S., Moreno, M., and Mayor, R. (2009). The posteriorizing gene Gbx2 is a direct target of Wnt signalling and the earliest factor in neural crest induction. Development *136*, 3267–3278.

Li, J., Sutter, C., Parker, D.S., Blauwkamp, T., Fang, M., and Cadigan, K.M. (2007). CBP/p300 are bimodal regulators of Wnt signaling. EMBO J. *26*, 2284–2294.

Lien, W.-H., Polak, L., Lin, M., Lay, K., Zheng, D., and Fuchs, E. (2014). In vivo transcriptional governance of hair follicle stem cells by canonical Wnt regulators. Nat. Cell Biol. *16*, 1–15.

Liu, F., van den Broek, O., Destrée, O., and Hoppler, S. (2005). Distinct roles for Xenopus Tcf/Lef genes in mediating specific responses to Wnt/beta-catenin signalling in mesoderm development. Development *132*, 5375–5385.

Logan, C.Y., and Nusse, R. (2004a). The Wnt signaling pathway in development and disease. Annu. Rev. Cell Dev. Biol. *20*, 781–810.

Logan, C.Y., and Nusse, R. (2004b). The Wnt signaling pathway in development and disease. Annu. Rev. Cell Dev. Biol. *20*, 781–810.

Love, J.J., Li, X., Case, D.A., Giese, K., Grosschedl, R., and Wright, P.E. (1995). Structural basis for DNA bending by the architectural transcription factor LEF-1. Nature *376*, 791–795.

Lu, H., Li, Y., Wang, Y., Liu, Y., Wang, W., Jia, Z., Chen, P., Ma, K., and Zhou, C. (2014). Wnt-promoted Isl1 expression through a novel TCF/LEF1 binding site and H3K9 acetylation in early stages of cardiomyocyte differentiation of P19CL6 cells. Mol. Cell. Biochem. 1–10.

Lustig, K.D., Kroll, K.L., Sun, E.E., and Kirschner, M.W. (1996). Expression cloning of a Xenopus T-related gene (Xombi) involved in mesodermal patterning and blastopore lip formation. Development *122*, 4001–4012.

Ma, H., Nguyen, C., Lee, K.-S., and Kahn, M. (2005). Differential roles for the coactivators CBP and p300 on TCF/beta-catenin-mediated survivin gene expression. Oncogene *24*, 3619–3631.

MacDonald, B.T., Tamai, K., and He, X. (2009). Wnt/β-Catenin Signaling: Components, Mechanisms, and Diseases. Dev. Cell *17*, 9–26.

Malik, S., and Roeder, R.G. (2010). The metazoan Mediator co-activator complex as an integrative hub for transcriptional regulation. Nat. Rev. Genet. *11*, 761–772.

Mann, R.S., Lelli, K.M., and Joshi, R. (2009). Chapter 3 Hox Specificity. Unique Roles for Cofactors and Collaborators. Curr. Top. Dev. Biol. *88*, 63–101.

Marlow, F.L. (2010). Maternal Control of Development in Vertebrates. Colloq. Ser. Dev. Biol. *1*, 1–196.

Marson, A., Foreman, R., Chevalier, B., Bilodeau, S., Kahn, M., Young, R.A., and Jaenisch, R. (2008). Wnt Signaling Promotes Reprogramming of Somatic Cells to Pluripotency. Cell Stem Cell *3*, 132–135.

Mckendry, R., Hsu, S., Harland, R.M., and Grosschedl, R. (1997). LEF-1 / TCF Proteins Mediate Wnt-Inducible Transcription from the Xenopus Nodal-Related 3 Promoter. *431*, 420–431.

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

Meng, X., Brodsky, M.H., and Wolfe, S.A. (2005). A bacterial one-hybrid system for determining the DNA-binding specificity of transcription factors. Nat. Biotechnol. *23*, 988–994.

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

Merrill, B.J., Pasolli, H.A., Polak, L., Rendl, M., García-García, M.J., Anderson, K. V, and Fuchs, E. (2004). Tcf3: a transcriptional regulator of axis induction in the early embryo. Development *131*, 263–274.

Messenger, N.J., Kabitschke, C., Andrews, R., Grimmer, D., Núñez Miguel, R., Blundell, T.L., Smith, J.C., and Wardle, F.C. (2005). Functional specificity of the Xenopus T-domain protein Brachyury is conferred by its ability to interact with Smad1. Dev. Cell *8*, 599–610.

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

Mosimann, C., Hausmann, G., and Basler, K. (2009). Beta-catenin hits chromatin: regulation of Wnt target gene activation. Nat. Rev. Mol. Cell Biol. *10*, 276–286.

Mukhopadhyay, M., Shtrom, S., Rodriguez-Esteban, C., Chen, L., Tsukui, T., Gomer, L., Dorward, D.W., Glinka, A., Grinberg, A., Huang, S.P., et al. (2001). Dickkopf1 Is Required for Embryonic Head Induction and Limb Morphogenesis in the Mouse. Dev. Cell *1*, 423–434.

Naiche, L. a, Harrelson, Z., Kelly, R.G., and Papaioannou, V.E. (2005). T-box genes in vertebrate development. Annu. Rev. Genet. *39*, 219–239.

Najdi, R., Syed, A., Arce, L., Theisen, H., Ting, J.-H., Atcha, F., Nguyen, A. V, Martinez, M., Holcombe, R.F., Edwards, R.A., et al. (2009). A Wnt kinase network alters nuclear localization of TCF-1 in colon cancer. Oncogene *28*, 4133–4146.

Narlikar, G.J., Fan, H.Y., and Kingston, R.E. (2002). Cooperation between complexes that regulate chromatin structure and transcription. Cell *108*, 475–487.

Nateri, A.S., Spencer-Dene, B., and Behrens, A. (2005). Interaction of phosphorylated c-Jun with TCF4 regulates intestinal cancer development. Nature *437*, 281–285.

Newport, J., and Kirschner, M. (1982). A major developmental transition in early Xenopus embryos: I. characterization and timing of cellular changes at the midblastula stage. Cell *30*, 675–686.

Nguyen, H., Merrill, B.J., Polak, L., Nikolova, M., Rendl, M., Shaver, T.M., Pasolli, H.A., and Fuchs, E. (2009). Tcf3 and Tcf4 are essential for long-term homeostasis of skin epithelia. Nat. Genet. *41*, 1068–1075.

Niehrs, C. (2010). On growth and form: a Cartesian coordinate system of Wnt and BMP signaling specifies bilaterian body axes. Development *137*, 845–857.

Nishita, M., Hashimoto, M.K., Ogata, S., Laurent, M.N., Ueno, N., Shibuya, H., and Cho, K.W.Y. (2000). Interaction between Wnt and TGF- ᴺʟ signalling pathways during formation of Spemann ’ s organizer. *357*, 781–785.

Nowotschin, S., Liao, J., Gage, P.J., Epstein, J.A., Campione, M., and Morrow, B.E. (2006). Tbx1 affects asymmetric cardiac morphogenesis by regulating Pitx2 in the secondary heart field. Development *133*, 1565–1573.

Nusse, R. (2012). Wnt signaling. Cold Spring Harb. Perspect. Biol. *4*, 2012–2015.

Phillips, R.G., and Whittle, J.R. (1993). wingless expression mediates determination of peripheral nervous system elements in late stages of Drosophila wing disc development. Development *118*, 427–438.

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

Polakis, P. (2012). Wnt signaling in cancer. Cold Spring Harb. Perspect. Biol. *4*, 1837–1851.

Ramel, M.-C., and Lekven, A.C. (2004). Repression of the vertebrate organizer by Wnt8 is mediated by Vent and Vox. Development *131*, 3991–4000.

Rankin, S.A., Gallas, A.L., Neto, A., Gomez-Skarmeta, J.L., and Zorn, A.M. (2012). Suppression of Bmp4 signaling by the zinc-finger repressors Osr1 and Osr2 is required for Wnt/ -catenin-mediated lung specification in Xenopus. Development *139*, 3010–3020.

Ravindranath, A., and Cadigan, K.M. (2014). Structure-Function Analysis of the C-clamp of TCF/Pangolin in Wnt/ß-catenin Signaling. PLoS One *9*, e86180.

Reid, C.D., Zhang, Y., Sheets, M.D., and Kessler, D.S. (2012). Transcriptional integration of Wnt and Nodal pathways in establishment of the Spemann organizer. Dev. Biol. *368*, 231–241.

Rex, M., Hilton, E., and Old, R. (2002). Multiple interactions between maternally-activated signalling pathways control Xenopus nodal-related genes. Int. J. Dev. Biol. *46*, 217–226.

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

Rivera-Pérez, J.A., Mallo, M., Gendron-Maguire, M., Gridley, T., and Behringer, R.R. (1995). Goosecoid is not an essential component of the mouse gastrula organizer but is required for craniofacial and rib development. Development *121*, 3005–3012.

Ro, H., and Dawid, I.B. (2011). Modulation of Tcf3 repressor complex composition regulates cdx4 expression in zebrafish. EMBO J. *30*, 2894–2907.

Robertis, E.M. De (2006). Spemann ' s organizer and self- regulation in amphibian embryos. *7*, 102–108.

Robertis, E. De, and Larrain, J. (2000). The establishment of Spemann's organizer and patterning of the vertebrate embryo. Nat. Rev. … *1*.

De Robertis, E.M. (2006). Spemann's organizer and self-regulation in amphibian embryos. Nat. Rev. Mol. Cell Biol. *7*, 296–302.

De Robertis, E.M., and Kuroda, H. (2004). Dorsal-ventral patterning and neural induction in Xenopus embryos. Annu. Rev. Cell Dev. Biol. *20*, 285–308.

Roche, D., Almouzni, G., and Quivy, J.-P. (2006). Chromatin assembly of DNA templates microinjected into Xenopus oocytes. Methods Mol. Biol. *322*, 139–147.

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

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

Ryan, K., Garrett, N., Mitchell, A., and Gurdon, J.B. (1996). Eomesodermin, a key early gene in xenopus mesoderm differentiation. Cell *87*, 989–1000.

Ryu, S.L., Fujii, R., Yamanaka, Y., Shimizu, T., Yabe, T., Hirata, T., Hibi, M., and Hirano, T. (2001). Regulation of dharma/bozozok by the Wnt pathway. Dev. Biol. *231*, 397–409.

Sander, V., Reversade, B., and De Robertis, E.M. (2007). The opposing homeobox genes Goosecoid and Vent1/2 self-regulate Xenopus patterning. EMBO J. *26*, 2955–2965.

Sawa, H. (2012). Control of Cell Polarity and Asymmetric Division in C. elegans. Curr. Top. Dev. Biol. *101*, 55–76.

Scharf, S.R., and Gerhart, J.C. (1980). Determination of the dorsal-ventral axis in eggs of Xenopus laevis: complete rescue of uv-impaired eggs by oblique orientation before first cleavage. Dev. Biol. *79*, 181–198.

Schier, A.F., and Talbot, W.S. (2005). Molecular genetics of axis formation in zebrafish. Annu. Rev. Genet. *39*, 561–613.

Schneider, S., Steinbeisser, H., Warga, R.M., and Hausen, P. (1996). β-catenin translocation into nuclei demarcates the dorsalizing centers in frog and fish embryos. Mech. Dev. *57*, 191–198.

Schohl, A., and Fagotto, F. (2002). Beta-catenin, MAPK and Smad signaling during early Xenopus development. Development *129*, 37–52.

Schuijers, J., Mokry, M., Hatzis, P., Cuppen, E., and Clevers, H. (2014). Wnt-induced transcriptional activation is exclusively mediated by TCF/LEF. EMBO J. *33*, 146–156.

Shetty, P., Lo, M.-C., Robertson, S.M., and Lin, R. (2005). C. elegans TCF protein, POP-1, converts from repressor to activator as a result of Wnt-induced lowering of nuclear levels. Dev. Biol. *285*, 584–592.

Showell, C., Binder, O., and Conlon, F.L. (2004). T-box genes in early embryogenesis. Dev. Dyn. *229*, 201–218.

Shy, B.R., Wu, C.-I., Khramtsova, G.F., Zhang, J.Y., Olopade, O.I., Goss, K.H., and Merrill, B.J. (2013). Regulation of Tcf7l1 DNA binding and protein stability as principal mechanisms of Wnt/β-catenin signaling. Cell Rep. *4*, 1–9.

Sierra, J., Yoshida, T., Joazeiro, C.A., and Jones, K.A. (2006). The APC tumor suppressor counteracts beta-catenin activation and H3K4 methylation at Wnt target genes. Genes Dev. *20*, 586–600.

Singh, R., and Kispert, A. (2010). Tbx20, Smads, and the Atrioventricular Canal. Trends Cardiovasc. Med. *20*, 109–114.

Skirkanich, J., Luxardi, G., Yang, J., Kodjabachian, L., and Klein, P.S. (2011). An essential role for transcription before the MBT in Xenopus laevis. Dev. Biol. *357*, 478–491.

Smith, J.C. (2009). Forming and interpreting gradients in the early Xenopus embryo. Cold Spring Harb. Perspect. Biol. *1*, a002477.

Smith, W.C., and Harland, R.M. (1991). Injected Xwnt-8 RNA acts early in Xenopus embryos to promote formation of a vegetal dorsalizing center. Cell *67*, 753–765.

Smith, J.C., Price, B.M., Green, J.B., Weigel, D., and Herrmann, B.G. (1991). Expression of a Xenopus homolog of Brachyury (T) is an immediate-early response to mesoderm induction. Cell *67*, 79–87.

Sokol, S., Christian, J.L., Moon, R.T., and Melton, D.A. (1991). Injected Wnt RNA induces a complete body axis in Xenopus embryos. Cell *67*, 741–752.

Sokol, S.Y., Klingensmith, J., Perrimon, N., and Itoh, K. (1995). Dorsalizing and neuralizing properties of Xdsh, a maternally expressed Xenopus homolog of dishevelled. Development *121*, 1637–1647.

Song, H., Spichiger-Haeusermann, C., and Basler, K. (2009). The ISWI-containing NURF complex regulates the output of the canonical Wingless pathway. EMBO Rep. *10*, 1140–1146.

Spemann, H., and Mangold, H. (2001). Induction of embryonic primordia by implantation of organizers from a different species. 1923. Int. J. Dev. Biol. *45*, 13–38.

Standley, H.J., Destrée, O., Kofron, M., Wylie, C., and Heasman, J. (2006). Maternal XTcf1 and XTcf4 have distinct roles in regulating Wnt target genes. Dev. Biol. *289*, 318–328.

Stennard, F., Carnac, G., and Gurdon, J.B. (1996). The Xenopus T-box gene, Antipodean, encodes a vegetally localised maternal mRNA and can trigger mesoderm formation. Development *122*, 4179–4188.

Stennard, F., Zorn, A.M., Ryan, K., Garrett, N., and Gurdon, J.B. (1999). Differential expression of VegT and Antipodean protein isoforms in Xenopus. Mech. Dev. *86*, 87–98.

Suda, Y., Kokura, K., Kimura, J., Kajikawa, E., Inoue, F., and Aizawa, S. (2010). The same enhancer regulates the earliest Emx2 expression in caudal forebrain primordium, subsequent expression in dorsal telencephalon and later expression in the cortical ventricular zone. Development *137*, 2939–2949.

Sudou, N., Yamamoto, S., Ogino, H., and Taira, M. (2012). Dynamic in vivo binding of transcription factors to cis-regulatory modules of cer and gsc in the stepwise formation of the Spemann-Mangold organizer. Development *139*, 1651–1661.

Sun, Y., Kolligs, F.T., Hottiger, M.O., Mosavin, R., Fearon, E.R., and Nabel, G.J. (2000). Regulation of beta -catenin transformation by the p300 transcriptional coactivator. Proc. Natl. Acad. Sci. U. S. A. *97*, 12613–12618.

Tada, M., and Smith, J.C. (2001). T-targets: clues to understanding the functions of T-box proteins. Dev. Growth Differ. *43*, 1–11.

Takahashi, S., Yokota, C., Takano, K., Tanegashima, K., Onuma, Y., Goto, J., and Asashima, M. (2000). Two novel nodal-related genes initiate early inductive events in Xenopus Nieuwkoop center. Development *127*, 5319–5329.

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

Tam, W.-L., Lim, C.Y., Han, J., Zhang, J., Ang, Y.-S., Ng, H.-H., Yang, H., and Lim, B. (2008). T-cell factor 3 regulates embryonic stem cell pluripotency and self-renewal by the transcriptional control of multiple lineage pathways. Stem Cells *26*, 2019–2031.

Tanaka, K., Shouguchi-Miyata, J., Miyamoto, N., and Ikeda, J.-E. (2004). Novel nuclear shuttle proteins, HDBP1 and HDBP2, bind to neuronal cell-specific cis-regulatory element in the promoter for the human Huntington's disease gene. J. Biol. Chem. *279*, 7275–7286.

Tang, W., Dodge, M., Gundapaneni, D., Michnoff, C., Roth, M., and Lum, L. (2008). A genome-wide RNAi screen for Wnt/beta-catenin pathway components identifies unexpected roles for TCF transcription factors in cancer. Proc. Natl. Acad. Sci. U. S. A. *105*, 9697–9702.

Tao, Q., Yokota, C., Puck, H., Kofron, M., Birsoy, B., Yan, D., Asashima, M., Wylie, C.C., Lin, X., and Heasman, J. (2005). Maternal wnt11 activates the canonical wnt signaling pathway required for axis formation in Xenopus embryos. Cell *120*, 857–871.

Theil, T., Aydin, S., Koch, S., Grotewold, L., and Rüther, U. (2002). Wnt and Bmp signalling cooperatively regulate graded Emx2 expression in the dorsal telencephalon. Development *129*, 3045–3054.

Tiemessen, M.M., Baert, M.R.M., Schonewille, T., Brugman, M.H., Famili, F., Salvatori, D.C.F., Meijerink, J.P.P., Ozbek, U., Clevers, H., van Dongen, J.J.M., et al. (2012). The Nuclear Effector of Wnt-Signaling, Tcf1, Functions as a T-Cell-Specific Tumor Suppressor for Development of Lymphomas. PLoS Biol. *10*.

Todeschini, A.-L., Georges, A., and Veitia, R. a (2014). Transcription factors: specific DNA binding and specific gene regulation. Trends Genet. 1–9.

Trompouki, E., Bowman, T. V, Lawton, L.N., Fan, Z.P., Wu, D.-C., DiBiase, A., Martin, C.S., Cech, J.N., Sessa, A.K., Leblanc, J.L., et al. (2011). Lineage regulators direct BMP and Wnt pathways to cell-specific programs during differentiation and regeneration. Cell *147*, 577–589.

Uchiyama, H., Kobayashi, T., Yamashita, A., Ohno, S., and Yabe, S. (2001). Cloning and characterization of the T-box gene Tbx6 in Xenopus laevis. Dev. Growth Differ. *43*, 657–669.

Valenta, T., Lukas, J., and Korinek, V. (2003). HMG box transcription factor TCF-4's interaction with CtBP1 controls the expression of the Wnt target Axin2/Conductin in human embryonic kidney cells. Nucleic Acids Res. *31*, 2369–2380.

Valenta, T., Hausmann, G., and Basler, K. (2012). The many faces and functions of β-catenin. EMBO J. *31*, 2714–2736.

Verlinden, L., Kriebitzsch, C., Eelen, G., Van Camp, M., Leyssens, C., Tan, B.K., Beullens, I., and Verstuyf, A. (2013). The odd-skipped related genes Osr1 and Osr2 are induced by 1,25-dihydroxyvitamin D3. J. Steroid Biochem. Mol. Biol. *136*, 94–97.

Verzi, M.P., Hatzis, P., Sulahian, R., Philips, J., Schuijers, J., Shin, H., Freed, E., Lynch, J.P., Dang, D.T., Brown, M., et al. (2010). TCF4 and CDX2, major transcription factors for intestinal function, converge on the same cis-regulatory regions. Proc. Natl. Acad. Sci. U. S. A. *107*, 15157–15162.

Wang, L., and Coulter, D.E. (1996). bowel, an odd-skipped homolog, functions in the terminal pathway during Drosophila embryogenesis. EMBO J. *15*, 3182–3196.

Weise, A., Bruser, K., Elfert, S., Wallmen, B., Wittel, Y., Wöhrle, S., and Hecht, A. (2010). Alternative splicing of Tcf7l2 transcripts generates protein variants with differential promoter-binding and transcriptional activation properties at Wnt/beta-catenin targets. Nucleic Acids Res. *38*, 1964–1981.

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

Van de Wetering, M., Cavallo, R., Dooijes, D., van Beest, M., van Es, J., Loureiro, J., Ypma, A., Hursh, D., Jones, T., Bejsovec, A., et al. (1997). Armadillo coactivates transcription driven by the product of the Drosophila segment polarity gene dTCF. Cell *88*, 789–799.

Van de Wetering, M., Sancho, E., Verweij, C., De Lau, W., Oving, I., Hurlstone, A., Van der Horn, K., Batlle, E., Coudreuse, D., Haramis, A.P., et al. (2002). The β-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell *111*, 241–250.

White, J.A., and Heasman, J. (2008). Maternal control of pattern formation in Xenopus laevis. J. Exp. Zool. B. Mol. Dev. Evol. *310*, 73–84.

White, R.J., Sun, B.I., Sive, H.L., and Smith, J.C. (2002). Direct and indirect regulation of derrière, a Xenopus mesoderm-inducing factor, by VegT. Development *129*, 4867–4876.

Wittler, L., Shin, E., Grote, P., Kispert, A., Beckers, A., Gossler, A., Werber, M., and Herrmann, B.G. (2007). Expression of Msgn1 in the presomitic mesoderm is controlled by synergism of WNT signalling and Tbx6. EMBO Rep. *8*, 784–789.

Wu, C.-I., Hoffman, J. a, Shy, B.R., Ford, E.M., Fuchs, E., Nguyen, H., and Merrill, B.J. (2012). Function of Wnt/β-catenin in counteracting Tcf3 repression through the Tcf3-β-catenin interaction. Development *139*, 2118–2129.

Wylie, C.C., Snape, A., Heasman, J., and Smith, J.C. (1987). Vegetal pole cells and commitment to form endoderm in Xenopus laevis. Dev. Biol. *119*, 496–502.

Xanthos, J.B., Kofron, M., Wylie, C., and Heasman, J. (2001). Maternal VegT is the initiator of a molecular network specifying endoderm in Xenopus laevis. Development *128*, 167–180.

Xanthos, J.B., Kofron, M., Tao, Q., Schaible, K., Wylie, C., and Heasman, J. (2002). The roles of three signaling pathways in the formation and function of the Spemann Organizer. *4043*, 4027–4043.

Yanai, I., Peshkin, L., Jorgensen, P., and Kirschner, M.W. (2011). Mapping Gene Expression in Two Xenopus Species: Evolutionary Constraints and Developmental Flexibility. Dev. Cell *20*, 483–496.

Yang, J., Tan, C., Darken, R.S., Wilson, P.A., and Klein, P.S. (2002). Beta-catenin/Tcf-regulated transcription prior to the midblastula transition. Development *129*, 5743–5752.

Yao, J., and Kessler, D.S. (2001). Goosecoid promotes head organizer activity by direct repression of Xwnt8 in Spemann's organizer. Development *128*, 2975–2987.

Yi, F., Pereira, L., Hoffman, J. a, Shy, B.R., Yuen, C.M., Liu, D.R., and Merrill, B.J. (2011). Opposing effects of Tcf3 and Tcf1 control Wnt stimulation of embryonic stem cell self-renewal. Nat. Cell Biol. *13*, 762–770.

Yochum, G.S., Cleland, R., McWeeney, S., and Goodman, R.H. (2007). An antisense transcript induced by Wnt/beta-catenin signaling decreases E2F4. J. Biol. Chem. *282*, 871–878.

Yochum, G.S., Cleland, R., and Goodman, R.H. (2008). A genome-wide screen for beta-catenin binding sites identifies a downstream enhancer element that controls c-Myc gene expression. Mol. Cell. Biol. *28*, 7368–7379.

Yoshioka, K., Fukushima, S., Yamazaki, T., Yoshida, M., and Takatsuji, H. (2001). The plant zinc finger protein ZPT2-2 has a unique mode of DNA interaction. J. Biol. Chem. *276*, 35802–35807.

Zhang, J., and King, M.L. (1996). Xenopus VegT RNA is localized to the vegetal cortex during oogenesis and encodes a novel T-box transcription factor involved in mesodermal patterning. Development *122*, 4119–4129.