TARGET GENE SELECTION IN THE WINGLESS SIGNALING PATHWAY

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Molecular, Cellular and Developmental Biology)
in The University of Michigan
2009

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Chance favors the prepared mind.

Louis Pasteur
1822-1895

To my family with love

ACKNOWLEDGEMENTS

With the completion of my thesis ahead, my heart is overflowing with gratitude. Although I cannot acknowledge all the people who have supported me, I deeply appreciate their help.

First, I'd like to express my gratitude to my mentor, Ken Cadigan. As an academic advisor, he has taught me a lot about science. However, what I have learned and have gotten out of the Cadigan lab is more than biology itself. Before I joined his lab, I had spent one year in a lab as a graduate student. The experience gave me lot of frustration, making me seriously doubt my competence for science. Ken has changed my doubt into strong motivation. Most of the time in his lab, he has listened to my opinion and discussed with me any problems I had. He has also encouraged me to continue in science, telling me that I could be a good scientist. Whatever I do in the future, I won't forget the great experiences in the Cadigan lab. "Ken, thank you so much!"

I also have been supported by terrific people in Cadigan lab. Especially, Jinhee Chang, my best friend. Conversations with her, from science to gossip, was a good reason for me to stay in the lab. I would feel very lonely if Hui Li, Yunyun Ni and Chandan Bhambhani were not around me in this last fall of Ann Arbor. I'd like to thank Martha Synder, another coffee addict like me, who has edited my coarse writing into a sophisticated thesis. I would also like to thank all the previous Cadigan Lab members for their scientific and emotional help.

. Lots of people at the University of Michigan should be acknowledged as well. I'd like to thank my thesis committee, John Schiefelbein, Gyorgyi Csankovszki, Yukiko Yamashita and Scott Barolo, for their thoughtful advice. I appreciate Eric Fearon and Sean Morrison who helped me get work in the Fearon Lab as a technician before entering the Ph.D. program. The people from the Csankovszki lab, the Wittkopp lab and the Collins lab have given me great comments on my research. I'd like to thank the people in the MCDB office, especially Mary Carr who is my mom in the USA. Lastly, I'd like to thank my Korean friends and their family in MCDB.

I am grateful for the financial assistance from the American Heart Association predoctoral fellowship as well.

I'd like to thank my friends in Korea who always believed in me, although we couldn't see each other for a long time. I am very blessed with a supportive family: my parents, parents-in-law, grandparents, two younger sisters and her family. Hearing their voice through my cell phone was a great happiness in daily life.

Finally, I would like to thank my husband, Injune. Although we have only been married for five and half years, I couldn't imagine my life without him. He is my love, a good friend, a colleague at science and another academic advisor. Since he was always together with me during my Ph.D. course, I was able to overcome some difficulties I faced without too much frustration and to step forward. Love you and thank you.

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CHAPTER 1

GENERAL INTRODUCTION

WINGLESS/WNT SIGNALING

Identification of Wnt family proteins

A multicellular organism develops from a single cell, a fertilized egg. During this process of organismal development, cells derived from the fertilized egg need to choose their correct cell fate in part by communicating to their neighbors. Among the messengers that mediate the cell-cell communication are the Wnt family of proteins. The name Wnt is a compound of the first ones discovered: *wingless* (*wg*) in *Drosophila* and *int-1* in mouse (Nusse and Varmus, 1992). As the name implies, *wg* mutant flies containing a hypomorphic allele of *wg* (wg^1) often lack wings (Sharma and Chopra, 1976). Mouse *int-1* was originally identified as a proto-oncogene induced by nearby integration of MMTV (mouse mammary tumor virus), and therefore was named *int-1* (integration site 1) (Nusse and Varmus, 1982). Later, it was discovered that *wg* is the *Drosophila* homolog of *int-1*, thus implicating Wnt familiy proteins in both development and disease (Rijsewijk et al., 1987). Subsequent studies identified additional Wnt family proteins that are defined by conserved primary sequences including 21 specifically spaced cysteins (Nusse and Varmus, 1992). They are conserved from Cnidarians to human (Logan and Nusse, 2004)(for more information, see Role Nusse's Wnt

Homepage: http://www.stanford.edu/~rnusse/wntwindow.html).

Role of Wingless signaling in *Drosophila* Development

Drosophila has 7 Wnt genes including *wg* (Llimargas and Lawrence, 2001). Wg is the best-characterized fly Wnt. Studies with *wg* null mutants showed that Wg plays important roles in the development of various tissues throughout the fly development (Bejsovec, 2006; Nakagoshi, 2005; Nusslein-Volhard and Wieschaus, 1980; Speese and Budnik, 2007; Tao and Schulz, 2007; Vincent and C, 2003; Zaffran and Frasch, 2002).

In the epidermis of the *Drosophila* embryo, Wg has two temporally distinct functions in segmentations (Vincent and C, 2003). First, Wg is required for establishing the anterior-posterior (A-P) boundary in each segment by regulating the expression of *engrailed* (*en*) (Figure 1.1A). As a secreted protein, Wg at the anterior of the parasegment stimulates the neighboring cells to induce the expression of *en*, but only at the posterior, because *sloppy-paired* (*slp*), a repressor of *en*, is present at the anterior (Cadigan et al., 1994). *En* in turn, induces the expression of *Hedgehog* (*Hh*) that is required for sustaining *wg* expression at the anterior. This positive feedback loop results in a stable A-P boundary in each segment. Later, Wg signaling specifies smooth cuticle cells (naked cuticle) separating denticle belts in each segment. This Wg function is achieved by actively repressing the expression of *shaven baby* (*svb*), a gene that directs the formation of denticles (Payre et al., 1999) (Figure 1.1B).

Wg is also required for other processes such as the heart development during embryogenesis (Tao and Schulz, 2007; Zaffran and Frasch, 2002). The *Drosophila* heart, called the dorsal vessel, is derived from the dorsal mesoderm where heart precursors are

specified. By further differentiation into distinct sub-types, the precursors give rise to the dorsal vessel that resembles the vertebrate heart at early developmental stages (Tao and Schulz, 2007). Wg signaling has been shown to play important roles both in the specification of the heart precursors, and in the determination of heart lineage, by regulating target genes such as *sloppy-paired*, *even-skipped* and *lbl* (Jagla et al., 1998; Knirr and Frasch, 2001; Lee and Frasch, 2000).

During the larval stages, Wg functions in the development of the adult organs such as fly wings (Klein, 2001; Martinez Arias, 2003; Sharma and Chopra, 1976; Zecca et al., 1996). In the developing wing of fly larvae, *wg* is expressed in a narrow stripe of cells along the dorsal-ventral (D-V) axis, and is thought to function as a morphogen (Cadigan, 2002; Martinez Arias, 2003). In the regions adjacent to *wg* expression domain, Wg activates the expression of the short-range targets, *senseless* and *achaete* (Martinez Arias, 2003; Parker et al., 2002). Wg also induce the expression of the long-range targets, *distalless* and *vestigial,* in broader regions. By regulating the expression of the proper target genes, Wg contributes the normal wing formation in flies.

The Wg/Wnt signaling pathway in cells

Wnt proteins can function through distinct signaling pathways (Logan and Nusse, 2004; Montcouquiol et al., 2006). However, Wg has been shown to function only through ß-catenin. This conserved signaling pathway, hereafter referred to as the Wg/Wnt signaling pathway, will be a focus of this thesis.

The major determinant of Wg/Wnt signaling is the stability of β-catenin/Armadillo (β-cat/Arm, Arm is the *Drosopihla* homolog of β-cat) (Figure 1.2). In

the absence of Wg stimulation, the β-cat/Arm in the cytosol is phosphorylated by a degradation complex containing adenomatous polyposis coli protein (APC), Axin and Glycogen synthases kinase 3 (GSK3) (Ding and Dale, 2002). The phosphorylated β-cat/Arm is ubiquitinated and degraded by the proteasome. Under these conditions, proteins of the TCF/LEF1 (TCF) family are thought to repress Wg/Wnt signaling target genes with the help of co-repressors, such as Groucho (Cavallo et al., 1998) (Figure 1.2A).

When Wg/Wnt stimulates cells through a Frizzled/low-density-lipoprotein-related (LRP) receptor complex, the degradation complex is inhibited, allowing the accumulation of hypo-phosphorylated β-cat/Arm in the cytosol (Cadigan and Liu, 2006; van Noort et al., 2002). The stabilized protein then translocates to the nucleus. Once in the nucleus, β-cat/Arm binds to TCF proteins, converting TCF from a transcriptional repressor into an activator (Parker et al., 2007). Together with additionally recruited co-activators, this TCF/Arm complex induces the transcription of the Wg/Wnt pathway targets (Parker et al., 2007) (Figure 1.2B).

In the Cadigan lab, we studies the Wg/Wnt signaling pathway in both fly cell lines and flies. The methods to activate Wg/Wnt signaling in cultured cells will be described. The first method is to treat cells with Wg-conditioned medium prepared with S2 cells which constitutively secrete Wg proteins into the culture medium (Blauwkamp et al., 2008). The second method is to manipulate positive or negative regulators in Wg/Wnt signaling (Blauwkamp et al., 2008). For example, the over-expression of a stabilized form of Arm, which cannot be degraded, can effectively activate Wg/Wnt signaling in cultured cells. Alternatively, depletion of a negative regulator of β-cat/Arm

stabilization, Axin, can stimulate the pathway efficiently. These methods efficiently activate Wg/Wnt signaling in cultured cells.

TCF AS A TRANSCRIPTION FACTOR IN WNT/WG-ß CATENIN SIGNALING

Historical Perspective

TCF family members were first identified as T cell-specific DNA binding proteins that can regulate enhancers of T-cell specific genes, such as T cell receptor α or *CD3* (Travis et al., 1991; van de Wetering et al., 1991; Waterman et al., 1991). Later, TCF family proteins were rediscovered as key transcription factors in Wnt/Wg-ß catenin signaling that play essential roles throughout development beyond controlling the expression of T cell specific genes (Behrens et al., 1996; Molenaar et al., 1996).

Before TCF was introduced to Wg/Wnt signaling, it had been already known that β-cat/Arm mediates Wg/Wnt signaling (Noordermeer et al., 1994; Peifer et al., 1991). However, how β-cat/Arm in the cytosol controls gene expression in response to Wg/Wnt stimulation remained unclear, partially because β-cat/Arm has been widely known as an adhesion molecule (Peifer et al., 1992). In 1996, the Birchmeier and Clevers groups demonstrated that TCF from human (TCF-1), mouse (LEF-1) and xenopus (XTCF-3) can interact with β-catenin in yeast two-hybrid screens (Behrens et al., 1996; Molenaar et al., 1996). The functional interaction of mouse and Xenopus TCF with β-catenin was shown in *Xenopus* embryos (Behrens et al., 1996; Molenaar et al., 1996).

The importance of TCF in Wg/Wnt signaling during development was further demonstrated in *Drosophila* (Brunner et al., 1997; van de Wetering et al., 1997). Biochemical analysis revealed that fly TCF (*pangolin*) interacts with Arm. Pangolin was

shown to function downstream of Arm (Brunner et al., 1997; van de Wetering et al., 1997). *pangolin* (*pan*) mutant embryos showed a segment polarity phenotype similar to *wg* mutant embryos' although the phenotype is not as extreme as that of *wg* mutants (Brunner et al., 1997; van de Wetering et al., 1997) (Figure 1.3). In addition, in *pan* mutant embryos, the reduction in the expression of the Wg target gene, En (Figure 1.1a) was observed (van de Wetering et al., 1997). These results strongly suggested that TCF proteins play critical roles in Wg/Wnt signaling, connecting β-cat/Arm in the cytosol to the DNA in the nucleus.

There are a few reports that other transcription factors such as Pitx2 or Sox 17 can control gene expression by interacting with ß-catenin as well (Kioussi et al., 2002; Sinner et al., 2004). Although this mechanism could be used for the fine regulation of target genes in diverse developmental contexts, it remains unknown how frequently Wnt signaling control the expression of target genes independent of TCF.

Conserved domains in TCF family proteins

The function of TCF family members in Wg/Wnt signaling is highly conserved from hydra to human (Arce et al., 2006). Human and mouse have four TCF family members (*TCF-1*, *LEF-1*, *TCF-3* and *TCF-4*), each of which can produce diverse isoforms by alternative splicing or by using different promoters (Arce et al., 2006; Hovanes et al., 2000). *Drosophila,* on the other hand, have only one TCF, *pan* (Arce et al., 2006). Analysis of genomic organization such as splice sites and comparison of protein primary sequences, have revealed that the vertebrate TCF-1E isoform is the most

similar to fly TCF (Atcha et al., 2007; Dooijes et al., 1998; van de Wetering et al., 1997) (Figure 1.4).

There are three highly conserved regions in TCF family proteins. The first conserved region is the β-catenin binding domain (about 50 amino acids) at the N-termini (Figure 1.4) (Behrens et al., 1996; Graham et al., 2000; Molenaar et al., 1996; van de Wetering et al., 1997). This domain shows about 60% sequence identity among TCF family proteins (Arce et al., 2006). Expression of TCFs lacking this domain caused dominant-negative effects on endogenous Wg/Wnt signaling (Molenaar et al., 1996; van de Wetering et al., 1997). LEF1 containing mutations of six amino acids in the β-catenin binding domain failed to activate a WRE (Hsu et al., 1998). In addition, the *pan* mutant allele that has one amino acid substitution in this domain suppressed the abnormal phenotype caused by ectopic expression of Wg protein, showing the functional importance of this domain in Wg/Wnt signaling *in vivo* (Brunner et al., 1997).

The second region is the HMG (high-mobility group) domain. TCF family proteins exhibit about 95 ~ 99% protein sequence identity in this domain (Figure 1.4) (Arce et al., 2006). While another subclass of HMG domain proteins was shown to recognize DNA conformation with no or weak sequence specificity, TCF proteins have been shown to bind DNA in a sequence-specific manner through their HMG domain (Giese et al., 1991; Landsman and Bustin, 1993; van de Wetering et al., 1991). As expected from the sequence conservation in HMG domains, the consensus HMG domain binding sites (CTTTGAT) identified *in vitro* were very similar among TCF proteins (Figure 1.5) (Atcha et al., 2007; Hallikas et al., 2006; van Beest et al., 2000; van de Wetering et al., 1997). The HMG domain of LEF-1 was shown to bind the DNA minor

groove and bend DNA up to 130 degrees (Giese et al., 1992; Love et al., 1995). A basic region downstream of the HMG domain were also shown to be critical for the binding and bending of DNA (Love et al., 1995). Critical amino acid residues required for binding and bending are conserved in other TCF proteins, implying that the DNA binding mechanism by the HMG domain could be conserved among TCF family members.

The third conserved region is the C-clamp motif that is located at the C-terminus of the HMG domain (Figure 1.4) (Atcha et al., 2007). The C-clamp motif (29 amino acids) is present in the "E" isoforms of TCF proteins and is highly conserved from *Hydra* to humans (Figure 1.5) (Hovanes et al., 2000). The sequence identity of the motif between fly TCF and human TCF-1E is about 86%. The studies of the promoters of Wnt target genes showed that the LEF-1 and CDX1 promoters are activated specifically by "E" isoforms of TCF-1 and TCF-4, implying the important role of "E" tail in the gene expression of subsets of Wg/Wnt target genes (Atcha et al., 2003; Hecht and Stemmler, 2003). In *Drosophila*, a TCF mutant allele (pan^{13a}) that contains one amino acid substitution in the fifth position of the C-clamp (A374V) caused a weak segment polarity phenotype that is consistent with some reduction in Wg signaling activity, suggesting that the C-clamp of TCF is critical for the activity of endogenous Wg signaling (van de Wetering et al., 1997). In addition, the segmentation defect caused by a constitutively active form of Arm was largely rescued in a pan^{13a} mutant background (van de Wetering et al., 1997). Studies with human TCF-1E suggested that the C-clamp motif enables TCF to bind to an extended sequence beyond the CTTTGAT site recognized by HMG domain (Atcha et al., 2007).

TCF is a repressor in the absence of Wg/Wnt signaling

It has been widely accepted that TCF proteins serve not only as transcriptional activators in the presence of Wg/Wnt signaling but also as transcriptional repressors in the absence of the signaling (Figure 1.2) (Parker et al., 2007). Here, the repressor function of TCF will be described by mainly focusing on fly TCF.

The idea that TCF proteins repress the expression of Wg/Wnt target genes in the absence of Wg/Wnt stimulation has been supported by genetic analysis and the examination of Wg/Wnt response elements (WREs). A *pan* null mutant showed a segment polarity phenotype consistent with TCF being a transcriptional activator in the pathway (van de Wetering et al., 1997). However, the phenotype was not as severe as that of a *wg* mutant, even when both maternally- and zygotically-driven Pan was removed (Schweizer et al., 2003). In addition, the phenotype of *wg*;*pan* double mutants was less severe than the one of wg mutants (Figure 1.1)(Cavallo et al., 1998). These results are consistent with the repressor function of TCF in the absence of Wnt/Wg signaling.

Analyses of WREs also support the idea of default repression by TCF. A WRE of a homeotic gene, *Ultrabithorax* (*Ubx*) mediates Wg-dependent transcription in the visceral mesoderm of fly embryos (Riese et al., 1997). Mutations of TCF sites in this enhancer significantly reduced the expression of a reporter gene in cells near the Wg expression domain, but increased the expression of the reporter gene in cells away from the Wg source (Riese et al., 1997). A similar effect was observed with the *even-skipped* mesodermal enhancer in fly and with the *siamois* promoter in *Xenopus* (Brannon et al., 1997; Knirr and Frasch, 2001).

On the other hand, some TCF binding sites were shown to work only for default repression but not for activation by Wnt/Wg signaling. The *decapentaplegic* (*dpp*) enhancer is active at specific regions of the *Drosophila* visceral mesoderm (Yang et al., 2000). The mutation of TCF sites in the *dpp* enhancer caused ectopic expression of the reporter gene outside of the endogenous expression domain (Yang et al., 2000). However, no significant reduction was observed where the *dpp* enhancer is normally active (Yang et al., 2000). Although there is a little difference in the mechanism, the results from the mutagenesis analysis of WREs mentioned above support a view of the default repression by TCF outside of the endogenous expression domain of each gene.

TCF achieves the repressor function by forming protein complex with co-repressors. Among them, Grg/TLE (Groucho-related proteins/Transductin-Like Enhancer of Split (TLE)) family proteins are the best characterized co-repressors. Grg/TLE proteins which lack a known DNA binding domain are known to interact with a variety of sequence specific DNA binding proteins and repress the expression of target genes (Chen and Courey, 2000). The implication of Grg/TLE in Wg/Wnt signaling was first seen in a yeast two-hybrid screen for mouse proteins interacting with human TCF-1 (Roose et al., 1998). The result that human TCF-1 interacts with vertebrate Grg made it possible to test whether the *Drosophila* homolog Groucho (Gro) is required for TCF-mediated repression (Cavallo et al., 1998). Similar to vertebrate Grg, Gro physically and functionally interacts with fly TCF (Cavallo et al., 1998; Levanon et al., 1998). Loss of function mutations in *gro* suppressed the cuticle phenotype in *wg* and *arm* mutants (Cavallo et al., 1998). In addition, the reduction in maternally driven Gro significantly suppressed the loss of Wg signaling phenotype caused by excess expression of TCF or by

ectopic expression of a dominant negative form of TCF (Cavallo et al., 1998). Gro was suggested to achieve this co-repressor function, at least partly, by recruiting histone deacetylases (HDAC) (Billin et al., 2000; Chen and Courey, 2000; Chen et al., 1999).

The repression of Wnt/Wg target genes is also mediated by other co-repressors that act in parallel with TCF (Arce et al., 2006; Liu et al., 2008; Parker et al., 2007). For example, the co-repressor C-terminal binding protein (CtBP) and the chromatin remodeler ISWI were shown to bind to WREs independent of TCF and repress Wg target genes together with TCF (Fang et al., 2006; Liu et al., 2008). These proteins seem to repress Wg target genes in a gene-specific manner.

As described above, it is clear that TCF can repress the expression of Wg/Wnt target genes in the absence of signaling. However, this seems not to be the case for all of the targets. For example, in the WREs of vertebrate Wnt target genes, such as *Brachyury* and *c-Myc,* and *Drosophila* Wg target genes, such as *sloppy-paired*, *nkd* and *notum*, mutations in TCF binding sites only reduced the expression of a reporter gene where WREs are normally active, with no de-repression (He et al., 1998; Lee and Frasch, 2000; Yamaguchi et al., 1999). TCF binding sites identified in these WREs seem to be more dedicated to activation than to repression. Eliminating *pan* in subset of cells within the *Drosophila* wing imaginal disc reduced the expression of Wg signaling-regulated genes such as *Distalless* (*Dll*) and *vestigial* (*vg*) within the endogenous *Dll* and *vg* expression domain (Schweizer et al., 2003). However, no up-regulation of *Dll* or *vg* expression was observed outside of the expression domain (Schweizer et al., 2003). These could be because the activation or repression of each WRE involves other activators or repressors in addition to TCF.

TCF activates transcription in the presence of Wg/Wnt signaling

Wg/Wnt stimulation results in the stabilization of cytosolic β-cat/Arm that consequently translocates to the nucleus (Figure 1.2). In the nucleus, β-cat/Arm is thought to convert TCF from a repressor to an activator. Consistent with this, mutations of TCF binding sites in WREs often showed reduction in Wg/Wnt responsiveness (Brannon et al., 1997; He et al., 1998; Knirr and Frasch, 2001; Lee and Frasch, 2000; Riese et al., 1997; Yamaguchi et al., 1999). The β-cat/Arm interacting domain identified does not overlap with the Grg/TLE binding region in TCF (Roose et al., 1998; van de Wetering et al., 1997). However, it was shown that binding of β-catenin to TCF displaces the Grg/TLE co-repressor from TCF *in vitro* (Daniels and Weis, 2005). Consistent with *in vitro* results, activated Wnt signaling was shown to induce both the recruitment of β-catenin to the *c-Myc* WRE and elimination of TLE from the WRE (Sierra et al., 2006).

In addition to releasing Wnt/Wg signaling target genes from their default of repression, β-cat/Arm recruits co-activators such as the Legless (Lgs)-Pygopus (Pygo) complex to its N-terminus and CREB-binding protein (CBP)/p300 at the C-terminus to activate the transcription (Logan and Nusse, 2004; Parker et al., 2007). *lgs* and *pygo* were identified as additional components in Wg signaling in *Drosophila* genetic screens and/or in yeast two hybrid screens (Belenkaya et al., 2002; Kramps et al., 2002; Parker et al., 2002; Thompson et al., 2002). Molecular analysis of these proteins showed that Lgs act as an anchor to connect Pygo to Arm, forming of TCF-Arm-Lgs-Pygo complex in the presence of Wg signaling (Kramps et al., 2002; Stadeli and Basler, 2005). *lgs* or *pygo* null mutants show a segment polarity phenotype that is very similar to *wg* null embryos

(Belenkaya et al., 2002; Kramps et al., 2002; Parker et al., 2002; Thompson et al., 2002). In addition, genetic analysis of *pygo* mutants from embryos to adults showed that all the Wg signaling readouts examined (eg. morphological changes and transcriptional regulation) require the function of endogenous Pygo (Belenkaya et al., 2002; Kramps et al., 2002; Parker et al., 2007; Thompson et al., 2002). Although mutations in *pygo* do not always show the complete loss of Wg signaling phenotype (Jessen et al., 2008; Parker et al., 2007), the genetic analysis strongly supports the essential roles of Pygo (likely TCF-Arm-Lgs-Pygo complex) in Wg signaling in *Drosophila*. On the other hand, *pygo* genes in mammals seem not to be as essential as in *Drosophila*, though some developmental phenotypes in *pygo* deficient mice are consistent with a loss of Wnt signaling (Jessen et al., 2008; Li et al., 2007a).

Despite the importance of Pygo as a co-activator in Wg signaling, how Pygo positively regulates the transcription of Wg target genes remains unclear (Carrera et al., 2008; Jessen et al., 2008). The N-terminal homology domain (NHD) domain of Pygo was shown to be required for the transcriptional activation of target genes by Wg signaling (Hoffmans et al., 2005). Recently, it was suggested that NHD domain recruits the mediator complex subunits, *kohtalo* and *skuld*, likely connecting TCF-co-activator complex to the target promoter (Carrera et al., 2008). Whether Pygo can directly bind to the mediator subunits remains to be determined. Pygo also contains plant homeodomain (PHD) fingers that have been linked to chromatin remodeling processes (Bienz, 2006; Jessen et al., 2008). However, so far, there is no report suggesting the role of Pygo in chromatin remodeling.

There are other co-activators that play important roles in the target gene activation by Wg/Wnt signaling. For example, histone acetyltransferases (HAT), CBP/p300, were also shown to bind to β-cat/Arm (Daniels and Weis, 2002). The role of CBP/p300 in the Wg/Wnt signaling-mediated transcription might be different between flies and mammals. In *Drosophila*, loss of CBP caused a mutant phenotype similar to elevated levels of Wg signaling, probably by interacting with the HMG domain of TCF (Waltzer and Bienz, 1998). In contrast, in mammals, CBP has been shown to positively regulate the transcription of Wnt targets (Hecht et al., 2000; Miyagishi et al., 2000; Sun et al., 2000; Takemaru and Moon, 2000). Recently, Li et al., reported the activator function of *Drosopihla* CBP and the repressor function of human CBP, showing a dual role for CBP in Wg/Wnt signaling (Li et al., 2007b). CBP/p300 was shown to have intrinsic HAT activity (Ogryzko et al., 1996). Consistent with this, CBP was suggested to be responsible for Wg signaling induced-acetylation of the histone 3 and histone 4 subunits in Wg target genes (Parker et al., 2007). However, in mammalian cells, p300 lacking p300 HAT activity was able to activate a *siamois* promoter in the similar manner to wild type p300 (Hecht et al., 2000), suggesting that CBP/p300 might affect the activation of some WREs by Wg/Wnt signaling independent of its HAT activity as well.

TARGET GENE SELECTION IN WNT/WG SIGNALING

TCF binding sites in WREs

High affinity TCF binding sites (CTTTGAT) have been identified by *in vitro* screens (Figure 1.5) (Atcha et al., 2007; Hallikas et al., 2006; van Beest et al., 2000; van de Wetering et al., 1997). Studies with colorectal cancer cells showed that 84% of β-

catenin bound regions and 70% of TCF-4 bound regions contain these consensus TCF binding sites (Hatzis et al., 2008; Yochum et al., 2007). Consistently, functional TCF binding sites matching the consensus have been identified in many WREs (Barolo, 2006) (eg. (Brannon et al., 1997; Riese et al., 1997; Yamaguchi et al., 1999)). Multimerized consensus TCF binding sites upstream of a minimal promoter have been shown to recapitulate Wnt signaling in many tissues in transgenic mice and zebrafish (Barolo, 2006). These results support a view that TCF binding sites are sufficient to define a stretch of DNA as WREs.

The existence of TCF binding sites in a genomic DNA, however, does not always guarantee the transcriptional regulation by Wg/Wnt signaling. Multiple copies of TCF binding sites taken from native WREs did not respond to endogenous Wg signaling in transgenic *Drosophila* where only one or two copies of a transgene were integrated (Barolo, 2006; Riese et al., 1997). Detailed analysis of WREs in a Wg target gene, *nkd*, showed that not all the genomic regions containing TCF binding sites can recruit TCF and function as WREs (Parker et al., 2008). Some functional TCF binding sites identified in WREs were quite different from the consensus sites generated *in vitro* (eg. TTTTGTC in *slp* WRE) (Lee and Frasch, 2000). With these criteria, potential TCF binding sites can be identified throughout the genome with very high frequency. These results have raised questions of what constitutes authentic TCF binding sites and how Wg/Wnt signaling causes TCF to locate its proper targets.

Genome-wide search for WREs

Availability of genome sequences has stimulated interest in identifying WREs (or Wg/Wnt signaling target genes) through genome-wide screening (Vlad et al., 2008). Two approaches have been used: an experimental approach to identify genomic regions that interacts with TCF or β-catenin, and a bioinformatical approach to search the entire genome for TCF binding sites *in silico*. Here, current reports using each approach will be described.

Hatzis et al., and Yochum et al., used Chromatin immunoprecipitation (ChIP) to identify WREs (Hatzis et al., 2008; Yochum et al., 2007). TCF-4 or β-catenin bound genomic regions were recovered from human colorectal cancer cells, respectively. The majority of TCF-4 or β-catenin bound regions contains consensus TCF binding sites. Comparisons of the outcomes from the two studies, however, showed only an 18% overlap, even though both studies used human colorectal caner cell lines (LS174T for Hatzis et al. and HCT116 for Yochum et al.). The small overlap could be from the difference in methods used to analyze the immunoprecipitates (DNA microarray (Chip)(Hatzis et al., 2008) versus serial analysis of gene expression (SAGE)(Yochum et al., 2007)), further emphasizing the necessity of the validation process.

A bioinfomatical approach to identify unknown WREs also has been done (Hallikas et al., 2006). Hallikas et al., have developed a computational program called enhancer element locator (EEL) to identify the enhancers regulated by Hedgehog and Wnt signaling in mammals. First, EEL aligns human and mouse orthologous gene pairs. Instead of aligning the entire DNA sequences at once as other DNA alignment program do (Elgar and Vavouri, 2008), EEL finds all binding sites of the transcription factors with

considering the degeneracy in their binding sites, and aligns them. Next, EEL scores the aligned sequences by considering the binding affinity of TCF, clustering of TCF sites and the conserved pattern of TCF binding sites. This approach succeeded in identifying several known enhancers in *Drosophila* and mammals. EEL also predicted 132 candidates for mouse WREs. However, only six of them (4.5%) were located close to previously reported TCF target genes, and <0.15% of sites occupied by TCF-4 were predicted by EEL (Hatzis et al., 2008). This implies that there could be additional information beyond TCF binding sites to be considered in *in silico* identification of WREs.

TARGET GENE SELECTION BY OTHER TRANSCRIPTION FACTORS

Specific recognition of DNA by transcription factors is essential for precise gene regulation. Most of transcription factors are known to recognize short DNA sequences (4~8 bp). Therefore, a single DNA binding domain is not sufficient for recognition of transcriptional targets among the vast amount of genome. To overcome this difficulty, transcription factors often employ strategies to recognize larger and more specific DNA sequences. In this section, the strategies will be described in a little detail by using specific examples.

Oligomerization (eg. tetramerization of p53)

Famous as a tumor suppressor, p53 controls various processes including DNA repair, cell-cycle arrest and apoptosis by regulating the transcription of its target genes (Riley et al., 2008). p53 is a well-known example of DNA binding proteins that can

specifically recognize DNA by forming an oligomer (Riley et al., 2008). Consensus p53 binding sites were first identified by two independent groups (el-Deiry et al., 1992; Funk et al., 1992). The consensus site contains two copies of a 10 bp motif, often called a half site, separated by 0-21 bp (5`-RRRCWWGYYY-3`; R is a purine, W is either A or T and Y is a pyrimidine). A DNA binding domain of p53 contacts each quarter site (RRRCW) (el-Deiry et al., 1992). Subsequent biochemical and structural studies have shown that p53 exists mostly as dimers in solution, but forms a stable tetramer on a 20 bp consensus site suggesting that there is a reciprocal interaction between p53 and specific DNA sequences (Kitayner et al., 2006; Weinberg et al., 2004).

Cooperative binding with other proteins (eg. Ultrabithorax-Extradenticle complex)

Hox family proteins play important roles in determining morphological identities along anterior-posterior (AP) axis. Despite their different *in vivo* functions, lots of Hox proteins were shown to bind the same conserved TAAT motif with high-affinity (Svingen and Tonissen, 2006). In some cases, Hox proteins such as Ultrabithorax (Ubx), overcome this problem by interacting with co-factors such as Extradenticle (Exd), another homeodomain protein. In *Drosophila*, mutations in *exd* caused defects in segment identities and the reduction in Ubx target gene expression by affecting the activity of Ubx (Peifer and Wieschaus, 1990). Ubx was shown to cooperatively interact with Exd at particular target sites (Chan et al., 1994). This interaction was suggested to increase the affinity and specificity of the Ubx/Exd complex and its target sequences (Chan et al., 1994; Joshi et al., 2007). By adopting additional DNA binding domains from a cofactor, Exd, Ubx could specifically recognize proper target sites in the nucleus.

Cooperative binding by multiple DNA binding motifs (eg. POU domain proteins)

Some proteins contain more than one DNA binding motif that cooperatively binds to a proper transcription factor response element. The POU domain protein family (for Pit, Oct and UNC) is a well-known example of this strategy (Herr and Cleary, 1995; Phillips and Luisi, 2000). Members of the POU family are shown to regulate diverse developmental and homeostatic processes such as neurogenesis (Banerjee-Basu and Baxevanis, 2001; Friedrich et al., 2005; Phillips and Luisi, 2000). The POU DNA binding domain contains two structurally independent sub-domains that function as a DNA binding unit: the POU-specific domain (POU_S) at the N-terminus and the POU class homeodomain (POU_H) at the C-terminus. Studies done on a POU protein, Oct-1, revealed that the POU domain binds to an octamer sequence (ATGCAAAT) in the histone H2B promoter where POU_S and POU_H bind to ATGC or AAAT, respectively (Klemm et al., 1994). The binding of POU_S and POU_H to octamer sequences were shown to be cooperative (Klemm and Pabo, 1996). Similar to the interaction among different DNA binding proteins mentioned above, two DNA binding motifs in one transcription factor also could enhance the specificity of protein-DNA interactions.

RATIONALE AND SPECIFIC AIMS

Wg/Wnt signaling controls diverse biological events mostly through regulating the gene expression. A central focus of my thesis research has been to understand how TCF, a major transcription factor of the pathway, find its proper targets in the nucleus.

Chapter II: To understand the characteristics of *nkd*-WREs.

nkd is known to be activated by Wg signaling in a variety of tissues in *Drosophila* (Zeng et al., 2000). There is no other pathway which is known to regulate *nkd* expression. Therefore, we assumed that unlike WREs of tissue-specific Wnt/Wg target genes, *nkd* could have a single WRE that responds to Wg signaling in diverse tissues.

We found that *nkd* rather has several WREs which are activated by Wg signaling in multiple tissues, in distinct but overlapping patterns. As expected, TCF binding sites were necessary for the activity of *nkd*-WREs. These studies also found that the activity of identical TCF sites can differ depending on the context around the TCF binding sites within WREs.

This work has been published in *Developmental Biology* (2008) (Chang et al., 2008a). I am a second author on this paper. My contribution will be mentioned at the end of Chapter II.

Chapter III: To explore the importance of Helper sites in WREs and to determine the molecular mechanism by which Helper sites work

In order to determine what makes a TCF site functional, I decided to study one of the *nkd*-WREs, *nkd*-IntE in more detail. The further examination of *nkd*-IntE identified a new cis-regulatory element named the TCF Helper site (Helper site) that is required for the activation of WREs in several different WREs. Helper sites were shown to greatly enhance the ability of TCF sites to respond to the pathway, likely by direct interaction with the C-clamp motif in TCF. A genome-wide search for TCF-Helper site clusters identified two potential WREs.

This work has been published in Current Biology (Chang et al., 2008b).

Chaper IV: To further test the function of Helper sites and C-clamp *in vivo*

The function of Helper sites in WREs and the C-clamp of fly TCF has been further studied in *Drosophila*. Two WREs found through an *in silico* search (Chpater III) have been examined in transgenic reporter flies. Consistent with previous results (ChapterIII), ChIP with transgenic *Drosophila* embryos revealed that Helper sites are as important as TCF binding sites for TCF-WREs interaction, strongly suggesting that the C-clamp of TCF could play important roles in developmental processes mediated by Wg signaling (Schweizer et al., 2003; van de Wetering et al., 1997). This possibility will be tested by performing TCF protein rescue assays in flies. Endogenous TCF will be depleted by expressing dsRNA against 3`UTR of TCF in several developmental stages. The defect caused by TCF depletion will be rescued by expressing either wild type or C-clamp mutant TCF. The current experiments and results will be presented in Chapter IV.

FIGURES

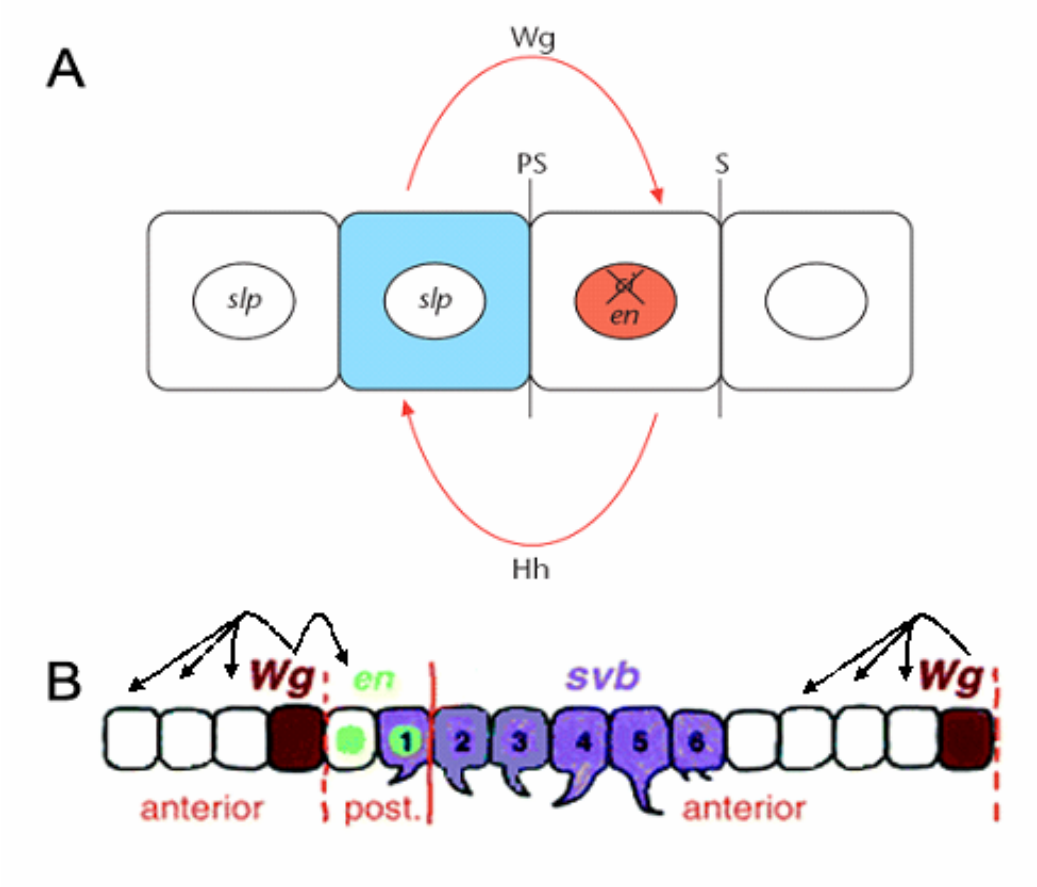

Figure 1.1. Wg play important roles in patterning segment in *Drosophila* embryo. (A) Schematic diagram showing the role of Wg in establishing the anterior-posterior boundary within each segment (S). Taken from (Vincent and C, 2003). *wg* is expressed at the anterior of the parasegment (PS) and induces *en* expression only at the posterior where slp, a repressor for *en* does not exist. En in turn induces the expression of *hh* that is required for sustaining *wg* expression at the anterior. (B) Schematic diagram of the ventral epidermis showing the expression pattern of *wg*, *en* and *svb*. Taken from (Payre et al., 1999) Wg represses the expression of *svb* in cells at the anterior and one cell at the posterior. *svb* expressing cells produce denticle.

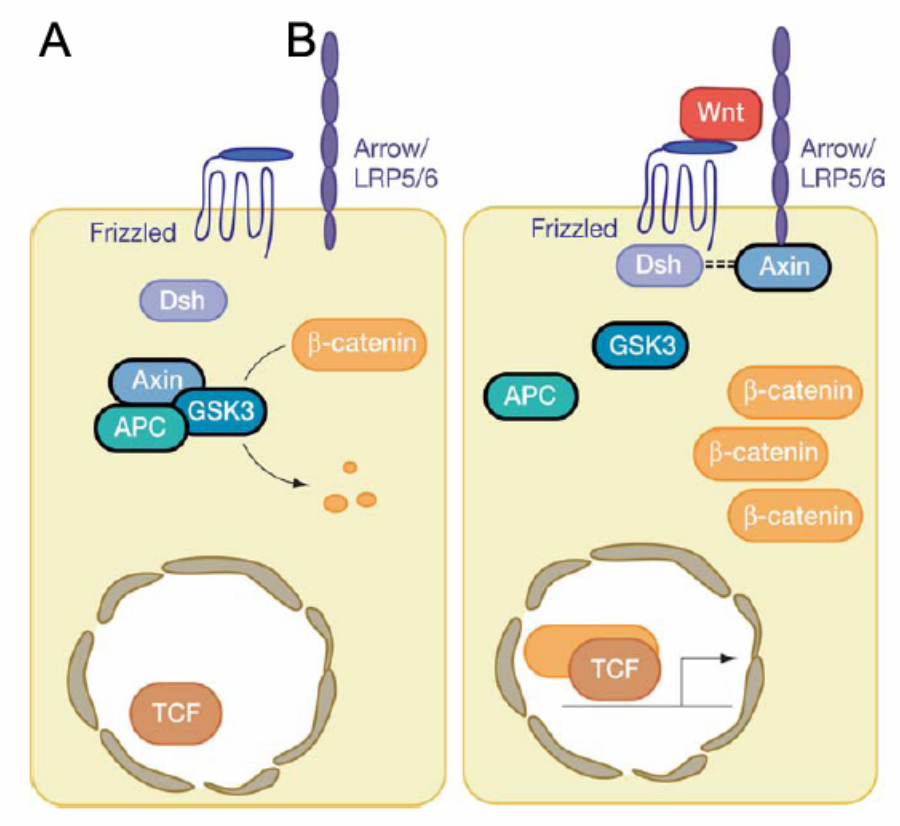

Figure 1.2. Simple model of Wnt/Wg signaling in the absence (A) or presence (B) of Wnt. Taken from (Logan and Nusse, 2004). (A) In the absence of Wnt/Wg stimulation, ß-catenin/Arm in the cytosol is constantly degraded. Under this condition, Wnt/Wg signaling target genes are repressed by TCF proteins. (B) Wnt stimulation disables the activity of destruction complex which includes Axin, APC and GSK3, stabilizing ß-catenin/Arm in the cytosol, and, consequently, in the nucleus. In the nucleus, ß-catenin/Arm binds to TCF proteins and activates the expression of Wnt/Wg signaling target genes.

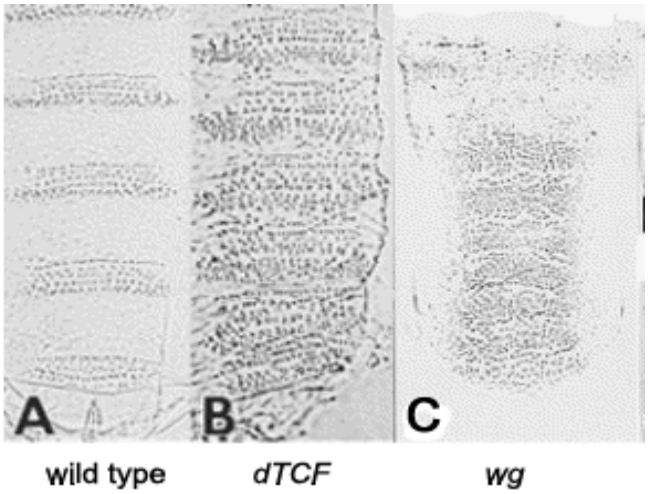

Figure 1.3. Mutations in fly *TCF* show a segment polarity phenotype in *Drosophila* embryos. (A-C) Taken from (van de Wetering et al., 1997). (A) In wild type embryos, each segment contains the denticles at the anterior and naked cuticles at the posterior (Figure 1.1B). (B) The epidermis of *pan* mutants forms a lawn of denticles with no naked cuticle but the phenotype is not as extreme as that of wg mutants. The head segments which are lost in *wg* mutant embryos were not affected. (C) The *wg* mutant embryos show a severe segment polarity phenotype.

Figure 1.4. Fly *TCF* is similar to human *TCF1-E* and *C. elegans POP-1*. Taken from (van de Wetering et al., 1997). (A) Sequence alignment of TCF proteins (H: human TCF-1E, D: *Drosophila* TCF, P: *C. elegans POP-1*). Boxed regions show that β-catenin binding domain, HMG box and C-clamp motif in order. Fly TCF has two isoforms (dTCF-A and dTCF-B) that differ in the HMG box. (B) Schematic diagrams show the structure of TCF proteins. The three conserved regions are colored.

	Optimal TCF binding sites	Reference
HMG domain of dTCF	CCTTTGATCTT	(van de Wetering et al, 1997)
HMG domain of human TCF-1	CCCTTTGATCTT	(van Beest et al., 2000)
Full length Human TCF-4 (lacking first 30 amino acids)	CCTTTGATC/G	(Hallikas et al., 2006)
Full length LEF-1	TTTCCTTTGATCTT	(Atcha et al., 2007)
Full length TCF-1E	TTTTCTTTGATCTT$-(n_{0-3})-$GCCG	(Atcha et al., 2007)
Consensus High affinity TCF/LEF binding sites	**CTTTGAT**	

Figure 1.5. Consensus TCF binding sites were generated by sequence alignment of high affinity TCF binding sites identified by independent *in vitro* selections. In order to know the *in vitro* selection procedure for each experiment, see the reference cited above.

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CHAPTER II.

REGULATION OF THE FEEDBACK ANTAGONIST NAKED CUTICLE BY WINGLESS SIGNALING

ABSTRACT

Signaling pathways usually activate transcriptional targets in a cell type-specific manner. Notable exceptions are pathway-specific feedback antagonists, which serve to restrict the range or duration of the signal. These factors are often activated by their respective pathways in a broad array of cell types. For example, the Wnt ligand Wingless (Wg) activates the *naked cuticle* (*nkd*) gene in all tissues examined throughout *Drosophila* development. How does the *nkd* gene respond in such an unrestricted manner to Wg signaling? Analysis in cell culture revealed regions of the *nkd* locus that contain Wg response elements (WREs) that are directly activated by the pathway via the transcription factor TCF. In flies, Wg signaling activates these WREs in multiple tissues, in distinct but overlapping patterns. These WREs are necessary and largely sufficient for *nkd* expression in late stage larval tissues, but only contribute to part of the embryonic expression pattern of *nkd*. These results demonstrate that *nkd* responsiveness to Wg signaling is achieved by several WREs which are broadly (but not universally) activated by the pathway. The existence of several WREs in the *nkd* locus may have been

necessary to allow the Wg signaling-Nkd feedback circuit to remain intact as Wg expression diversified during animal evolution.

INTRODUCTION

Throughout development, some signaling pathways are used in a reiterated manner to achieve the proper regulation of gene expression. For example, the *Drosophila* gene *wingless* (*wg*), which encodes a member of the Wnt family of secreted signaling proteins, has numerous essential roles in embryogenesis and larval stages (Couso et al., 1993; Klingensmith and Nusse, 1994). Because Wg signaling so profoundly affects cell fate, its expression is tightly controlled both spatially and temporally (Couso et al., 1993; Sanson, 2001). To achieve this complex pattern of expression, the *Wg* locus contains multiple enhancers, each active in different tissues and controlled by various signal and local inputs (Costas et al., 2004; Lessing and Nusse, 1998;Neumann and Cohen, 1996; Pereira et al., 2006).

The Wg ligand is the trigger for an evolutionarily conserved signaling cascade that promotes nuclear accumulation of the fly β-catenin, Armadillo (Arm). Nuclear Arm binds to the transcription factor TCF, converting it from a transcriptional repressor to an activator of Wg targets (Parker et al., 2007; Stadeli et al., 2006). Consistent with its multitude of functions, Wg signaling regulates gene expression in a cell and tissue-specific manner. For example, in the embryo, epidermal Wg activates Engrailed (En) and Hedgehog expression in the epidermis (Sanson, 2001) but mesodermal Wg regulates a different set of targets in the visceral and cardiac mesoderm (Bilder and Scott, 1998; Riese et al., 1997). In wing imaginal discs,Wg signaling regulates a different set of

targets (Cadigan, 2002). The specificity of Wg activation is thought to occur through combinatorial regulation with other factors or signaling pathways. For example, theWg Response Element (WRE) responsible for cardiac expression of *even-skipped* (*eve*) is also directly regulated by Decapentaplegic (Dpp) and RAS signaling (Halfon et al., 2000; Knirr and Frasch, 2001; Han et al., 2002). A high degree of specificity is also observed in vertebrate cell culture, where microarray analysis revealed minimal overlap between Wnt targets in different cell types (Vlad et al., 2008).

Many developmental signaling pathways activate the expression of feedback antagonists, which limit their range of action. These include *naked cuticle* (*nkd*) and *notum*/*wingful* for Wg signaling (Gerlitz and Basler, 2002; Giraldez et al., 2002; Zeng et al., 2000), as well as *patched*, *daughters against dpp* and *argos* for the Hedgehog, Dpp and Epidermal Growth Factor signaling pathways, respectively (Alexandre et al., 1996; Golembo et al., 1996; Tsuneizumi et al., 1997). In contrast to the cell-specific nature of most target genes, these feedback antagonists are ubiquitously activated by their respective signaling pathways regardless of cell type. The regulatory cis-acting sequences controlling these universal responders have not been characterized. Do universal enhancers activate expression of these antagonists in all cell types, or do these targets require multiple tissue-specific enhancers? To address this question, we examined the regulation of *nkd* by Wg signaling.

nkd has all the essential features of a signal-induced feedback antagonist. Its expression requires Wg signaling and it is often expressed in a slightly broader pattern than Wg, owing to the ability of secreted Wg to diffuse to neighboring cells (Zeng et al., 2000). *nkd* encodes an EF hand protein that is thought to antagonize Wg signaling

through binding to Dishevelled, a protein which mediates Wg dependent stabilization of Arm (Rousset et al., 2001). Loss of *nkd* in fly embryos results in elevated Arm levels and ectopic activation of Wg signaling, causing a dramatic reprogramming of epidermal cell fate (Waldrop et al., 2006; Zeng et al., 2000).

Our lab previously identified a WRE in the first intron of the *nkd* gene that is bound by TCF and activated by Wg signaling in cell culture (Fang et al., 2006; Li et al., 2007; Parker et al., 2008). In this report, we used chromatin immunoprecipitation (ChIP) to identify a region upstream of the *nkd* gene that is also bound by TCF. This region contains two overlapping WREs (UpE1 and UpE2). Mutagenesis of TCF-binding sites in these WREs and the intronic WRE (IntE) demonstrate direct regulation by the pathway through TCF. Each of these WREs is activated by Wg signaling in patterns very similar to that of *nkd* transcripts. The WREs are active in broad, partially overlapping patterns in larval imaginal discs, and the sum of the three can largely account for the entire *nkd* pattern in late third instar larva. In contrast, these WREs only partially recapitulate the embryonic *nkd* pattern. When deleted from the endogenous *nkd* locus, loss of IntE has a minimal effect on *nkd* expression whereas loss of a region containing UpE1/UpE2 displays a dramatic reduction in imaginal disc expression. However, neither deletion affected embryonic expression of *nkd*. Our data demonstrate that multiple WREs are needed for *nkd* to respond to Wg signaling in all tissues. The overlapping specificity of these WREs may provide robustness to the Wg-Nkd feedback circuit in the various cells where it operates. In addition, multiple *nkd*-WREs may have been required for the activation of *nkd* to be maintained as the expression of the Wg ligand became more elaborate during animal evolution.

RESULTS

The *nkd* locus contains several WREs that are directly activated by Wg signaling in cultured cells

As previously reported (Fang et al., 2006; Li et al., 2007; Parker et al., 2008), expression of *nkd* is induced in *Drosophila* Kc167 (Kc) cells upon stimulation with Wg-conditioned media (Wg-CM) or RNAi depletion of *Axin*. RNAi knockdown of either *TCF* or *arm* significantly reduced this *nkd* induction (Figure 2.1A, B). When the *nkd* transcription unit and surrounding DNA (see cartoon in Figure 2.1C) were assayed for TCF binding via ChIP, TCF was highly enriched in the region containing an intronic WRE (IntE) and this binding was enhanced upon Wg-CM treatment (Fang et al., 2006; Parker et al., 2008). Thus, IntE is likely to be a major WRE in mediating Wg-dependent activation of *nkd* expression in Kc cells.

Occasionally, more modest TCF binding was also observed ~10 kb upstream of the *nkd* transcriptional start site (TSS) in Wg-CM treated Kc cells (Parker et al., 2008). TCF binding to this upstream region (UpE) was much more pronounced when cells were treated with *Axin* RNAi, reaching levels similar to those seen at IntE (Figure 2.1D). Cells simultaneously depleted for Axin and TCF lost TCF binding at both locations, demonstrating the specificity of the TCF antisera (Figure 2.1D). As observed previously (Fang et al., 2006; Li et al., 2007; Parker et al., 2008), no significant binding of TCF was found at the ORF (Figure 2.1D).

To test whether UpE is a functional WRE, a genomic fragment (1084 bp) containing this region was cloned into a *hsp* 70 core promoter/luciferase reporter. Such

reporters can be assayed for Wg responsiveness by cotransfection with a stabilized form of Arm (Arm*) (Fang et al., 2006; Parker et al., 2008). This UpE fragmentwas activated almost 1000-fold by co-expression with Arm* (Figure 2.2A), indicating that it possesses a high level of WRE activity.

To further localize the WRE activity in UpE, it was split into three parts and tested in reporter assays. None of the smaller fragments (#1, #2 or #3) had high WRE activity (Figure 2.2A). However, regions containing the 5′ (UpE1) or 3′ (UpE2) two thirds of UpE were dramatically activated by Arm* (Figure 2.2A). These data suggest that the UpE region contains two WREs, albeit with overlapping sequences.

UpE1, UpE2 and IntE all contain multiple predicted TCF-binding sites (Figure 2.2) that are conserved among the sequenced *Drosophila* species, including the distantly related *D. virilis* (Figure 2.3B, C). Examination of the nine conserved TCF sites reveals a consensus of SCTTTGW(S = G or C;W= A or T) very similar to the preferred binding site of fly TCF (CCTTTGAT) (van deWetering et al., 1997). In the 869 bp IntE previously identified (Fang et al., 2006), all three sites are clustered at the 3′ end of the fragment (Figure 2.2B). A 255 bp fragment containing all three TCF sites still possessed a high level of WRE activity, though less than that of the 869 bp fragment (Figure 2.2B).

To determine whether the conserved TCF sites in these WREs are functional, they were destroyed by site-directed mutagenesis. Individual mutation of the five TCF sites in UpE2 demonstrated that three of the five contributed to WRE activity (Figure 2.4A). Individual mutations in any of the three TCF sites in IntE (255 bp) reduced WRE activity (Figure 2.4B). Simultaneous mutation of three TCF sites in UpE1 resulted in a large reduction in Wg responsiveness (Figure 2.2A). More emphatically, mutation of two TCF

sites in UpE2 or all three sites in IntE completely abolished the activity of these WREs (Figure 2.2A, B). Together with the ChIP data, the mutagenesis results demonstrate that these WREs are directly activated by TCF-Arm in Kc cells.

UpE1, UpE2 and IntE are highly responsive to Arm* in Kc cells (Figure 2.2), which are derived from embryonic hemocytes (Goto et al., 2001). These WREs are also activated by Arm* in two other fly cell lines, S2R +and Clone8 (Table 2.1), derived from embryonic hemocytes and wing imaginal disc epithelia cells, respectively (Peel et al., 1990; Yanagawa et al., 1998). In addition, these WREs were also highly activated by Wg-CM treatment (Table 2.1).

The TCF binding and reporter gene data suggest that the UpE1 and UpE2 act as WREs for the *nkd* locus but it is also possible that they activate other nearby genes. To explore this, three genes that are upstream of the *nkd* TSS (*mkp3*, *CG3797* and *Acp76A*) and two downstream of the 3´ end of the gene (*CG18136* and *CG3808*) were tested for Wg responsiveness. The only gene whose expression was altered by *Axin* RNAi was *CG3808*, which showed a 1.8 fold increase (*nkd* was activated 56 fold in the same experiment; data not shown). This suggests that UpE1 and UpE2 mediate transcriptional activation of the *nkd* gene by Wg signaling.

The *nkd*-WREs identified from cultured cells are also directly activated *in vivo* by Wg signaling

Wg is expressed in many embryonic tissues (e.g., Figure 2.5A, F) and larval imaginal discs (e.g., Figure 2.5K, P, U). In all tissues examined, *nkd* transcripts are found in patterns similar to that of Wg (e.g., Figure 2.5E, J, O, T, Y). In some tissues the nkd

pattern is broader than that of Wg (compare Figure 2.5A and U with Figure 2.5E and Y), consistent with nonautonomous activation by the secreted Wg ligand (Zeng et al., 2000).

To determine whether the WREs from the nkd locus reflect the endogenous *nkd* pattern in fly tissues, *nkd*-UpE1, UpE2 and IntE (the 869 bp fragment) were cloned into the pH-Pelican lacZ reporter (Barolo et al., 2000) and introduced into the fly genome by P-element transgenesis. All three reporters were expressed in patterns reminiscent of *nkd* transcript distribution. UpE1 was active in multiple imaginal discs (Figure 2.5L, Q, V) but displayed no activity during embryogenesis (Figure 2.5B, G). UpE2 partially recapitulates the epidermal striped *nkd* pattern during germband extension (Figure 2.5C) but was not expressed in older embryos (Figure 2.5H). UpE2 also has activity in several imaginal discs (Figure 2.5M, R, W). IntE did not express LacZ reporter at germband extension (Figure 2.5D), but was active in the mesoderm and endoderm in older embryos (Figure 2.5I). This reporter also displayed activity in late third instar imaginal discs (Figure 2.5N, S, X). In summary, each reporter recapitulated part of the endogenous *nkd* pattern.

The expression patterns of the *nkd*-WREs in various fly tissues are summarized in Table 2.2. In the larval eye disc, the activities of all three reporters are very similar (Figure 2.5V–X). However, in all other tissues the WREs display a fair degree of specificity. While all three WREs were active in the leg discs, UpE1 activation was largely restricted to the columnar epithelia of the disc (Figure 2.5Q) while IntE was mostly active in the peripodial membrane (Figure 2.5S). UpE2 was active in both cell types (Figure 2.5R). In the wing pouch, hinge region and antennae primordia, UpE1 and UpE2 are active (though UpE2 is significantly stronger) while IntE is very weak or not

detected (Figure 2.5L–N, V-X). Conversely, in older embryos, IntE is active in several mesodermal and endodermal tissues (Figure 2.5I) similar to *nkd* (Figure 2.5J) but UpE1 and UpE2 have no activity (Figure 2.5G, H). In the notum, UpE1 is most active (Figure 2.5L), IntE has intermediate expression (Figure 2.5N) while UpE2 is not active (Figure 2.5M). These data demonstrate that these WREs have partially overlapping patterns, but that they are also selectively used in many tissues.

All the lacZ expression patterns described for the *nkd*-WREs are consistent with activation by Wg signaling. In several cases this was demonstrated experimentally. When the IntE reporter was crossed into a *wg* null mutant background, the expression pattern was lost in several tissues (compare Figure 2.6B with Figure 2.6A), except for the activity at the leading edge of the migrating dorsal epithelia (arrow in Figure 2.6B). Wg regulation of these reporters in the wing imaginal disc was tested by expressing a dominant-negative form of TCF (TCF^{DN}) in the posterior part of the wing pouch, via en-Gal4. TCF^{DN} is known to potently inhibit Wg signaling (van de Wetering et al., 1997), as exemplified by inhibition of Sens (arrowheads in Figure 2.6L; compare to Figure 2.6K), a known Wg target (Parker et al., 2002). UpE1 and UpE2 expression was markedly reduced by TCF^{DN} (arrowheads in Figure 2.6F and I). Conversely, expression of an active form of Arm (Arm^{S10}) (Pai et al., 1997) via *dpp*-Gal4 caused a dramatic increase in UpE1 expression in the wing pouch (arrows in Figure 2.6G). Similar activation by$Arm^{S10}$was observed forUpE2 and IntE (data not shown). In all cases examined, loss of Wg signaling dramatically reduced *nkd*-WRE activity while activation of the pathway increased reporter expression.

To demonstrate that UpE2 and IntE are directly regulated by TCF in fly tissues, the functional TCF sites identified in cell culture (Figure 2.2A, B) were destroyed in the WRE-lacZ reporters. In the case of IntE, a shorter (255 bp) transgene was the starting point. IntE (255 bp) has an identical pattern to the longer IntE (869 bp) but the expression is less robust in most tissues (Figure 2.6C and data not shown). Mutation of the three TCF sites in IntE (255 bp) abolished the expression of LacZ in stage 13 embryos (Figure 2.6D) and various imaginal discs (data not shown). In the case of UpE2, altering two TCF sites drastically reduced lacZ expression in the wing disc (Figure 2.6J) and all other imaginal discs examined (data not shown). Thus, IntE and UpE2 are directly activated by Wg signaling in several fly tissues.

UpE and IntE are not sufficient to recapitulate the embryonic *nkd* pattern

The patterns of UpE1, UpE2 and IntE appear to cover most of the endogenous *nkd* pattern in the imaginal discs of late third instar larva. However, the sum of these WREs accounts for only part of the endogenous embryonic pattern of *nkd* (Figure 2.5). To test whether the UpE region and IntE might act synergistically in the embryo, transgenic animals containing the entire UpE (1084 bp) and IntE (869 bp) cloned into the pH-Pelican vector (Figure 2.7A) were created and monitored for lacZ expression.

In the wing imaginal discs, the combined WRE reporter was active in all the locations observed with the individual UpE1, UpE2 and IntE reporters (Figure 2.7B). However, the pattern was slightly more than the sum of the three individual WREs. For example, the combined reporter had elevated lacZ expression on either side of the Wg stripe at the dorsal/ventral boundary (Figure 2.7B). In addition, the level of expression in

the wing pouch and hinge region was consistently elevated in the combined reporter. Because of the elevated lacZ expression in this portion of the wing disc, the expression in the notum appears weaker in the combined reporter compared to the UpE1 reporter (compare Figure 2.7B to L). However, the level of expression of both reporters in the notum appears roughly similar (data not shown). The expression of the combined WRE reporter in the eye/antennal and leg imaginal discs is similar to that of endogenous *nkd* and appears to be the sum of the three individual WRE reporters (data not shown).

In embryos, the combined WRE reporter was active in weak stripes at germband extension (Figure 2.7C), a pattern similar to UpE2 (Figure 2.5C). In older embryos, the pattern of the combined WRE reporter (Figure 2.7D) was similar to that found in IntE embryos (Figure 2.5I). The additive nature of the expression patterns indicates no detectable cooperative interactions between UpE and IntE, suggesting that at least one other WRE exists in the *nkd* locus which might account for the missing embryonic pattern.

The data obtained from reporter assays strongly support the model that UpE1, UpE2 and IntE are bona fide WREs of *nkd*. To determine whether UpE or IntE were required for expression of endogenous *nkd*, two deletions in the gene were created. Using transposons inserted in the locus that contain Flp recombinase recognition sites (FRTs) (Parks et al., 2004), a deletion removing approximately 13 kb of sequence upstream of the *nkd* TSS (Δ*UpE*; Figure 2.9A) was engineered (see Materials and methods and Figure 2.9 for more details). Imprecise excision of a P-element in IntE was used to generate the Δ*IntE* allele, which lacks approximately 3 kb of intronic sequence including IntE (Figure 2.9B).

To test the effect of the Δ*UpE* and Δ*IntE* alleles on *nkd* expression, they were placed over *Df(3L)ED4782*, a large (175 kb) deficiency removing *nkd* and several surrounding genes. These transheterozygous backgrounds produced viable fertile adults at about the same frequency as *+/Df(3L)ED4782* individuals (data not shown). Since mutations in the *nkd* gene are embryonic lethal (Zeng et al., 2000), the viability of Δ*UpE/Df(3L)ED4782* and Δ*IntE/Df(3L) ED4782* indicates that UpE and IntE are dispensable for *nkd* expression in the embryo. Consistent with this, no detectable reduction in nkd transcript level was detected from Δ*UpE/Df(3L) ED4782* and Δ*IntE/Df(3L)ED4782* embryos as judged by in situ hybridization (data not shown).

Unlike the situation in the embryo, Δ*UpE* did affect *nkd* expression in larval tissues. *In situ* hybridization revealed that Δ*UpE/Df(3L) ED4782* larvae had a marked reduction in *nkd* transcript levels compared to*+/Df(3L)ED4782* controls in the wing and leg imaginal discs (Figure 2.9C, D, F, G). A similar reduction was observed in the eyeantennal discs (data not shown). qRT-PCR quantification revealed that Δ*UpE/Df(3L)ED4782* wing discs had 41% (S.D.+26%) of the *nkd* mRNA found in control wing discs. In contrast to Δ*UpE*, loss of IntE did not detectably change *nkd* expression in the imaginal discs, as judged by in situ hybridization (Figure 2.9E, H) and qRT-PCR of RNA from wing imaginal discs (data not shown). With the important caveat that the Δ*UpE* allele deletes almost 12 kb of sequence besides UpE, the data obtained are consistent with the idea that UpE is a bona fide WRE of *nkd*. IntE, on the other hand is dispensable for expression of endogenous *nkd* in the tissues that were examined.

DISCUSSION

We previously identified binding of TCF to a region in the *nkd* intron that corresponds to IntE (Fang et al., 2006; Parker et al., 2008). In this report, we find that TCF is also highly enriched on the chromatin about 10 kb upstream of the *nkd* TSS (UpE) when the Wg pathway is chronically activated by *Axin* RNAi (Figure 2.1). Wg signaling also increases the binding of TCF to the region containing IntE (Figure 2.1), but this effect occurs within a few hours of pathway stimulation (Fang et al., 2006; Parker et al., 2008). The increase in TCF-binding in the UpE region is not due to increased TCF, since the expression of TCF and its nuclear localization are not affected by *Axin* RNAi (Chang et al., 2008). Rather, we postulate that widespread histone acetylation at the nkd locus upon Wg stimulation (Parker et al., 2008) allows subsequent recruitment of TCF to the UpE region in Kc cells.

Further analysis of the UpE region with reporter gene assays revealed the presence of two overlapping stretches of DNA (UpE1 and UpE2) that confer a high degree of responsiveness to Wg signaling (Figure 2.2A). Mutagenesis of two to three predicted TCF-binding sites in UpE1, UpE2 and IntE largely abolished their ability to respond to Wg signaling (Figure 2.2A, B). While this suggests that TCF sites within a WRE act in a redundant manner, mutation of individual sites indicates that some sites contribute more than others. For example, in UpE2, the TCF 2 and TCF 3 sites share the same sequence (Supplemental Figure 2.3A), but mutation of TCF 2 reducesWg activation while mutation of TCF 3 does not (Supplemental Figure 2.4A). These data suggests that the exact sequence of the TCF-binding site is likely not as important as the context in which they are located within the WRE.

When tested in flies, UpE1, UpE2 and IntE are all activated by Wg signaling in several tissues in patterns that partially recapitulate that of the endogenous *nkd* gene (Figure 2.5, 2.6 and Table 2.2). In the leg and eye imaginal discs, all three WREs are active. In addition, each WRE has unique tissue-specific activities. For example, in later embryogenesis IntE is the only WRE that is active. Even though UpE1 and UpE2 share more than 400 bp of sequence (Figure 2.2A), only UpE2 is active in the embryonic epidermis. UpE1 and UpE2 are both active in the wing and antennal imaginal discs, (IntE shows no or minimal expression in these tissues), but UpE1 is expressed strongly in the notum whereas UpE2 is not. Each WRE is active in multiple tissues but also contains information that confers tissue-specific Wg responsiveness.

The basis for the tissue specificity of the various *nkd*-WREs is not clear at present. It could be that different TCF sites within each WRE are utilized in different tissues. However, our data in cell culture argue that multiple TCF sites are required in each WRE in a partially redundant manner (Figure 2.2 and 2.4). In addition, the same TCF sites that are required for UpE2 and IntE activity in Kc cells are required for WRE activation in all tissues examined (Figure 2.6D, J; data not shown). Therefore, we favor the view that the tissue specificity for the different WREs is derived from the presence of other cis-acting elements that work with the TCF sites to allow activation by Wg signaling.

When all three WREs are placed within a single reporter construct the resulting pattern largely recapitulates that of the endogenous *nkd* gene in imaginal discs at the late third larval instar stage (Figure 2.7B; data not shown). However, we have not examined the regulation of our WRE reporters at earlier larval stages, where Wg is also expressed (Williams et al., 1993; Neumann and Cohen, 1996). Even at the late larval stage

examined, the pattern of the reporters does not completely match that of endogenous *nkd* in the wing disc. Wg is expressed in a double ring pattern in the hinge region (Figure 2.5K; the proximal ring indicated by the arrow) and *nkd* transcripts are also found in a double ring (Figure 2.9C; arrow). However, expression of WRE reporters in the proximal ring is weak (Figure 2.5M) and often not present (Figure 2.7B). This suggests the existence of at least one other WRE for the wing imaginal disc.

In the embryo, it is even more obvious that additional regulatory information for *nkd* expression remains to be identified. In the embryonic epidermis the pattern of the combined WRE construct is only a subset of the endogenous *nkd* pattern and is equal to the sum of the IntE and UpE2 WREs (Figure 2.7C, D; data not shown). This suggests the presence of at least one other WRE that is active in the embryonic epidermis. Consistent with this, deletions of genomic fragments containing IntE or UpE do not affect the expression of *nkd* in embryos or the viability of the animals when heterozygous with a *nkd* deficiency (data not shown).

In the wing and leg imaginal discs, loss of UpE results in a significant decrease in *nkd* transcript levels (Figure 2.9D, G). In contrast, the IntE deletion had *nkd* expression in the normal range (Figure 2.9E, H). Even with the large UpE deletion, there is still significant *nkd* expression in the wing disc (41% of wild type; see Results). These data could be evidence for redundancy between IntE and UpE in these tissues. It is also possible that additional WREs exist that contribute to imaginal disc expression, which are still present in the IntE and UpE deletions.

Our data indicate that *nkd* does not contain a universally responding WRE that is activated by Wg signaling in all tissues. Rather there are at least several WREs that can

respond to the pathway in multiple, overlapping tissues. It appears that only limited multi-tissue responsiveness can be obtained with any individual WRE. In the absence of a universal WRE, the strategy of having multiple WREs responding to Wg signaling in each tissue may be required to ensure the robustness of the Wg-Nkd feedback circuit. Whether this is the case for the regulation of other Wnt feedback antagonists or those acting in other signaling pathways remains to be determined.

The finding that the *nkd* locus does not contain a universal WRE raises the question of how the Wg-Nkd relationship could remain intact during animal evolution as the Wg expression pattern became more elaborate. We postulate that the existence of several WREs with broad tissue specificity could have ensured that when an enhancer evolved that expressed Wg in a new location, at least one of the existing nkd-WREs would be able to respond to the pathway in that tissue. This precludes the need to have a tissue-by-tissue *de novo* synthesis of *nkd*-WREs every time Wg was expressed in a new pattern. Retaining the feedback inhibition of Wg signaling by Nkd may have allowed Wg to be used more readily during the diversification of animal body plans.

EXPERIMENTAL PROCEDURE

Drosophila cell culture

Kc167 (Kc) and S2R+cells were cultured in the Schneider's *Drosophila* media (Invitrogen) containing 5% or 10% FBS at 25 °C, respectively. Clone8 cells were cultured as described (http://flyrnai.org/RNAi_index.html).

RNAi, Wg-conditioned media (Wg-CM) treatment and qRT-PCR

Double-stranded RNA (dsRNA) corresponding to *control*, *Axin*, *arm*, and *TCF* was synthesized as described (Fang et al., 2006). The Wg pathway in cultured cells was activated by depleting *Axin* or adding Wg-CM as described (Chang et al., 2008; Fang et al., 2006; Li et al., 2007). Briefly, 1 or control dsRNA were added to each well. When *Axin* dsRNA was combined with another RNA duplex, 10 mg of each dsRNA was used. Cultures were incubated with dsRNAs for 6 days before harvesting for qRT-PCR analysis.

Wg-CM was prepared using stable p*Tub-wg* S2 cells, kindly provided by Dr Roel Nusse from Stanford University. Wg-CM was collected from dense cultures (typically 7–10 million cells/ml) lacking hygromycin. Usually 200 ml of unconcentrated Wg-CM was added to 600 ml cell suspensions containing 1–3 million cells. As a control, media collected from S2 cells was used. Cells were treated for 5 h with control media or Wg-CM before harvesting for qRT-PCR analysis.

After the treated cells were collected, total RNA was isolated with Trizol reagent (Invitrogen) and cDNA synthesis was performed using Superscript III reverse transcriptase (Invitrogen) according to themanufacturer's protocols. Additional details of the qRT-PCR and the primer sequences used are described (Fang et al., 2006).

Chromatin immunoprecipitation (ChIP)

ChIP analysis was performed essentially as described (Fang et al., 2006) except that the dimethyl 3,30-dithio-bis(propionimidate) dihydrochloride treatment was eliminated. Typically 3×106 cells and 5–10 ml of TCF antibody were used per ChIP and all precipitated DNA samples were quantified by qPCR. Data are expressed as the

percent of input DNA. The specific primer pairs for UpE, IntE and ORF correspond to #N1, #N5, and #N0 primer sets described previously (Fang et al., 2006).

Plasmids

Luciferase reporter constructs containing various *nkd*-WREs were made by incorporating MluI/XmaI PCR fragments into a *pGL3-Basic* vector (Promega) containing the *Drosophila hsp* 70 minimal promoter. *hsp* 70 promoter was cloned into pGL3-Basic via PCR using 5′ ATCTCGAGCTCGAGATCTGAGCGCCGGAGT3′ (XhoI site is underlined) and 5′ATAAGCTTAAGCTTCCCAATTCCCTATTCAGAGTTCTC3′ (HindIII site is underlined) primers. The specific primers to amplify the UpE and IntE genomic fragments are the following: UpE, 5′ TCCTACGCGTGGCTGGGCTCGATGC AGATAA3′ and 5′AATTCCCGGGGGGCCGCTGTCGGCCAACTG3′; UpE1, ′TCCT ACGCGTGGCTGGGCTCGATGCAGATAA3′ and 5′GGTGCCCGGGTTTGTAGTT TGCGGTGGT3′; UpE2, 5′AATTACGCGTCAG GAGGTCTGCCAACTTAAGTAG3′ and 5′ AATTCCCGGGGGGCCGCTGTCGGCCAAC TG3′; IntE (869 bp) ′TTAGACGCGTGCTCTCGGGCCAC3′ and ′CCAGCCCGGGTTCCTCAAAGCA ACC3′; IntE (255 bp) ′GCCACGCGTATAGTTTGTGTATAGTT3′ and 5′ CCCAGCCCGGGTTCCTCAAAGCAACC3′. The MluI (ACGCGT) and XmaI sites (CCCGGG) are underlined. Deletions of the WREs (UpE #1, #2, #3, and IntE 558 bp) were made by standard PCR cloning or subcloning.

TCF-binding sites in the reporter constructs were destroyed using quick change site-directed mutagenesis (Stratagene). Base substitutions were A to C or T to G (or vice

versa). For UpE1 and UpE2 all eight nucleotides of the TCF-binding site (SSTTTGWW) were substituted while the 4th, 6th and 7th positions were altered in IntE.

For analysis in fly tissues, WREs were cloned into the pH-Pelican lacZ reporter (Barolo et al., 2000) and introduced into the fly genome by P-element transgenesis (Bestgene Inc.). The combined *nkd* WRE has a 1084 bp UpE fragment upstream of a 869 bp IntE fragment.

Transfection and reporter gene assays

Transient transfections and reporter assays were done essentially as previously described (Fang et al, 2006, Li et al., 2007). Briefly, a mixture of plasmids containing 100 ng luciferase reporter, 5 ng *pAclacZ* (Invitrogen) and 100 ng of *pAc-Arm** (Fang et al., 2006) were cotransfected with 1 x 10^6 cells. The *pAc-Arm** is a derivative of *pAc5.1* expression vector (Invitrogen) encoding a constitutively active form of Arm which has Thr^{52}, Ser^{56} substituted with Ala (Freeman and Bienz, 2001). The empty *pAc5.1* vector was used to normalize the DNA content or as controls. Cells were harvested 3 days after transfection for further reporter assays.

Luciferase and β-galactosidase activities were assayed using the Tropix Luc-Screen and Galacto-Star kits (Applied Biosystems) and quantified with a Chameleon plate luminometer (Hidex Personal Life Science). Transfection efficiency was normalized using the *pAclacZ β-galactosidase* activities. When Wg-CM was used to activate Wg signaling instead of co-expression of Arm*, cells were transfected with the same amount of reporter and *pAcLacZ* and incubated for 2 days before the cells were treated with Wg-CM for 24 h prior to harvesting for the reporter assays.

Drosophila genetics

Fly stocks were maintained on standard medium at 25 °C unless otherwise indicated. The P[*En-Gal4*] and P[*Dpp-Gal4*] are as described (Li et al., 2007). The dominant-negative TCF transgene (P[*UAS-TCF*^{DN}]) and constitutively active arm (P[*UAS-armS10*]) were obtained from M. Peifer (Pai et al., 1997; van de Wetering et al., 1997). *wg*^{CX4} is a molecular null (van den Heuvel et al., 1993). *Df(3L)ED4782*, a large (175 kb) deficiency lacking the entire *nkd* locus and a hypomorphic allele *nkd*$^{l(3)4869}$ (Zeng et al., 2000) were obtained from Bloomington Stock Center. Homozygous *Df(3L)ED4782* embryos and *Df(3L)ED4782/nkdl(3)4869* transheterozygotes display loss of cuticular denticles characteristic of *nkd* loss-of-function. Experiments with *En-Gal4* and *Dpp-Gal4* were carried out at 25 °C.

A 13 kb deletion lacking UpE (*ΔUpE*) was generated by mitotic recombination using *hsFLP* and *PBac{RB}e00194* and *P{XP}d09466* chromosomes (Parks et al., 2004). Two transposon insertions, *PBac{RB}e00194* and *P{XP}d09466* (obtained from the Exelixis stock center, Harvard Medical School) were outcrossed to *w*^{1118} flies for three generations before isogenization, removing at least one linked lethal from each line. In the dysgenic cross, males with darker eye color than any of single transposon line were obtained (see Supplemental Fig. 5) and molecular mapping with PCR confirmed the deletion. The *ΔUpE* allele is homozygous semi-lethal but *ΔUpE/Df(3L)ED4782* transheterozygotes are viable and fertile. This indicates that the semi-lethality of homozygous *ΔUpE* is due to a linked mutation(s).

A 3 kb genomic deletion removing IntE was created by imprecise excision of the *P*[*KG0529*] transposon (obtained from Bloomington Stock Center) as described

previously (Zhou et al., 2003). The *P*[*KG0529*] linewas outcrossed to w^{1118} flies for three generations before isogenization. The deletion was characterized using PCR and the relevant PCR bands were sequenced to confirm the deletion breakpoints.

Immunostaining, in situ hybridization and microscopy

Immunostaining and in situ hybridization of fly embryos and imaginal discs were performed as described previously (Lin et al., 2004; Parker et al., 2002), using rabbit anti-LacZ (1:500) (Abcam Inc.), guinea-pig anti-Sens (1:500) (Fang et al., 2006) and mouse anti-Wg antisera (1:100) (Developmental Studies Hybridoma Bank at the University of Iowa). Cy3-and Alexa 488-conjugated secondary antibodies were from Jackson Immunochemicals and Molecular Probes, respectively. Samples were examined using Leica confocal microscope DM6000B-CS (Leica) and processed in Adobe Photoshop 8.0.

Probes for in situ hybridization of nkd transcripts were made by PCR of genomic DNA with the following oligos: 5´GAATTAATACGACTCACTATAGGGAGAGCT GCTGGTCAGCGAACGTGACAATAA3´ and 5´GAATTAATACGACTCACTA TAGGGAGACAGACCCGTGGGCAACTTCTTCAGTTT3´. Underlined sequences are T7 promoter sites. Antisense dioxygenin probes were synthesized using the Ambion T7 Megascript kit with the Roche DIG RNA labeling mix. Samples for *in situ* analysis were photographed with a Nikon Eclipse800 compound microscope using DIC optics.

Quantification of nkd transcripts in wing imaginal discs

20 wing imaginal discs were collected from late 3rd instars of transheterozygotes *+/Df(3L)ED4782*, *ΔUpE/Df(3L)ED4782*, and Δ*IntE/Df(3L)ED4782* flies. After pelleting,

the discs were homogenized with 1.5 pellet pestles (South Jersey Precision Tool and Mold Inc.) in 200 µl Trizol reagent (Invitrogen). After addition of another 300 µl of Trizol, the samples were processed according to the manufacturer's protocols. Total RNA was resuspended in 10 µl of RNAase-free water and 2 µg of total RNAwas used to synthesize cDNAusing Superscript III (Invitrogen) according to the manufacturer's protocol. *nkd* and *β-tubulin* 56B transcripts were measured by qRT-PCR with primers used for cell culture experiments (Fang et al., 2006). The level of *nkd* transcript was normalized by the level of *β-tubulin* 56B. The value for *nkd* transcripts from *+/Df(3L)ED4782* flies was normalized to 1 and the relative level of transcripts in Δ*UpE* or Δ*IntE* was determined. The error represents the standard deviations from four independent experiments.

ACKNOWLEDGMENTS

We thank members of the Cadigan lab for helpful discussions and Chandan Bhambhani in particular for careful reading of the manuscript. This work was supported by NIH Grant R01CA95869 to K.M.C.

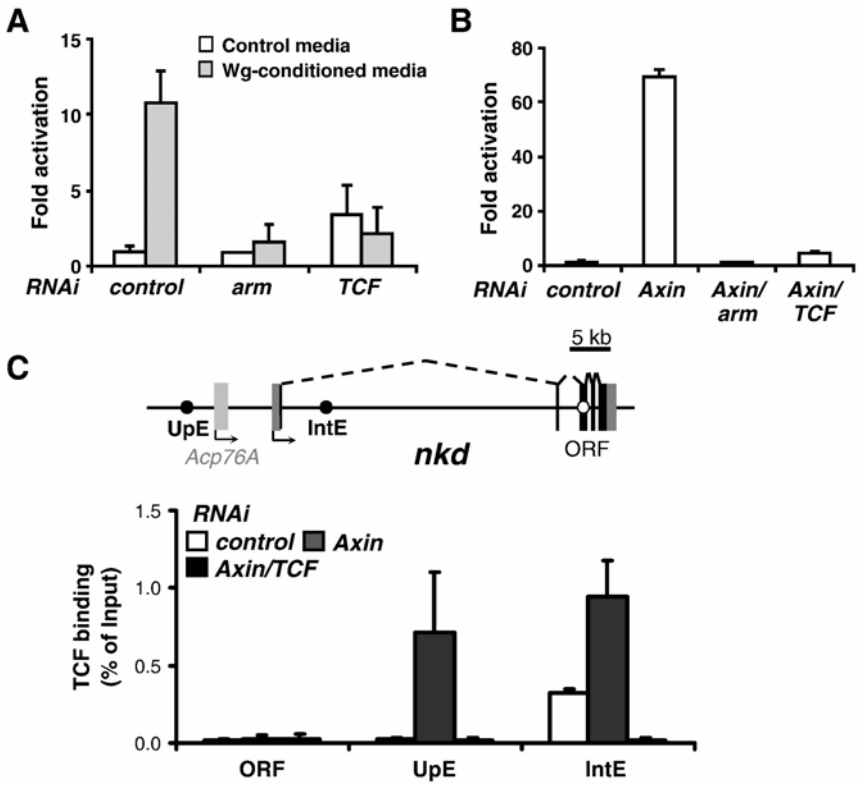
THIS CHAPTER WAS PUBLISHED AS:

Chang, J.L., **Chang, M.V**, Cadigan K.M. 2008. Regulation of the feedback antagonist naked cuticle by Wingless signaling. Dev. Biol. 321, 446-454.

In this project, I generated the data as below.

Figure 2.2B
Figure 2.4B
Figure 2.5D, I, N, S, X
Figure 2.6A, B, C, D
Table 2.1: The reporter assay data with IntE in S2R+, Clone8 and Kc 167
Table 2.2: The analysis results of the IntE reporter

FIGURES



Figures 2.1. TCF is recruited to two regions in or near the *nkd* transcription unit uponWg stimulation of fly Kc cells. (A) Stimulation withWg-CM for 5 h resulted in elevated *nkd* expression, which was greatly reduced by RNAi depletion of *arm* or *TCF*. (B) Activation of Wg signaling by RNAi depletion of *Axin* for 6 days caused a huge increase in *nkd* transcript levels in an Arm and TCF-dependent manner. Transcript levels were determined by qRT-PCR as described in Materials and methods. Fold activation is relative to *nkd* expression in cells treated with control RNAi and control media in panel A and control RNAi alone in panel B. Each bar is the mean of triplicates from cultures at each condition, with the standard deviation indicated. (C) Schematic of the *nkd* locus showing the location of three regions (UpE, IntE and ORF) assayed for TCF occupancy. Acp76A is not expressed at detectable levels in Kc cells. (D) Binding of TCF in Kc cells with the indicated RNAi treatments. In the absence ofWg signaling (*control* RNAi), TCF is bound to the region containing IntE but not UpE or ORF. In cells where the pathway is activated (*Axin* RNAi), there is strong binding to both UpE and IntE. TCF binding is dramatically lowered in *Axin*, TCF depleted cells, indicating that the ChIP signal is specific for TCF. Each bar represents the mean of duplicate ChIP samples with the standard error indicated. The data shown are representative examples from more than three separate experiments.

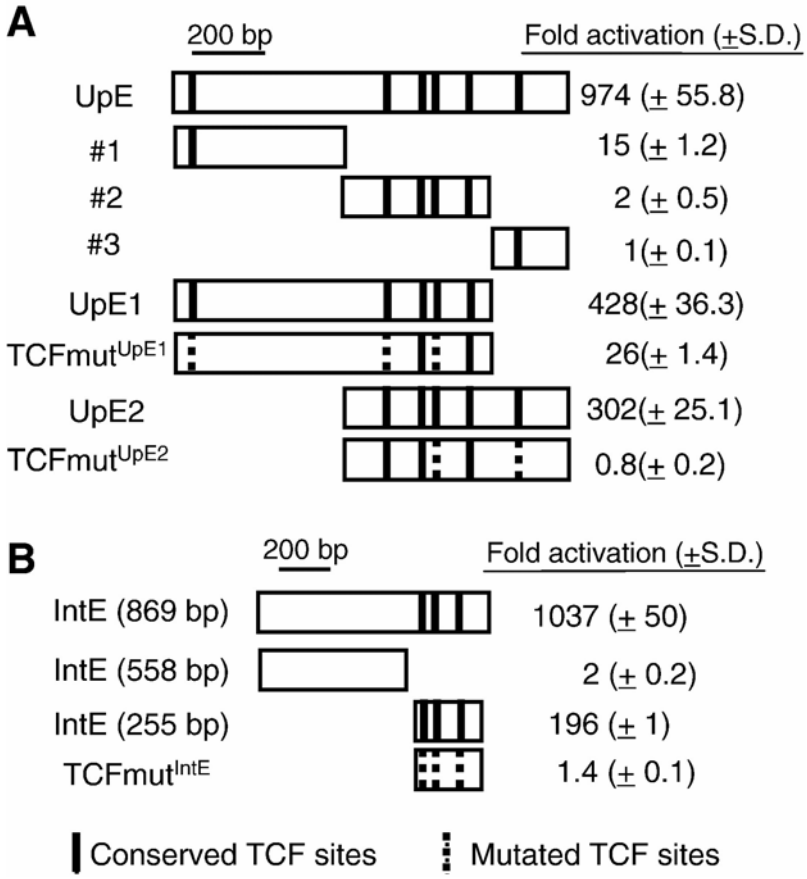


Figure 2.2. Dissection of the UpE and IntE regions reveals WREs that contain functional TCF binding sites. (A) When cloned upstream of a *hsp70* core promoter/luciferase reporter, UpE activated luciferase expression when co-expressed with Arm*. UpE was divided into three fragments (#1 – 3), none of which were highly responsive to Arm*. However, two overlapping stretches (UpE1 and UpE2) possessed strong WRE activity. Vertical lines in the boxes represent the predicted TCF sites that are conserved in 12 *Drosophila* species, while the dotted lines denote mutated TCF sites. UpE1 and UpE2 both require a subset of the TCF sites for Arm* responsiveness. (B) The IntE genomic region contains a 255 bp WRE that requires three TCF-binding sites for Arm* responsiveness. Fold activation is relative to luciferase expression without Arm* expression for each reporter construct. Each result is the mean of triplicate transfections, with the standard deviation indicated in parenthesis. The data shown are a representative example from more than three separate experiments.

A

TCF sites	WREs	
	UpE	IntE
1	GCTTTGAT	GCTTTGTC
2	GCTTTGAA	GCTTTGAG
3	GCTTTGAA	GCTTTGAC(rev)
4	CCATTGAT	
5	GCTTTGTA	
6	GCTTTGTT(rev)	
Consensus	(G/C)(G/C)TTTG(A/T)(A/T)	

Figure 2.3. The sequences and conservation of the TCF sites in the nkd-WREs. Predicted TCF sites that fit the consensus (SSTTTGWW) with one substitution allowed and are conserved in 12 *Drosophila* species (alignment was done via http://genome.ucsc.edu/) are shown. (A) Six TCF sites from UpE and three from IntE (255 bp) were identified. TCF sites in red represent TCF sites that were functional in the reporter assays (Figure 2.4). "(rev)" indicates the site is on the reverse strand. (B, C) Alignments of TCF sites and flanking sequences between *D. melanogaster* and *D. virilis*. We found there is a high correlation between phylogenetic conservation and functionality of the TCF sites. While 3 non-conserved TCF sites in UpE or IntE did not contribute to WRE activity (data not shown), 7 of 9 conserved TCF sites are required for UpE or IntE to be fully activated by Wg signaling. Despite their high similarity to the consensus TCF sequence, the conserved T3 and T5 sites in UpE are dispensable for activation in the reporter assays.

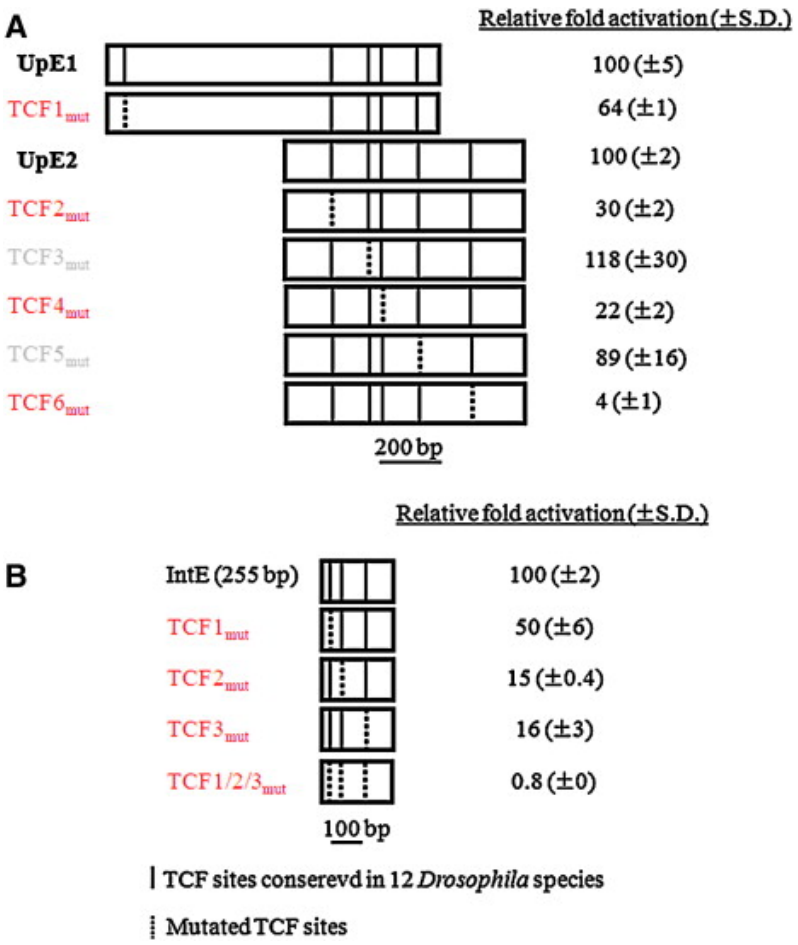


Figure 2.4. Not every TCF-binding site is functional or contributes to WRE activity equally. The indicated reporter constructs were activated by Arm* in Kc cells. The fold activation of UpE1, UpE2 and IntE (255 bp) was normalized to 100. (A) Compared to the UpE1 reporter, the TCF1mut construct showed a 36% reduction in the Wg signaling-responsiveness. The functionality of other TCF sites was tested in the context of UpE2. While the TCF2mut, 4mut and 6mut constructs had reduced WRE activity to different degrees, TCF3mut or 5mut were similar to wild type UpE2. Despite the different contributions of individual TCF sites to WRE function, there is no significant difference in their sequences or conservation (Figure 2.3). (B) In the IntE (255 bp) WRE, three TCF sites contribute to Wg signaling-responsiveness. Individual mutation of these TCF sites reduces the activation of IntE by Arm* and mutation in all three TCF sites completely abolishes the response All experiments shown here were done with triplicate samples (+ S.D.) and the results were reproducible in more than three separate experiments.

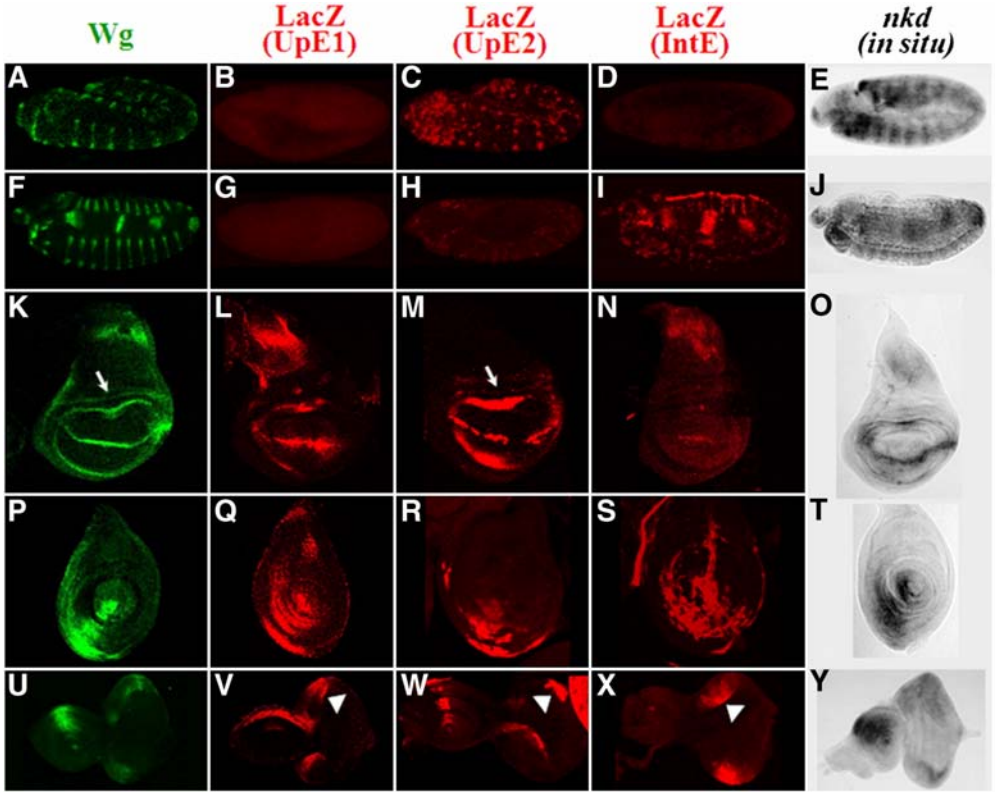


Figure 2.5. ***nkd*-WRE reporter transgenes are expressed in partially overlapping patterns similar to nkd transcript distribution.** The patterns of Wg protein are shown in green, the LacZ patterns of UpE1, UpE2 and IntE reporter transgenes shown in red while nkd transcripts are in grey. Patterns are shown for embryos at stage 11 (A–E), and stage 14 (F–J). Late third instar wing (K–O), leg (Q–S) and eye-antennal (U–Y) imaginal discs are also shown. Arrows indicate the proximal ring of Wg expression (K) and faint lacZ expression in the same region of the UpE2 reporter (M). LacZ expression in eye imaginal discs is marked with arrowheads (V–X). The WRE reporters show overlapping expression domains containing a subset of the endogenous *nkd* pattern. Three independent lines of each WRE reporter showed similar results.

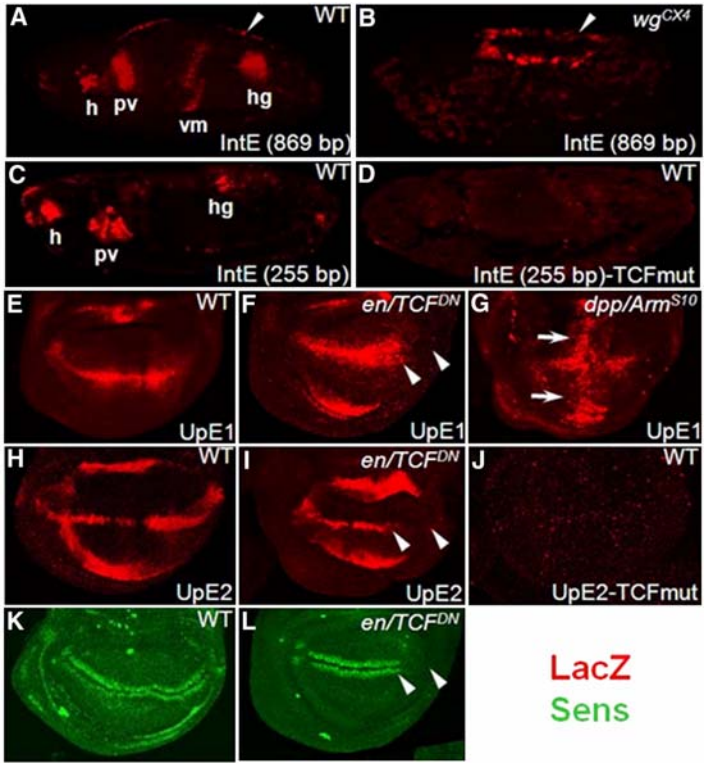


Figure 2.6. The *nkd*-WRE reporters are positively regulated by Wg signaling. (A) Stage 14 embryo containing the *nkd*-IntE reporter stained for LacZ (red). Several expression domains consistent with positive regulation byWg signaling are evident, including regions of the head (h), proventriculus (pv), visceral mesoderm (vm) and hindgut (hg). (B) IntE pattern in a wg mutant embryo. Most of the pattern is absent, except for the dorsal domain indicated by white arrowheads. (C) Stage 14 embryo with a smaller (255 bp) IntE reporter shows staining in a subset of the larger fragment. (D) IntE (255 bp) staining is abolished when the three TCF-binding sites indicated in Fig. 2B are mutated. (E–L) The *nkd*-UpE1 and UpE2 WREs requireWg signaling in late third instar wing imaginal discs. (F, I, L) Expression of a dominant negative form of TCF (TCF^{DN}) in the posterior compartment of the wing pouch (via *En-Gal4*; marked with arrowheads). TCF^{DN} inhibits expression of UpE1 (F), UpE2 (I) and theWg readout Sens (L). The broader expression of lacZ in the anterior compartment of UpE1 discs (F) is likely due to distortion of disc morphology caused by TCF^{DN} expression. The slightly elevated expression of lacZ in the posterior compartment of UpE2 discs (H) is not always observed (see Fig. 3M). (G) Expression of a stable form of Arm (Arm^{S10}) along the anterior/posterior boundary of the wing pouch (via *Dpp-Gal4*; white arrows) results in marked expansion of *nkd*-UpE1 expression. The decrease in lacZ expression at the dorsal/ventral boundary is likely due to distortion of patterning in the *Dpp/*Arm^{S10} discs. (J) Mutation of the two TCF-binding sites in UpE2 (the same ones indicated in Figure 2.2A) abolishes reporter expression in the pouch and hinge regions of the wing imaginal discs. For the TCF mutant constructs, three independent lines were examined with identical results as those shown.

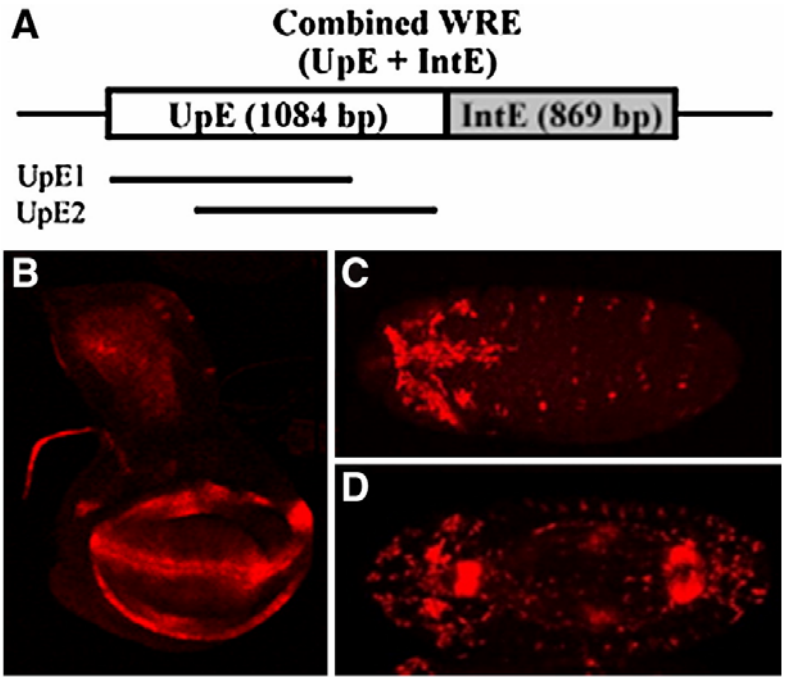


Figure 2.7. The combination of UpE and IntE largely recapitulates the endogenous *nkd* pattern in late larval third instar wing imaginal discs but not in embryos. (A) The combined WRE contains the overlapping regions of UpE1 and UpE2 upstream of the 869 bp IntE in the pH-Pelican lacZ vector. (B) The expression of the combined WRE reporter in the wing disc is very similar to the endogenous *nkd* pattern (Figure 2.5O) except for the proximal ring in the hinge (arrow in Figure 2.9C). The combined reporter appears to be the sum of the three individual WRE reporters in regard to spatial pattern (compare with Figure 2.5L–N). In regard to expression level, the combined reporter is greater in the wing hinge and pouch. The decrease gain of the confocal lasar in this image makes the notum staining less apparent (compare Figure 2.7B to 2.5L). (C, D) Ventral views of stage 11 (C) and stage 14 (D) embryos. The pattern is additive of the UpE2 and IntE WREs (Figure 2.5C, I and 2.6A) and does not fully recapitulate the endogenous *nkd* pattern (Figure 2.5E, J).

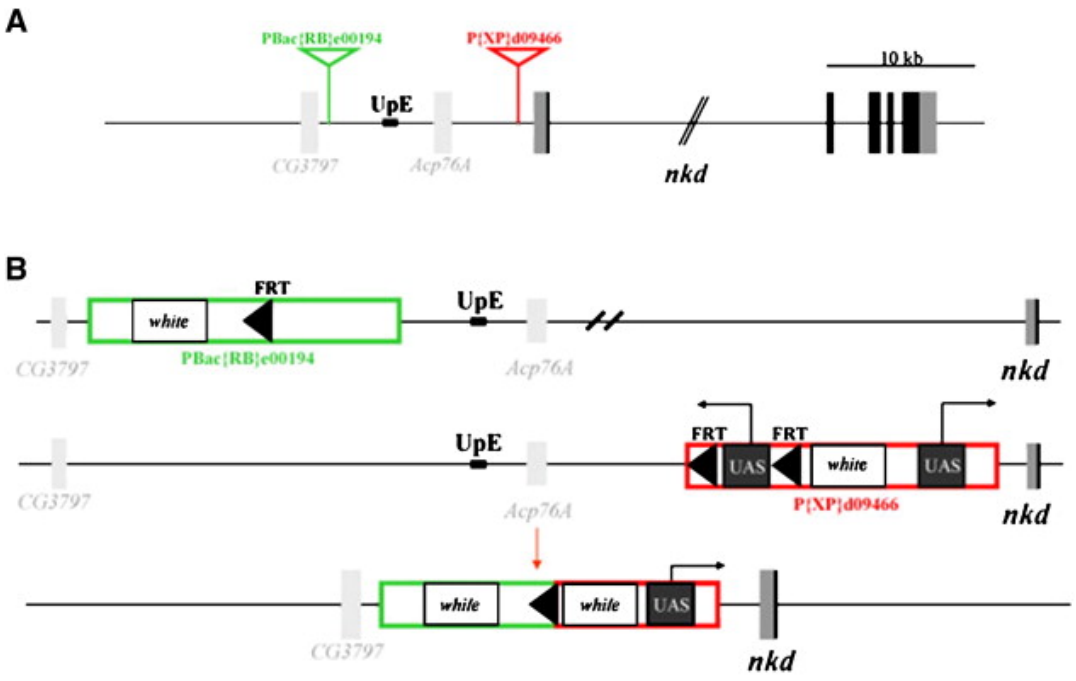


Figure 2.8. Schematic showing the generation of the *ΔUpE* deletion. (A) Two insertions, *PBac{RB}e00194* and *P{XP}d09466*, were used to generate *ΔUpE*. (B) The FRT site present in *PBac{RB}e00194* was recombined to one of the two FRTs present in *P{XP}d09466*. As a consequence, the intervening sequences having UpE and other flanking sequences were removed from one chromosome and the UpE region and Acp76A gene were duplicated on the other chromosome. Flies having the ΔUpE chromosome were selected based on the enhanced eye color and the mutation was confirmed by PCR. To test whether the *P{XP}d09466* transposon insertion influences *nkd* transcription, nkd transcripts in wild type and in *P{XP}d09466* were compared via *in situ* hybridization and no noticeable variation was detected (data not shown).

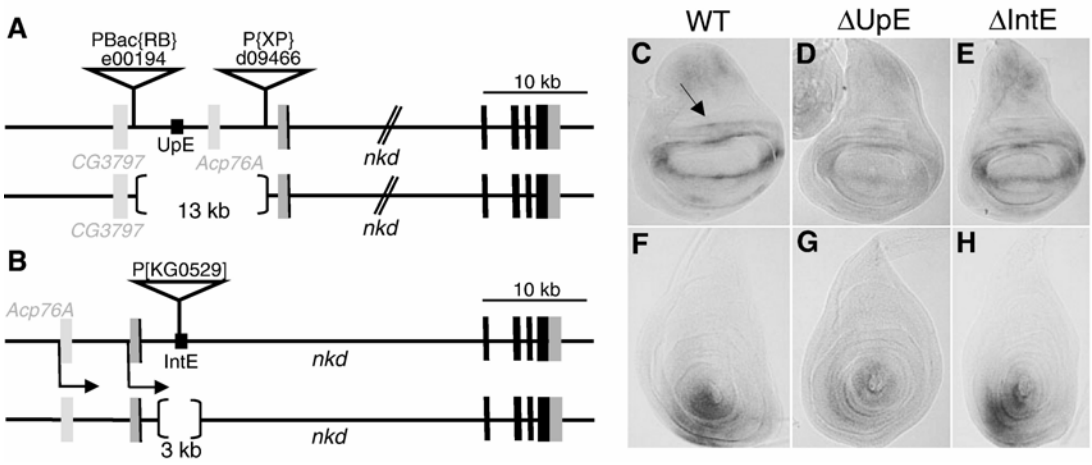


Figure 2.9. Deletion of IntE does not affect *nkd* expression, but a large deletion removing UpE reduces *nkd* expression in wing and leg imaginal discs. (A, B) Cartoon of the *ΔUpE* and *ΔIntE* deletions. See Materials and methods and Figure 2.8 for details of the deletion construction. (C-H) *nkd* transcripts in wing (C–E) and leg (F–H) imaginal discs from *+/Df(3L)ED4782* (C, F) *ΔUpE/Df(3L)ED4782* (D, G) and *ΔIntE/Df(3L)ED4782* (E, H) transheterozygotes. *Df(3L)ED4782* is a large deficiency removing the entire nkd locus. No noticeable decrease of *nkd* expression is detected from imaginal discs (E, H) or embryos (data not shown) when IntE is deleted. *ΔUpE/Df(3L)ED4782* transheterozygotes showed no decrease in *nkd* expression in the embryo (data not shown) but displayed a marked reduction in the wing and leg imaginal discs (D, G). This reductionwas consistently observed in three independent in situ analyses and was also confirmed by qRT-PCR (see text).

Table 2.1

Fold activation of WREs

	Arm*		Wg-CM
WREs	S2R+	Clone8	Kc167
UpE1	225 (±26)	40 (±2)	32 (±5)
UpE2	270 (±12)	348 (±18)	20 (±4)
IntE(255 bp)	66 (±6)	109 (±2)	126 (±8)

Table 2.2

Summary of lacZ expression of the UpE1, UpE2 and IntE *nkd*-WRE reporters

Tissues	*nkd*-WREs		
	UpE1	UpE2	IntE
Early embryos (stages 10–12)	−	+	−
Late embryos (stages 13–14)	−	−	+
Eye imaginal discs	+	+	+
Antennal imaginal discs	+	+	−
Leg imaginal discs (epithelia)	+	+	+/−
Leg disc (peripodal membrane)	+/−	+	+
Wing disc (notum)	+	−	+
Wing disc (D/V boundary)	+	+	+
Wing disc (hinge-distal ring)	+	+	−

The relative strength of expression in several tissues is indicated. All imaginal discs are from late third instar larva. See text for more details.

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CHAPTER III

ACTIVATION OF WINGLESS TARGETS REQUIRES BIPARTITE RECOGNITION OF DNA BY TCF

ABSTRACT

Specific recognition of DNA by transcription factors is essential for precise gene regulation. In Wingless (Wg) signaling in *Drosophila*, target gene regulation is controlled by TCF. The HMG domain of TCF is known to bind to specific sequences, but whether this interaction is sufficient for recognition of Wingless targets is controversial. Here, we identified a new cis-regulatory element named the Helper site that is essential for the activation of several Wg Response Elements (WREs) in various fly tissues. TCF binding to its established site is enhanced by the presence of a Helper site. This increased binding requires the C-clamp of TCF, which is also essential for the transcriptional activation of several WREs. Our data support a model where TCF recognizes DNA through a bipartite mechanism involving both the HMG domain and C-clamp. This information was used to identify additional WREs through a genome-wide search for TCF-Helper site clusters.

INTRODUCTION

Many aspects of development and physiology require precise regulation of an

organism's transcriptome. This is achieved in large part through sequence-specific recognition of DNA by transcription factors. In order to find their appropriate targets among the vast excess of genomic chromatin, these proteins employ various strategies to enhance DNA recognition. For example, the p53 tumor suppressor binds DNA as a homotetramer, with each subunit contacting a quarter site. Thus a typical p53 binding site contains 20 bases of specific DNA information (Riley et al., 2008). Many transcriptional factors enhance their specificity for DNA through cooperative binding with other transcription factors, e.g., HOX proteins and Extradenticle/Pbx (Mann and Affolter, 1998; Moens and Selleri, 2006). POU proteins contain two distinct DNA binding domains, a POU-specific domain and a homeodomain, allowing recognition of 8-9 bp motifs (Herr and Cleary, 1995). In all these cases, a single DNA binding domain is not sufficient for recognition of transcriptional targets.

This report is concerned with target gene identification in Wnt/β-catenin (Wnt/β-cat) signaling, which controls many aspects of normal development and adult tissue homeostasis in animals (Klaus and Birchmeier, 2008; Logan and Nusse, 2004). The key determinant in this pathway is the stability of β-cat, called Armadillo (Arm) in flies. In the absence of signaling, β-cat/Arm is constantly degraded. When Wnt stimulates cells, β-cat/Arm turnover is blocked and the protein accumulates in the nucleus where it binds to members of the TCF/LEF1 (TCF) family of transcription factors. This TCF-β-cat/Arm complex then recruits co-activators to induce the transcription of Wnt target genes (Parker et al., 2007; Stadeli et al., 2006).

TCFs contain a High Mobility Group (HMG) domain that is necessary and sufficient for DNA binding (Laudet et al., 1993). This domain makes extensive minor

groove contacts with the DNA and results in a large bend in the DNA (Love et al., 1995). These HMG domains preferentially bind to a consensus of CTTTGATS (S = G/C) (Laudet et al., 1993; van de Wetering et al., 1997). Functional motifs matching this consensus have been identified in many Wnt target genes (Brannon et al., 1997; Chamorro et al., 2005; He et al., 1998; Tetsu and McCormick, 1999; Yamaguchi et al., 1999). Placing multiple copies of this TCF site upstream of a minimal promoter results in reporters that are specifically activated by Wnt/β-cat signaling, suggesting that the HMG domain of TCFs is sufficient for target gene recognition (Barolo, 2006).

The fruit fly *Drosophila* offers a relatively simple system to study regulation of Wnt targets by TCF. Flies contain one TCF family member, sometimes referred to as Pangolin (but hereafter referred to as TCF). TCF is required for regulation of many targets by Wingless (Wg, a fly Wnt) in embryonic and larval development (Brunner et al., 1997; van de Wetering et al., 1997). While the HMG domain of fly TCF binds to typical sites with high affinity (van de Wetering et al., 1997), studies of several Wnt response elements (WREs) in Wg target genes have found that functional TCF binding sites can vary significantly from the consensus (Chang et al., 2008a; Lee and Frasch, 2000; Riese et al., 1997). Given the degeneracy of DNA recognition by TCF, potential binding sites are found with high frequency throughout the genome. This suggests that additional sequence information may exist to specify which TCF sites can act as WREs.

The loose consensus of TCF binding sites has presented a challenge in the identification of WREs using bioinformatics. An algorithm called enhancer element locator (EEL) based on TCF binding affinity, density of sites and phylogenetic conservation has been used to identify WREs in the mammalian genome (Hallikas et al.,

2006). While EEL found several well-known WREs, it failed to identify many others. In addition, there was minimal overlap between the potential WREs identified by EEL and the sites bound by TCF4 in a human cell line determined by a genome-wide Chromatin immunoprecipitation (ChIP) screen (Hatzis et al., 2008). At present, *in silico* approaches to identify WREs are hampered by the large number of potential TCF sites in the genome.

One mechanism to enhance the specificity of TCF binding to WREs could be combinatorial interactions with other transcriptional factors. Consistent with this, activation of several WREs in flies and vertebrates have been found to require inputs from other signaling pathways (e.g., (Halfon et al., 2000; Han et al., 2002; Knirr and Frasch, 2001; Nishita et al., 2000; Riese et al., 1997). However, there is no direct evidence that TCF binding to WREs is influenced by interactions with other DNA-binding proteins.

In this report, we identified a new cis-regulatory element named the TCF Helper site (Helper site) that is essential for activation of several WREs in cell culture and in flies. As the name implies, this motif potentiates the ability of TCF binding sites to mediate transcriptional activation by Wg signaling. The presence of a Helper site increased the binding affinity of TCF to a classic TCF site. This enhanced binding was dependent on the presence of the C-clamp, a domain recently identified to enhance DNA binding of some vertebrate TCF isoforms (Atcha et al., 2007). Activation of several WREs containing functional Helper sites was C-clamp dependent. Interestingly, there is no apparent spacing or orientation requirement for TCF-Helper site pairs. Despite this, we were able to identify WREs in known Wg targets as well as two new WREs through a genome-wide search for clusters of TCF-Helper site clusters. Our data argue that for

many targets, DNA binding by the HMG domain of TCF is not sufficient for transcriptional regulation, likely because the protein cannot distinguish WREs from random TCF sites. This problem is overcome by a physical interaction between the C-clamp and the Helper site which increases the affinity of TCF to bona fide WREs. This bipartite recognition of DNA enables TCF to locate and activate WREs.

RESULTS

Helper sites are crucial for activation of WREs in the *nkd* locus by Wg signaling

Our laboratory has previously characterized the regulation of the *naked cuticle* (*nkd*) gene by Wg signaling in the *Drosophila* cell line Kc167 (Kc). ChIP studies revealed that TCF is preferentially bound to a region of the first intron of *nkd* approximately 5 kb downstream of the transcription start site (TSS) (Fang et al., 2006; Parker et al., 2008). In addition, TCF is localized to a region 10 kb upstream of the TSS (Chang et al., 2008a). These two TCF bound regions contain WREs which are highly responsive to pathway stimulation in reporter assays. These WREs contain consensus sites for the HMG domain of TCF (hereafter referred to as TCF binding sites) that are essential for Wg activation, indicating direct regulation by the pathway (Chang et al., 2008a; Li et al., 2007).

The *nkd* locus encompasses over 50 kb of DNA and contains numerous clusters of potential TCF binding sites (Parker et al., 2008). This suggests that the presence of TCF sites is not sufficient to account for the localized binding of TCF to the two regions of *nkd* mentioned above. In addition, several of the TCF site clusters not bound by TCF in ChIP assays are unresponsive to Wg signaling in reporter gene assays (Parker et al.,

2008) (Figure 3.1 and Table 3.1). These data argue for the presence of information in addition to TCF binding sites to specify a stretch of DNA as a functional WRE.

To identify the additional sequence information in WREs required for activation by Wg signaling, the WRE in the first intron of *nkd*, referred to as *nkd*-IntE was further studied. This WRE has been localized to 255 bp and contains three functional TCF sites (Chang et al., 2008a)(Figure 3.3A and 3.4A). Systematic mutagenesis of the entire *nkd*-IntE with non-overlapping 10 bp substitutions revealed the presence of other motifs besides the TCF sites that are required for activation by a stabilized form of Arm (Arm*; Figure 3.2 and 3.3). Among them, two motifs contain 7 nucleotides (GCCGCCA), which is hereafter referred to as a TCF Helper site (Helper site) (Figure 3.3B and 3.4A). Mutation of either Helper site significantly reduced activation of the WRE (Figure 3.3B) and simultaneous mutation of both motifs resulted in a 100-fold decrease in Arm*-responsiveness (Figure 3.3B and 3.4A).

The mutagenesis of the *nkd*-IntE found another element that is required for the activation (Region B in Figure 3.2). The element (CGGTTTGCTT) resembles the TCF sites (SSTTTGWW, S: G/C, W: A/T). However, the mutations of the element that are supposed to destroy the binding of TCF didn't reduce the activation of the *nkd*-IntE, suggesting the element doesn't serve as a TCF site (Figure 3.3). Currently, Cadigan lab is trying to understand the mechanism by which the new element (CGGTTTGCTT) contributes the activity of the *nkd*-IntE.

Our analysis of *nkd*-IntE suggests that WREs may require both TCF sites and Helper sites to respond to Wg signaling. Using an online algorithm Target Explorer (http://luna.bioc.columbia.edu/Target_Explorer/) (Sosinsky et al., 2003), the *nkd* locus

was searched for additional TCF/Helper site clusters (2 TCF sites- 2 Helper sites within 200 bp). Besides the one in IntE, a second cluster was identified in a WRE from the region upstream of the *nkd* TSS, referred to as *nkd*-UpE2. This WRE contains two TCF sites essential for activation by Wg signaling (Chang et al., 2008a)(Figure 3.4A). As seen for *nkd*-IntE, mutation of the two Helper sites in *nkd*-UpE2 resulted in a drastic reduction in activation by Arm* (Figure 3.4A).

To test the functional importance of Helper sites in *nkd*-WREs in fly tissues, *nkd*-IntE and *nkd*-UpE2 were cloned into the pH-Pelican LacZ reporter (Barolo et al., 2000) and introduced into the fly genome by P element transgenesis. The *nkd*-UpE2 and *nkd*-IntE reporters exhibit LacZ expression in partially overlapping patterns similar to *nkd* transcript distribution in the dorsal/ventral boundary and hinge region of the wing imaginal disc (Figures 3.4B and 3.4C) and at the dorsal and ventral edges of the eye disc (Figures 3.4F and 3.4G). Consistent with being direct targets of TCF, the activity of *nkd*-WREs was abolished by mutation of the TCF sites (Chang et al., 2008a)(Figures 3.4D and 3.4H). Strikingly, mutation of the Helper sites in these WREs also completely abolished their activity (Figures 3.4E and 3.4I). A similar requirement for the Helper sites was observed in other larval tissues where these WREs are normally active (Figure 3.5). These results demonstrate that Helper sites are indispensable for Wg responsiveness of *nkd*-WREs in a broad range of tissues.

Functional Helper sites are also present in WREs from other Wg targets

To determine whether Helper sites are required for the activation of WREs in other Wg target genes, a WRE of *Notum* (also called *wingful*, or *wf*) was examined (Figure

3.6A and 3.7A). *Notum* encodes a secreted negative-feedback inhibitor of Wg signaling and the gene is activated by Wg signaling in various tissues throughout *Drosophila* development (Gerlitz and Basler, 2002; Giraldez et al., 2002) and in Kc cells (Parker et al., 2008). *Notum*-UpEB` is a derivative of a 2.2 kb WRE called *wf-luc* that was previously identified (Hoffmans et al., 2005) (Figure 3.7A). *Notum*-UpEB` has several predicted TCF sites and Helper sites (Figure 3.6A). Mutations in the TCF sites or the Helper sites greatly compromised the activation of *Notum*-UpEB` by Arm* or by Wg conditioned medium (Wg-CM) in Kc cells (Figure 3.6A; data not shown).

In the context of a transgenic fly reporter construct, the *Notum*-UpEB` WRE directed LacZ expression in a pattern consistent with activation by Wg signaling in a broad range of tissues. For example, expression in the epidermis of stage 11 embryos mirrors that of Wg (Figures 3.6B and 3.6C) and the same is true in the pouch and notum of wing imaginal discs (Figures 3.6F and 3.6G). These expression patterns were largely TCF site-dependent (Figures 3.6D and 3.6H) and mutation of the three Helper sites also resulted in a drastic reduction in reporter expression (Figures 3.6E and 3.6I). TCF and Helper sites were also required for *Notum*-UpEB` activity in other imaginal discs (Figure 3.7). As with the *nkd* WREs, Helper sites are critical for this *Notum* WRE to respond to Wg signaling.

Nkd and Notum are feedback antagonists that are activated by Wg signaling in many (perhaps all) fly tissues throughout *Drosophila* development (Gerlitz and Basler, 2002; Giraldez et al., 2002; Zeng et al., 2000). However, most Wg target genes are regulated in a cell-specific manner at particular developmental stages (Cadigan, 2002;

Sanson, 2001). This raises the possibility that Helper sites are only found in broadly activated WREs.

To address this issue, a WRE from the *sloppy paired* (*slp*) locus (slp5`-2) was examined. As found for the *slp* genes, this WRE is directly regulated by Wg signaling in a tissue-specific manner, being activated by the pathway in the ectoderm and mesoderm of fly embryos at germband extension (Lee and Frasch, 2000). slp5`-2 has four predicted Helper sites. Mutation of the third Helper motif, which is adjacent to a functional TCF site (Lee and Frasch, 2000) caused a large reduction in the responsiveness of slp5`-2-luciferase reporter to Arm* in Kc cells (Figure 3.8A). Mutation of the other Helper sites had very mild or no effect (data not shown). In embryos, mutation of the third Helper site in slp5`-2 caused a large reduction in LacZ expression in the epidermis and mesoderm (Figures 3.8C versus 3.8F). The results extend the importance of Helper sites to tissue-specific targets of Wg signaling.

To better define what constitutes a functional Helper site, the eight identified motifs were aligned (http://weblogo.berkeley.edu) to generate a consensus (Figure 3.8H). To provide functional support for this consensus, the second Helper site in *nkd*-IntE was subjected to fine scale mutagenesis (Figure 3.8I). The substitution of the individual nucleotides in first four bases almost abolished the activity of the reporter (Figure 3.8I). In addition, positions 5-7 of this Helper motif were also found to be required for full WRE activity (Figure 3.8I). Thus all seven nucleotides of the motif contribute to its activity.

Helper sites augment TCF site-mediated transcriptional activation in response to Wg signaling

To learn more about the mechanism of how Helper sites function, a series of synthetic reporters were constructed using multiple copies of TCF sites and/or Helper sites cloned upstream of a *hsp*70 core promoter/luciferase reporter (Figure 3.9A). Multimerized TCF sites are known to be activated by Wnt signaling in a variety of assays (Barolo, 2006). Consistent with this, constructs containing three (3TCF) or six (6TCF) copies of TCF sites were substantially activated by Arm* (Figure 3.9A). In contrast, Helper sites alone (three, six or twelve copies) didn't respond to Arm* (Figure 3.9A and data not shown).

Although Helper sites were transcriptionally inert by themselves, they potently augmented the ability of TCF sites to respond to Wg signaling. TCF/Helper site pairs in three (3TH) or six (6TH) copies showed a much greater activation by Arm* in Kc cells than constructs with the same number of TCF sites (Figure 3.9A). Replacing the Helper sites in 3TH with random sequence (3TS) reduced the fold activation 50-fold, arguing that the Helper site effect was not due to spacing of the TCF sites. Similar data was obtained in clone 8 cells and when Wg-CM was used to stimulate the pathway (Figure 3.10). The presence of Helper sites clearly enhances the responsiveness of TCF sites to Wg signaling.

The ability of Helper sites to enhance TCF site activity was also observed in the transgenic fly reporter assay. A construct containing six TCF sites (6TCF) had some reporter expression in several tissues, most notably the embryonic epidermis (Figure 3.9D) and the presumptive notum of the wing imaginal disc (Figure 3.9M). This

construct had very little or no detectable expression in eye-antennal and leg imaginal discs (Figures 3.9G and 3.9J). A reporter with six copies of the Helper site did not exhibit significant expression in any tissue examined (data not shown). However, when Helper sites flank the TCF sites (6TH), a dramatic enhancement of reporter gene expression was observed. The pattern in embryos and eye-antennal discs was consistent with positive regulation by Wg (compare Figures 3.9B and 3.9E with 3.9C and 3.9F, repectively) and the 6TH pattern was completely missing in *wg* mutant embryos (data not shown). In leg discs, the 6TH reporter was active in the ventral portion of the tissue (Figure 3.9I), consistent with activation by Wg (Figure 3.9H), with the peripodial membrane expressing high levels of the reporter (Figure 3.9I). In the wing disc, 6TH has enhanced expression in the notum (Figure 3.9L) compared to 6TCF (Figure 3.9M). In the wing pouch, 6TH has strong expression throughout the pouch (Figure 3.9L), though the pattern is variable even within a single transgenic line (data not shown). The reason for this variation is not clear, but 6TH can be further activated by Arm* expression in the pouch (data not shown). As was found in cultured cells, the presence of Helper sites markedly enhances the ability of TCF sites to respond to Wg signaling.

Helper sites physically and functionally interact with the C-clamp domain of TCF

Fly TCF is similar to some vertebrate isoforms of TCF (e.g., TCF-4E and TCF-1E) which contain a Cysteine rich domain termed the C-clamp (Atcha et al., 2007). Human C-clamp exhibits non-specific DNA binding activity on its own, but in the context of the full length protein it allows the TCF isoforms to bind to an extended sequence (Atcha et al., 2007). This extended sequence (RCCG) is somewhat similar to the Helper site,

raising the possibility that the C-clamp of fly TCF interacts with the Helper site, thereby enhancing TCF site-mediated transcriptional activation.

This possibility was tested by electrophoretic mobility shift assay (EMSA). Because full length TCF could not be expressed recombinantly, a TCF fragment containing the HMG domain and C-clamp fused to glutathione-S-transferase (GST-TCF) was expressed and purified. GST-TCF binds to an oligonucleotide containing a classic TCF site (ATCAAAGG) and a Helper site (probe TH; see Figure 3.11A) much more efficiently than to an oligonucleotide containing the TCF site only (probe TS; Figure 3.11B). Substitutions of the first five amino acids in the C-clamp of GST-TCF (see Figure 3.12A) weakened the affinity of the protein for the TH probe (Figure 3.11C). These results indicate that the enhanced binding of GST-TCF to the TCF-Helper site probe is C-clamp dependent.

While the C-clamp is required for optimal binding to a TCF-Helper site tandem, disruption of the C-clamp actually increased binding to the probe containing only a TCF site (Figures 3.11C). The basis for this increased effect is not clear, though it is reproducible (e.g., Figure 3.11F).

To test the specificity of the interaction between GST-TCF and the TCF-Helper site probe, competition assays were performed. Binding to the labeled probe was significantly reduced by excess amounts of unlabeled TH oligonucleotides (Figure 3.11D). However, competitors containing mutations in either the TCF sites or Helper sites (or both motifs) did not reduce binding of GST-TCF to the TH probe (Figure 3.11D). These results confirm that both the TCF and Helper sites are required for the specific binding of TCF to the tandem TCF-Helper site.

Can TCF bind to the Helper site independently of an adjacent TCF site? Under the same conditions as previous experiments (Figures 3.11B-D), the interaction between GST-TCF and a Helper site only (SH) probe was not observed (data not shown). However, increasing the concentrations of both GST-TCF and SH probe allowed a weak interaction to be observed (Figure 3.11E). Mutations in the C-clamp of GST-TCF abolished the interaction, implying that the C-clamp interacts directly with the Helper site.

Helper sites are clearly important for several WREs to respond to Wg pathway activation, but interestingly, their spacing and orientation in regard to the TCF sites is variable (Figure 3.11G). To test whether our recombinant GST-TCF can bind to TCF-Helper site pairs in more than one orientation, we tested a TCF-Helper site oligonucleotide (T(r)H) where the TCF site orientation is reversed compared to the one used in Figure 3.11B-3.11E. Like the TH oligonucleotide, T(r)H was bound more efficiently than an oligo with a TCF site alone (TS) and the enhanced binding was C-clamp dependent (Figure 3.11F). This indicates that TCF can recognize more than one orientation of a TCF-Helper site pair, consistent with the lack of a consistent orientation in endogenous WREs.

The finding that the C-clamp increases the affinity of TCF-DNA interaction *in vitro* suggests that this motif is required for the activation of WREs containing functional Helper sites. To address this question, Kc cells with endogenous TCF depleted by RNAi were transfected with Arm*, various WRE reporter constructs and either wild-type TCF or TCF containing the C-clamp mutation (the transgenic TCFs are not targeted by the TCF dsRNA used for RNAi). For all reporters examined, expression of wild-type TCF rescued the defect in Arm* responsiveness caused by TCF RNAi (Figure 3.12B). In stark

contrast, the TCF C-clamp mutant did not have the ability to rescue activation of the *nkd*-IntE/luciferase and *wf-luc* reporters (Figure 3.12B), despite being expressed at similar levels as wild-type TCF (Figure 3.12C). The one exception was the reporter containing six multimerized TCF sites (6TCF), which was rescued by the mutant TCF (Figure 3.12B). Unlike the endogenous WREs, this synthetic enhancer does not contain Helper sites. These results support a model where the C-clamp of TCF plays an essential role in the activation of Helper site-dependent WREs by Wg signaling.

New WREs identified by genome-wide search for clusters containing both TCF sites and Helper sites

Our results demonstrate that Helper sites are essential for the activity of several WREs and that they likely function as a docking site for the C-clamp of TCF. This suggests that additional WREs can be identified *in silico* by searching for TCF-Helper site clusters. One challenge with this strategy is the fact that the spacing and orientation of Helper sites in relation to nearby functional TCF sites vary significantly among the known TCF-Helper site pairs (Figure 3.11G). The lack of a confined spacing/orientation for the TCF-Helper sites means that any genome-wide search is likely to suffer from a large degree of false positives.

Despite the difficulties outlined above, we attempted to identify new WREs using the web-based program Fly enhancer (http://genomeenhancer.org/fly) (Markstein et al., 2002). The entire fly genome was searched for clusters of TCF sites and Helper sites using stringent parameters in order to reduce the number of hits to a manageable number: two TCF sites (SSTTTGWW) and two Helper sites (GCCGCC) within 100 bases. 97

clusters were identified. These positives were further prioritized by organization (selecting hits where the TCF and Helper sites alternated), proximity of the TCF sites to Helper sites and phylogenetic conservation. After these secondary screens, seven clusters were selected for reporter assays in Kc cells (Figure 3.13). Fragments of approximately 500 bp surrounding the clusters were cloned upstream of a *hsp*70 core promoter/luciferase reporter. Two positives out of seven displayed more than 10-fold activation of luciferase expression in response to Arm* (Figures 3.13 and 3.14). These two TCF-Helper site clusters were further characterized.

Both positives are located in or near genes which are potential targets of Wg signaling. One positive (cluster 1) is located within the first intron of *ladybird late* (*lbl*), known to be regulated by Wg signaling in muscle progenitors of *Drosophila* embryos (Jagla et al., 1998) (Figure 3.14A). Cluster 3 is found 15.2 kb upstream of *pxb*, a gene that is expressed in the regions similar to Wg expression domain in the midline cells and late stage embryos (Inaki et al., 2002; Wheeler et al., 2006) (Figure 3.14B). Whether these genes are directly regulated by Wg signaling hasn't been examined.

To determine whether the TCF and Helper sites from clusters 1 and 3 are functional, they were altered and tested for Arm* responsiveness. Mutations in the TCF sites of either cluster compromised their ability to respond to Wg signaling, suggesting the identified clusters are directly regulated WREs (Figure 3.14C). Mutation of the Helper sites in these WREs also resulted in a large reduction in Wg responsiveness (Figure 3.14C). These results further highlight the functional importance of Helper sties in WREs and illustrate how they can be used to facilitate identification of WREs *in silico*.

DISCUSSION

Helper sites are essential for Wg signaling-mediated transcriptional regulation

TCF is required for Wg signaling in embryonic and larval tissues (Brunner et al., 1997; van de Wetering et al., 1997). The HMG domain of TCF is sufficient for binding to the CTTTGATS consensus (van de Wetering et al., 1997) and several studies have shown that similar sequences are required for WRE activation of several Wg targets (e.g.,(Chang et al., 2008a; Lee and Frasch, 2000; Riese et al., 1997)). In cell culture, synthetic reporters containing multimerized TCF binding sites are sufficient for transcriptional activation by Wg signaling (DasGupta et al., 2005; Lum et al., 2003). These studies are consistent with the notion that the presence of TCF sites is necessary and sufficient to specify a stretch of DNA as a WRE.

There are several problems with the TCF site only model for WRE function in flies. First, naturally occurring WREs do not have the high density clustering of TCF sites that are used in the synthetic WREs which are responsive to pathway stimulation in cultured cells (Barolo, 2006). Reporters containing 3 or 4 multimerized TCF sites are not active in transgenic fly assays (Barolo, 2006; Riese et al., 1997). Though we found that six copies can mediate some activation, it was much weaker than synthetic enhancers containing TCF and Helper sites (Figure 3.9). In the endogenous Wg target *nkd*, there are many TCF site clusters, but only two are occupied by TCF in Kc cells (Chang et al., 2008a; Parker et al., 2008). These TCF-bound regions correspond to the functional WREs referred to in this report as UpE2 and IntE (Figure 3.4). Other regions containing TCF sites did not have WRE activity in reporter gene assays (Figure 3.1). These

observations suggest that in the context of native genes, TCF sites are not sufficient for recruitment of TCF and subsequent activation of transcription by Wg signaling.

Through unbiased mutagenesis of *nkd*-IntE, we identified two motifs of identical sequence (Helper sites) that are essential for Wg signaling responsiveness (Figure 3.3 and 3.4). Helper sites are also present in several other WREs (*nkd*-UpE2, *Notum*-UpEB` and slp5`-2), where they are just as important as TCF sites for Wg responsiveness in cell culture and a variety of fly tissues from embryos to larvae (Figures 3.4, 3.5, 3.6, 3.7 and 3.8). Unlike TCF sites, multimerized Helper sites do not respond to Wg signaling (Figure 3.9A). However, Helper sites enhance the ability of TCF sites to be activated by the pathway (Figure 3.9B-M). These results suggest that Helper sites, defined by the consensus GCCGCCR (Figure 3.8H) are important cis-regulatory elements frequently used in Wg signaling in order to supplement the function of TCF sites.

Helper sites may be a common strategy for WRE function in *Drosophila*. If the search parameters are relaxed (one substitution from the consensus), then Helper sites are present near functional TCF sites in WREs from the *Ultrabithorax* (*Ubx*) and *even-skipped* (*eve*) loci (Knirr and Frasch, 2001; Riese et al., 1997) as well as *nkd*-UpE1 (Chang et al., 2008a). Since our knowledge of which sequences constitute a Helper site is still rudimentary, site-directed mutagenesis will be required to confirm whether these Helper sites are functional.

nkd-IntE contains another element (CGGTTTGCTT) that contributes the activation by Wg signaling a lot (Figure 3.2 and 3.3). Despite the important role of this element in the activation of the *nkd*-IntE, the mechanism by which this element works remains unknown. This element seems not to function as a TCF site (Figure 3.3), and

doesn't have any obvious transcription factor binding sites. Analysis of the function of the new element in the *nkd*-IntE in fly tissues and the fine mutagenesis of this element will be required to determine the function of this element.

Bipartite recognition of DNA by TCF

What is the mechanism by which Helper sites promote the activation of WRE by Wg signaling? EMSA data strongly argue that the presence of a Helper site adjacent to a classic TCF binding site greatly enhances the ability of TCF to bind to the DNA (Figure 3.11B). The enhanced binding to the TCF/Helper site pair was dependent on the presence of an intact C-clamp in TCF (Figure 3.11C). As the C-clamp was not required for binding of TCF to the TCF site alone (Figure 3.11C), this implies a direct physical interaction between Helper sites and C-clamp. Indeed, at high concentrations TCF is able to bind to the Helper site (with no TCF site present) in a C-clamp-dependent manner (Figure 3.11E). However, Helper sites alone cannot mediate Wg activation of transcription (Figure 3.9 and data not shown). This suggests that the low affinity interaction between the C-clamp and Helper site is not sufficient for TCF recognition of DNA. Rather, the data are consistent with high affinity DNA binding of TCF occurring through simultaneous HMG domain-TCF site and C-clamp-Helper site interactions.

The importance of C-clamp in activating Wg targets was confirmed in Kc cells by rescuing the signaling defects of *TCF* RNAi by transfected *TCF*. Mutation of the C-clamp did not prevent *TCF* from rescuing activation of a synthetic reporter containing multimerized TCF sites (Figure 3.12B). However, other reporters containing functional Helper sites were only activated by wild type *TCF* having an intact C-clamp (Figure

3.12B). These results suggest that the physical interaction between C-clamp and Helper sites increases the efficiency of TCF binding to WREs, thereby allowing Wg signaling to identify and regulate its target genes.

A role of the C-clamp in Wg target gene regulation is also supported by the phenotype of a *TCF* mutant allele (pan^{13a}) in fly embryos. This allele contains a missense mutation in the fifth position of the C-clamp (A374V; see Figure 3.12A) (van de Wetering et al., 1997). The pan^{13a} homozygote mutants show a weak segment polarity phenotype that indicates some reduction in Wg signaling activity. More strikingly, the ability of a constitutively active form of Arm to alter embryonic cell fate was largely abolished in a pan^{13a} mutant background (van de Wetering et al., 1997). We suspect that this point mutation may only partially compromise the activity of the C-clamp and thus underestimate the importance of this motif for TCF function.

Several transcription factors increase the specificity of their respective target sites by binding to DNA as homo or heterooligomers, e.g, p53 (Riley et al., 2008) and Hox proteins (Mann and Affolter, 1998; Moens and Selleri, 2006). In contrast, fly TCF appears to solve this problem by bipartite recognition of DNA, with the binding of both the HMG domain and C-clamp to their respective DNA sites effectively doubling the size of the TCF recognition site. In this way, TCF is similar to Pax3, which contains both a paired domain (PD) and a paired-class homeodomain (HD). The PD of Pax3 can bind a specific DNA sequence on its own, while the Pax3-HD cannot (Chalepakis et al., 1994). However, in the presence of both DNA binding motifs, the HD of Pax3 does make specific DNA contacts, increasing the overall affinity of Pax3 for the extended recognition site (Apuzzo and Gros, 2007; Chalepakis et al., 1994; Fujioka et al., 1996).

Thus, like TCF, Pax3 combines a stronger and weaker DNA-binding domain to achieve high affinity, high specificity binding to its cognate site.

One notable feature of the TCF-Helper site pairs present in WREs is that they are orientation-independent and have variable spacing (see Figure 3.11G). This is consistent with the *in vitro* findings that the Helper site can enhance binding of TCF to the traditional site in either orientation (compare Figure 3.11B with 3.11F). The HMG and C-clamp domains are separated by just 27 residues (Figure 3.12A) so it is difficult to see how binding to the TCF-Helper site could be so spatially flexible. The ability of the HMG domain to induce a sharp bend in the DNA (Love et al., 1995) may contribute to the ability of TCF to recognize the binding sites with different orientations.

The C-clamp appears to be widely used in TCF function in *Drosophila*, but is this the case in mammalian systems? The C-clamp was first identified in specific "E box" isoforms of the human *TCF1* and *TCF4* genes (TCF-1E and TCF-4E), where it enables these proteins to recognize an extended binding sequence (Atcha et al., 2007). The extended binding site (RCCG) has some similarity to the Helper site and is present in the *Lef-1* and *Cdx* promoters, which are Wnt signaling targets (Atcha et al., 2007). Activation of a Lef-1 reporter by TCF-1E is C-clamp dependent (Atcha et al., 2007). While the C-clamp is likely to be critical for the E-box isoforms to bind to DNA, most mammalian TCFs lack this domain. It is possible that TCFs which lack a C-clamp can bind DNA through their HMG domains with greater specificity than fly TCF. However, we think it is more likely that additional mechanisms to enhance HMG domain binding to the classic TCF site exist for all family members to achieve high specificity DNA recognition.

Helper sites aid in the *in silico* identification of WREs

Can knowledge of the Helper site be exploited to identify new WREs through computational means? A search through the *Drosophila* genome identified seven TCF-Helper site clusters, two of which possessed high levels of WRE activity in Kc cells (Figure 3.13 and 3.14). Mutation of either the TCF sites or Helper sites in these WREs severely reduced responsiveness to Wg signaling (Figure 3.14C). Interestingly, both these WREs lie near genes, i.e., *lbl* and *pxb*, that are known to be activated by Wg signaling or expressed in a striped patterns in the embryo consistent with activation by Wg signaling (Inaki et al., 2002; Jagla et al., 1998; Wheeler et al., 2006). We are currently investigating whether these WREs are responsible for activation of these potential Wg targets.

While this initial success is encouraging, more information is required to increase the chances of success in searching for WREs by sequence alone. The primary difficulty lies in the lack of consistency in the spacing and orientation of the TCF-Helper sites. Additional research may shed light on possible restrictions in orientation/spacing, and should further refine the Helper site consensus. Having said this, our existing knowledge of the Helper site consensus should be valuable in locating WREs within target genes already known to be activated by Wg, as evidenced by our success in identifying the *nkd*-UpE2 and *Notum*-UpEB` WREs (Figures 3.4 and 3.6). The characterization of additional TCF site-Helper site pair in other WREs should help elucidate the parameters by which this motif enhances TCF binding and transcriptional activation in response to Wg signaling.

EXPERIMENTAL PROCEDURES

Drosophila cell culture, RNAi and Wg-CM treatment

Kc cells were cultured as described previously (Parker et al., 2008). Clone 8 cells were cultured as described (http://flyrnai.org/DRSC-PRC.html#Cl8). RNAi-mediated gene knockdowns were performed as described (Blauwkamp et al., 2008). For the TCF rescue assays, double-stranded RNA corresponding to the TCF 3`UTR was added to the medium (10μg/10^6 cells). After 4 days, cells were diluted to 10^6 cells/ml and transfected with the plasmids indicated in the figure legends. Three days later, cells were harvested for reporter assays. The primer sequences to generate TCF dsRNA are provided in the supplemental data. Wg-CM and control medium were prepared as described (Fang et al., 2006). 10^6 Kc cells were treated with 500 μl of Wg-CM for 24 hr prior to harvesting.

Transient transfections, reporter gene assays and purification of transfected cells

Transient transfections and reporter assays were carried out as described (Blauwkamp et al., 2008; Fang et al., 2006). For reporter assays, a mixture of plasmids containing 100 ng of luciferase reporters, 100 ng of pAcArm* and 10 ng of pAcLacZ (Invitrogen) were co-transfected into 10^6 Kc cells. For the TCF rescue assays, 50 ng of luciferase reporters, 2 ng (for 6TCF) or 10 ng (for *nkd*-IntE and *wf-luc*) of pAcArm*, 20 ng of pAc TCF-V5 or pAc mutTCF-V5 and 10 ng of pAcLacZ were co-transfected. For all reporter assays shown, each result represents the mean of two independent transfections, with the standard deviation indicated. The data shown are representative of multiple independent experiments. Luciferase activity in the absence of Arm* was normalized to 1.0 for each construct unless otherwise mentioned in figure legends.

Purification of transfected cells was performed as described (Blauwkamp et al., 2008). Briefly, cells were co-transfected with 100 ng of pAc-IL2α (Ogawa et al., 2002) along with pAc TCF-V5 or pAc mutTCF-V5. Three days later, transfected cells were harvested with anti-CD25 magnetic beads (Dynabeads) and resuspended in protein loading buffer for western blot analysis using mouse anti-V5 antibody (Invitrogen) and anti-tubulin antibody (Sigma).

Plasmids

The pAcArm* construct was described previously (Blauwkamp et al., 2008). pAc TCF-V5 was generated by substituting the stop codon of pAc TCF (Blauwkamp et al., 2008) with Alanine (TAA ➔ GCA). Luciferase reporter constructs containing *nkd*-WREs, *Notum*-WREs, slp5-2` and clusters 1-7 are derivatives of pGL3-Basic Vector (Promega) containing a *hsp*70 minimal promoter (Blauwkamp et al., 2008). In order to mutate TCF sites or Helper sites in these WREs, QuickChange II (Stratagene) was used. Base substitutions were A to C or T to G (or vise versa). Three positions in the TCF binding sites (SSTTTGWW) or seven positions in Helper sites (eg. GCCGCCA) were substituted in all luciferase reporters except UpE2, cluster1 and cluster3. In UpE2, all eight nucleotides of TCF binding sites were mutated. In cluster1 and cluster3, only two positions in Helper sites (eg. GCCGCCA) were mutated. The underlined sequences indicate the positions mutated. Primer sequences to generate the WRE reporters can be found in the Table 3.2. Luciferase reporter constructs containing multimerized TCF sites or Helper sites were derivatives of pGL2-Basic Vector containing a *hsp*70 minimal promoter. For analysis in fly tissues, WRE/LacZ reporters were generated by cloning

WREs into pH-Pelican (Barolo et al., 2000) and introduced into fly genome by P-element transgenesis (performed by BestGene Inc.).

Immunostaining, in situ hybridization and microscopy

Immunostaining and in situ hybridization were performed as described (Parker et al., 2002). Briefly, rabbit anti-LacZ (1:500) (Abcam Inc.) and mouse anti-Wg antisera (1:100) (Developmental Studies Hybridoma Bank at the University of Iowa) were used. Samples were examined by using Leica confocal miscroscope DM6000 B (Leica) and processed in Adobe Photoshop 7.0. Probes for in situ hybridization of *nkd* transcript were generated by amplifying the genomic DNA. Primer sequences can be found in the supplemental data. Three to five independent transgenic lines of each construct were assayed and typical representative images are shown.

Electrophoretic mobility shift assay

The GST-TCF expression plasmid was generated by replacing the HMG box in GST-HMG expression plasmid (Lee and Frasch, 2000) with a TCF fragment containing both the HMG box and C-clamp (amino acids 271-408) using the EcoRI and XhoI restriction sites. Primer sequences to clone the TCF fragment are provided in the supplemental data. GST-TCF wild-type and C-clamp mutant proteins were expressed and purified from *Escherichia coli*. EMSA was performed by using the LightShift Chemiluminescent EMSA Kit (Pierce) and the Chemiluminescent Nucleic Acid Detection Module (Pierce) per the manufacturer's instructions. Briefly, proteins in 10% glycerol and DNA oligos were incubated with 50ug/ml poly (dI-dC), 0.05% NP-40, 5

mM $MgCl_2$ and 2µl of 50% glycerol in the presence of binding buffer (10 mM Tris–HCl, pH 7.5, 50 mM KCl, 1 mM DTT). For the competition assay, unlabeled DNA oligos were incubated with reaction mixtures containing proteins for 10 min prior to adding labeled DNA oligos. The concentrations of proteins and oligos used in each experiment were indicated in figure legends.

ACKNOWLEGMENTS

The authors are indebted to M. Waterman for sharing data concerning the C-clamp in vertebrate TCFs before publication. We also thank H. Li, Y. Ni and C. Bhambhani for discussions and careful reading of the manuscript. This work was supported by an AHA predoctoral fellowship to M. V. C. and NIH grant GM08894 to K. M. C.

THIS CHAPTER WAS PUBLISHED AS:

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, 1877-81.

In this Chapter, Chang, J. L. generated the data as below:
Figure 3.4A(the reporter assay results with the *nkd*-UpE2), B, C, D, E and F
Fgirue 3.5A

In this Chapter, Gangopadhyay, A. generated the data under my supervision as below:
Figure 3.6A

In this Chapter, Shearer, A. generated the data under my supervision as below:
Figure 3.8A

FIGURES

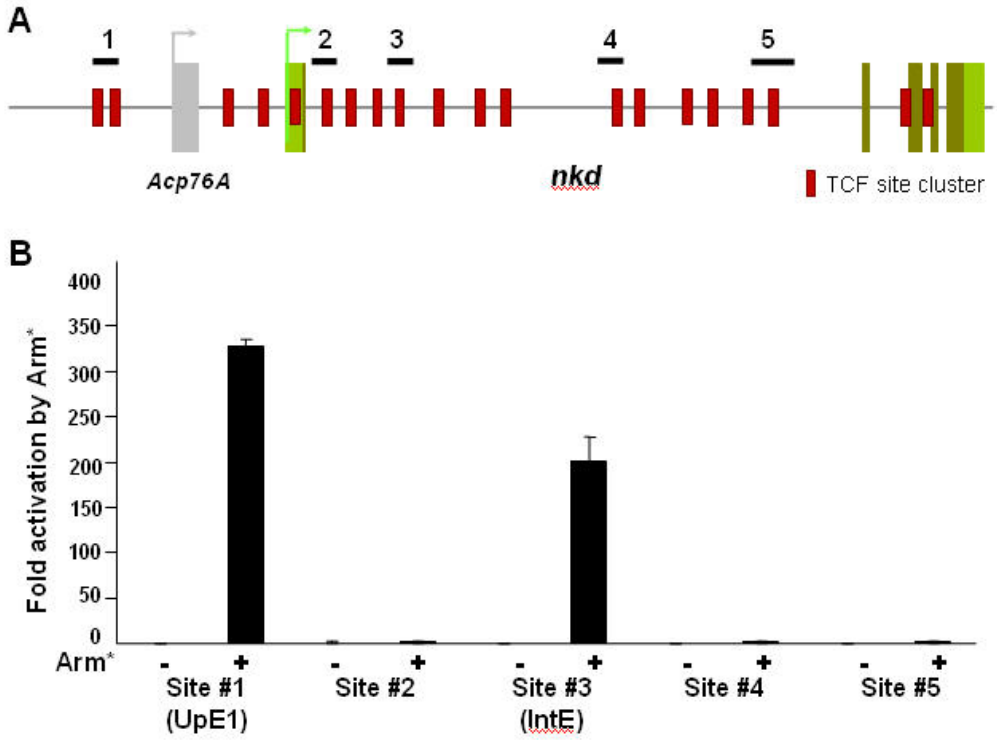


Figure 3.1. Not all TCF clusters respond to Wg signaling. (A) Schematic diagram of *nkd* loci showing the locations of TCF clusters (red boxes) and of genomic fragments (1-5, black lines) used for reporter assays (B). TCF clusters were identified by searching *nkd* loci using a stringent parameter (three TCF sites within 150 bases) with an online algorithm Target Explorer (Sosinsky et al., 2003). TCF is preferentially bound to Site 1 and Site 3 in Kc cells (Chang et al., 2008a; Fang et al., 2006; Parker et al., 2008). (B) Fragments of approximately 1000 bp surrounding the TCF clusters were cloned upstream of a *hsp*70 core promoter/luciferase reporter. Each reporter was co-transfected with an Arm* expression plasmid in Kc cells and luciferase activity was measured. Two reporters containing Site 1 or Site 3 where TCF bind are significantly activated by Arm*. However, Site 2, Site 4 and Site 5 not bound by TCF are unresponsive to Wg signaling, suggesting that not all genomic regions containing TCF sites can function as WREs.

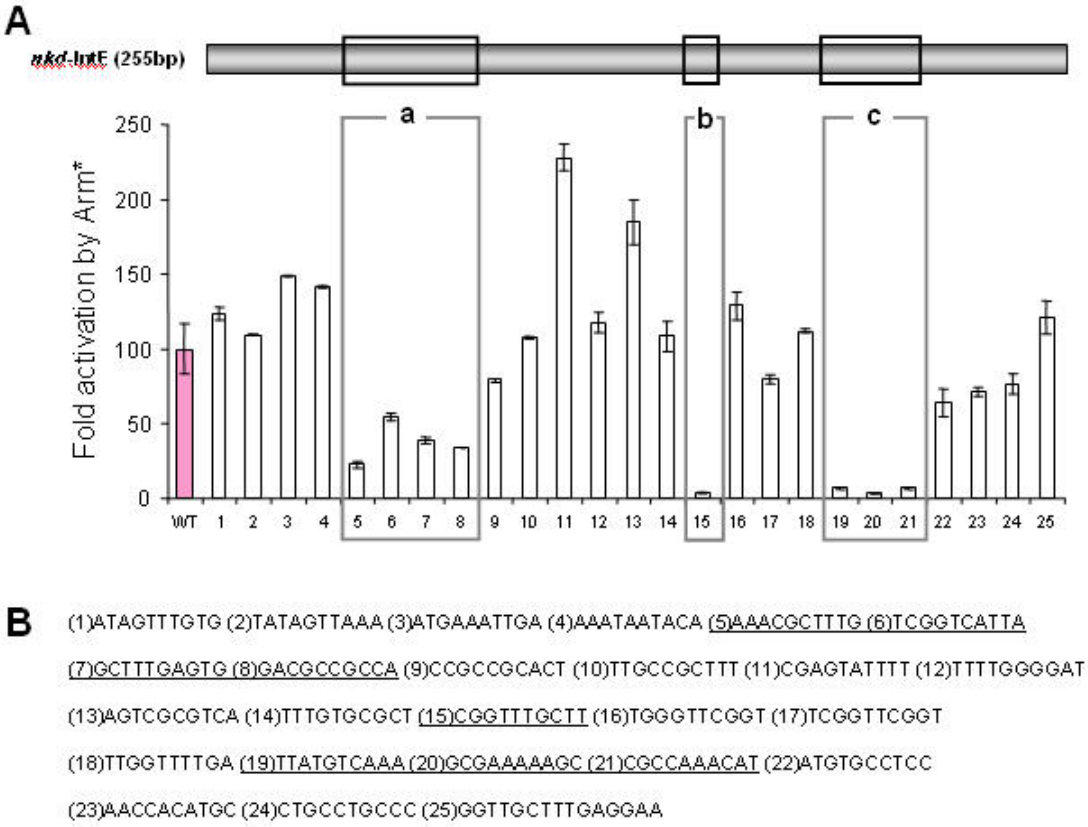


Figure 3.2. Systematic mutagenesis of *nkd*-IntE identified three regions required for Wg responsiveness. (A) Cartoon of the *nkd*-IntE showing three regions (the black outlined squares) that are necessary for Wg responsiveness, and reporter assay results with *nkd*-IntE and its mutants. The twenty five *nkd*-IntE mutant reporters (1-25) were generated by mutating the *nkd*-IntE reporter with non-overlapping 10 bp substitutions (B). Each reporter was co-transfected with an Arm* expression plasmid in Kc cells and luciferase activity was measured. This unbiased approach identified three regions (a, b and c) that are required for the activation of *nkd*-IntE. (B) The sequence of *nkd*-IntE showing the mutated nucleotides in each reporter. The sequences in three functional regions are underlined.

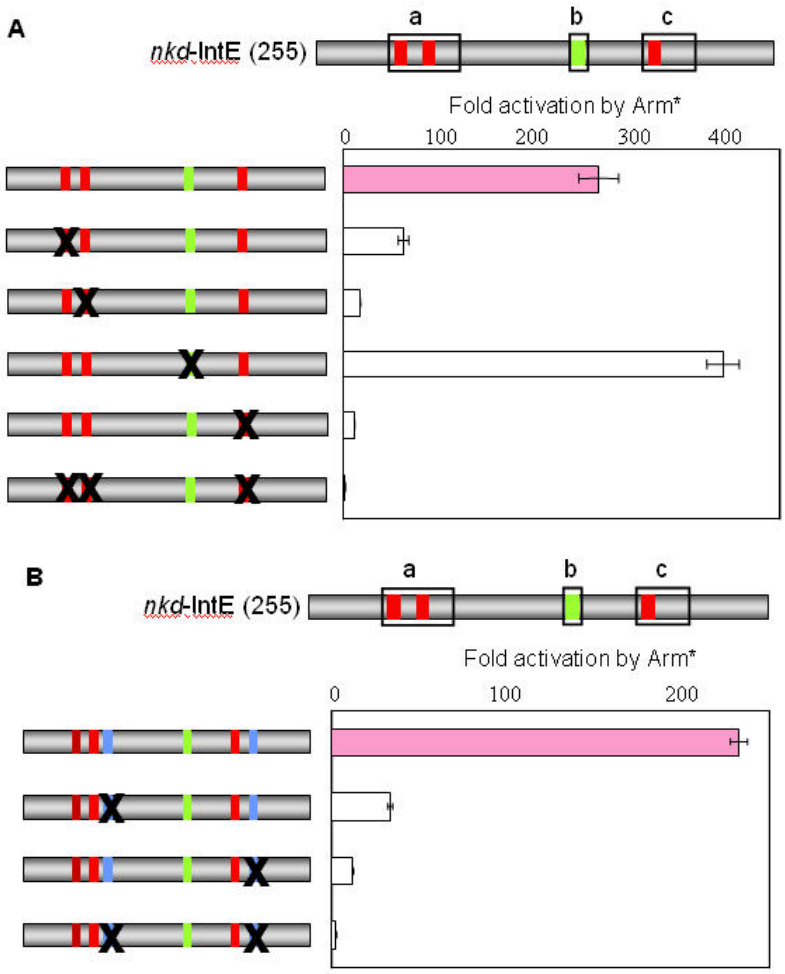


Figure 3.3. *nkd*-IntE has three TCF sites and two Helper sites essential for activation by Wg signaling. Cartoon of the *nkd*-IntE showing three regions (the black outlined squares) that are necessary for Wg responsiveness and its mutations. TCF sites in red, Helper sites in blue and the uncharacterized site in Region b in green. *nkd*-IntEs were cloned upstream of a hsp70 core promoter/luciferase reporter and were activated by Arm* in Kc cells. (A) Potential TCF sites closely matching the consensus (SSTTTGWW) were found in Region a (GCTTTGTC and GCTTTGAG), Region b (GGTTTGCT) and Region c (GTCAAAGC). Three point mutations (SSTTTGWW, mutated positions are underlined) that can prevent TCF from binding (data not shown) were generated in each potential TCF sites. Mutations of each TCF site in Region a and in Region c significantly reduced the activation of *nkd*-IntE. Simultaneous mutations of these TCF sites almost abolished the Wg responsiveness of *nkd*-IntE. Mutation of the predicted TCF site in Region b didn't affect the activation of *nkd*-IntE, suggesting Region b does not function as a TCF site. (B) Region a and Region b contain identical 7 nucleotide sequences (GCCGCCA) termed Helper sites. Mutation of either Helper site significantly reduced activation of *nkd*-IntE. Mutation of both motifs resulted in a 100-fold decrease in Arm*-responsiveness.

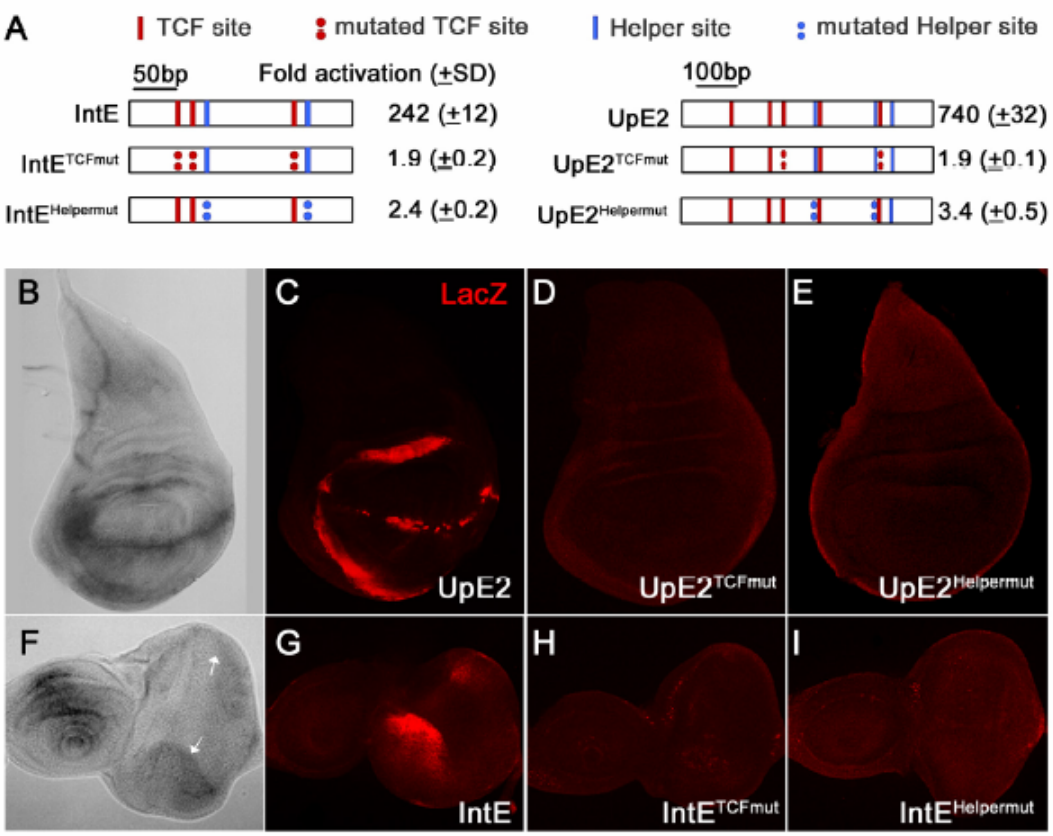


Figure 3.4. Helper sites are crucial for activation of *nkd*-WREs. (A) Cartoons of the *nkd*-IntE and UpE2 WREs showing predicted TCF sites, Helper sites and their mutations. UpE2 and IntE were cloned upstream of a *hsp*70 core promoter/luciferase reporter. These reporters were highly activated by Arm* in Kc cells. This activation was TCF and Helper site dependent. (B, F) Endogenous *nkd* transcripts in the wing (B) and eye-antennal (F) imaginal discs of third instar larvae detected by in situ hybridization. The white arrows indicate the dorsal and ventral regions of the presumptive eye where *nkd* is expressed. Confocal images of wing imaginal discs from P[UpE2-lacZ] flies (C-E) and eye-antennal discs from P[IntE-lacZ] flies (G-I) immunostained for LacZ. Both reporters are active in patterns that partially overlap that of *nkd* transcripts. The activity of UpE and IntE was abolished by mutations in the same TCF sites (D, H) and Helper sites (E, I) indicated in panel A.

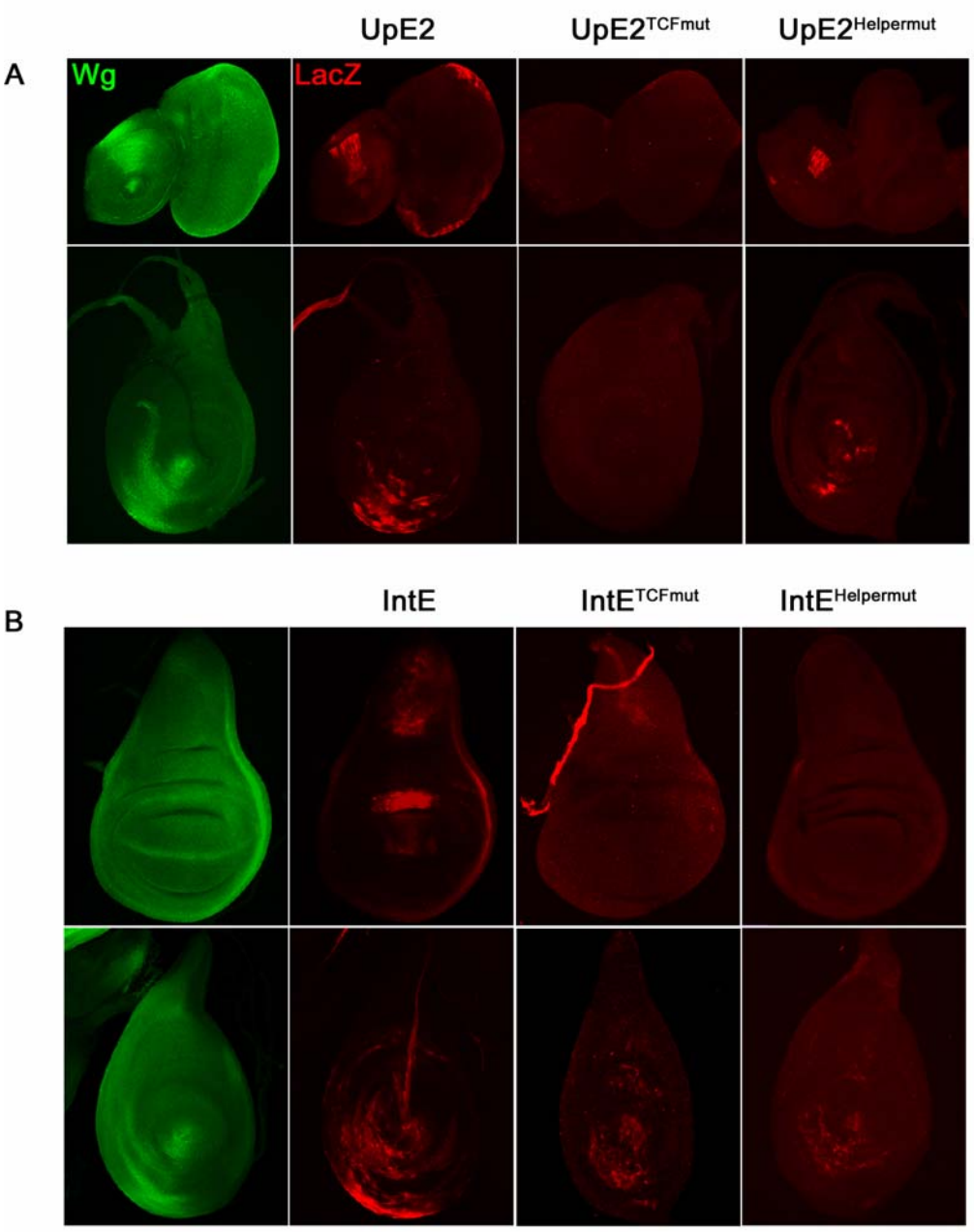


Figure 3.5. Helper sites are crucial for activation of *nkd*-WREs in various imaginal discs. Confocal images of imaginal discs in third instar larvae carrying UpE2/LacZ or IntE/LacZ reporters immunostained for Wg (green) and lacZ (red). UpE2 activates LacZ expression in eye-antenna discs (A. upper panels) and leg discs (A. lower panels) in a similar pattern to Wg. IntE induces the expression of LacZ in the wing (B. upper panels) and leg imaginal discs (B. lower panel) in partially overlapping patterns similar to Wg expression. Mutation of TCF sites or Helper sites significantly reduced LacZ expression in these tissues.

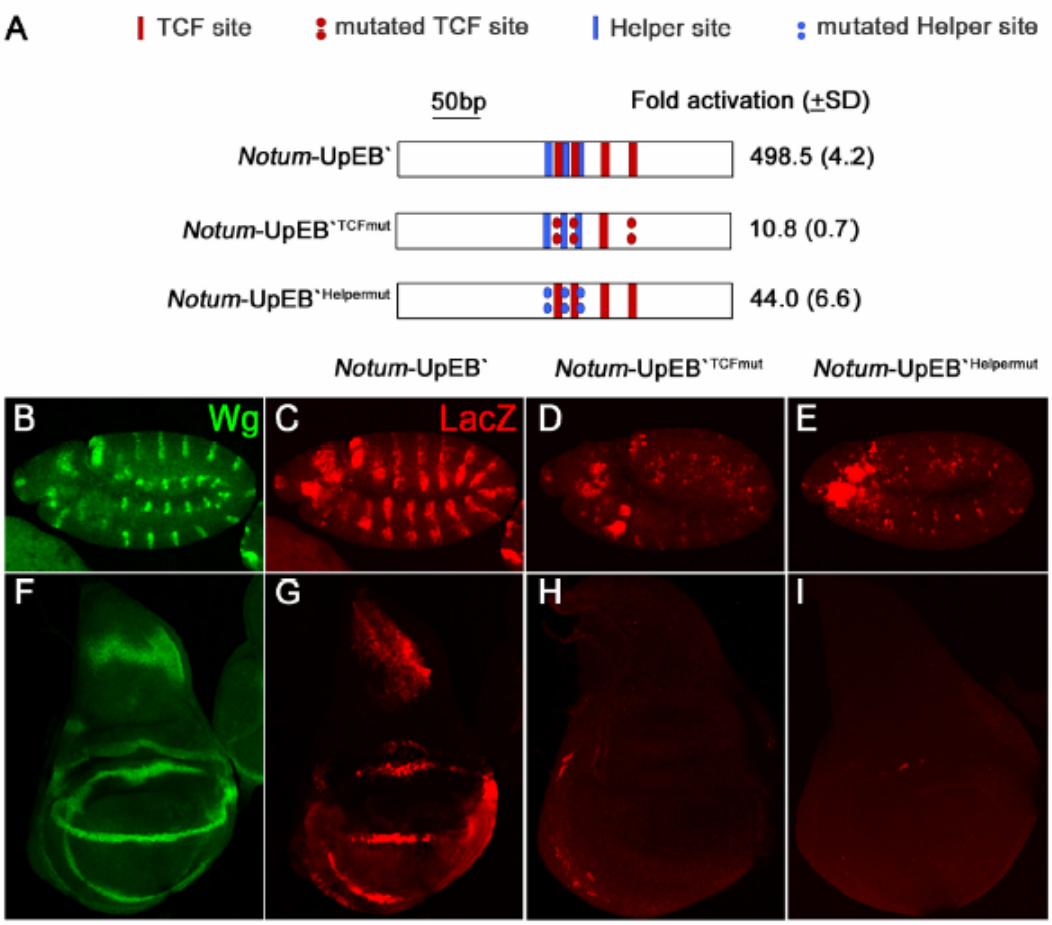


Figure 3.6. A *Notum*-WRE contains Helper sites that are essential for Wg signaling responsiveness. (A) Cartoon depicting the *Notum*-UpEB` reporter and a summary of Arm* responsiveness in Kc cells. Mutations in the TCF or Helper sites compromise the activation of *Notum*-UpEB` by Arm*. (B-E) Confocal images of stage 11 embryos of P[*Notum*-UpEB`] flies immunostained for Wg (green) and LacZ (red). The wild-type reporter is expressed in a similar pattern to Wg (B, C). Mutation of the TCF (D) or Helper sites (E) dramatically reduced LacZ expression in the segments but had little effect on domains of expression in the head. (F-I) Confocal images of third instar wing imaginal discs of P[*Notum*-UpEB`] flies immunostained for Wg (green) and LacZ (red). The reporter was expressed in a similar pattern to Wg (F, G) and was TCF and Helper site-dependent (H, I).

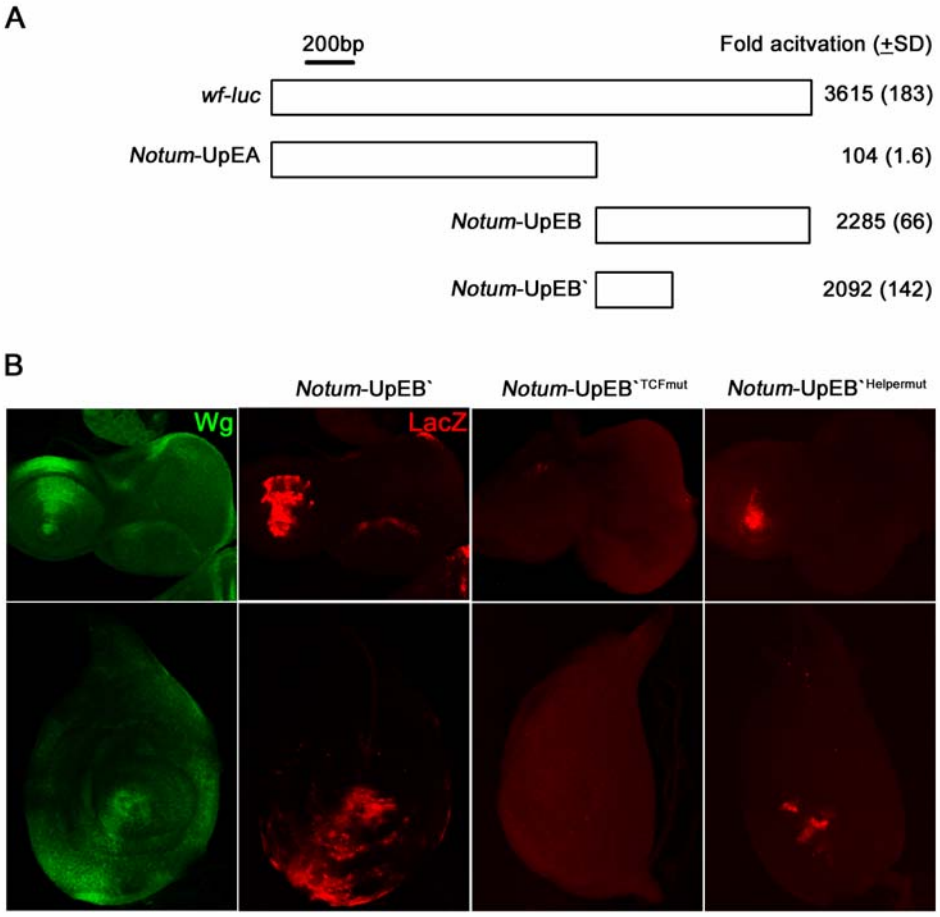


Figure 3.7. Helper sites are important for the activity of a *Notum*-WRE in fly tissues. (A) Cartoons of *Notum*-WREs and summary of reporter assay results in Kc cells. The 2.2 kB *wf-luc* construct previously identified (Hoffmans et al., 2005) is highly activated by Arm*. This fragment was divided into two halves (*Notum*-UpEA and *Notum*-UpEB). *Notum*-UpEB` is a 3` deletion of *Notum*-UpEB. The data shown are representative of multiple independent experiments. (B) The function of TCF sites and Helper sites in *Notum*-UpEB` was tested in the transgenic reporter flies. Eye-antenna discs (upper panels) and leg discs (lower panels) in third instar larvae carrying the *Notum*-UpEB` reporter were immunostained for Wg (green) and LacZ (red). Confocal images of these tissues are shown. *Notum*-UpEB` activates LacZ expression in the similar pattern to the Wg expression domain. This expression is significantly reduced by mutation of the Helper sites as well as mutation of three TCF sites.

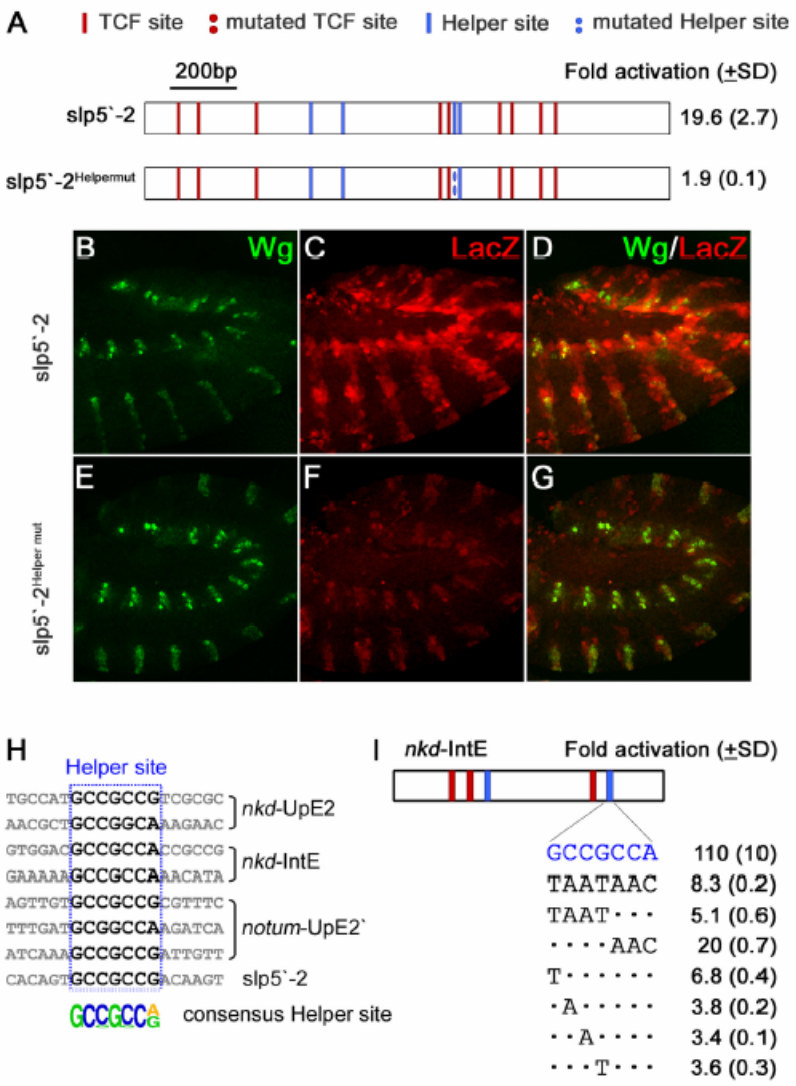


Figure 3.8. A tissue specific WRE from the *slp1* locus contains a functional Helper site and the Helper site consensus. (A) Cartoon depicting the *slp5`-2* reporter illustrating the presence of a Helper site that is essential for activation by Arm*. (B-G) Confocal images of P[*slp5`-2*-lacZ] embryos at late stage 11 immunostained for Wg (green) and LacZ (red). Consistent with a previous report (Lee and Frasch, 2000), the reporter was active in ectodermal stripes and the mesoderm (white arrows). Mutation of the single Helper site shown in panel A significantly reduced the LacZ expression in both germ layers. (H) Alignment of functional Helper sites from several WREs with the consensus sequence. (I) Dissection of a Helper motif from *nkd*-IntE by site-directed mutagenesis. The wild-type Helper sequence is shown in blue and mismatched bases for each mutant construct are indicated in black. These *nkd*-IntE reporters were activated by overexpressing Arm*. The data indicate that the first four positions are critical, and that positions 5-7 also contribute to Wg signaling responsiveness.

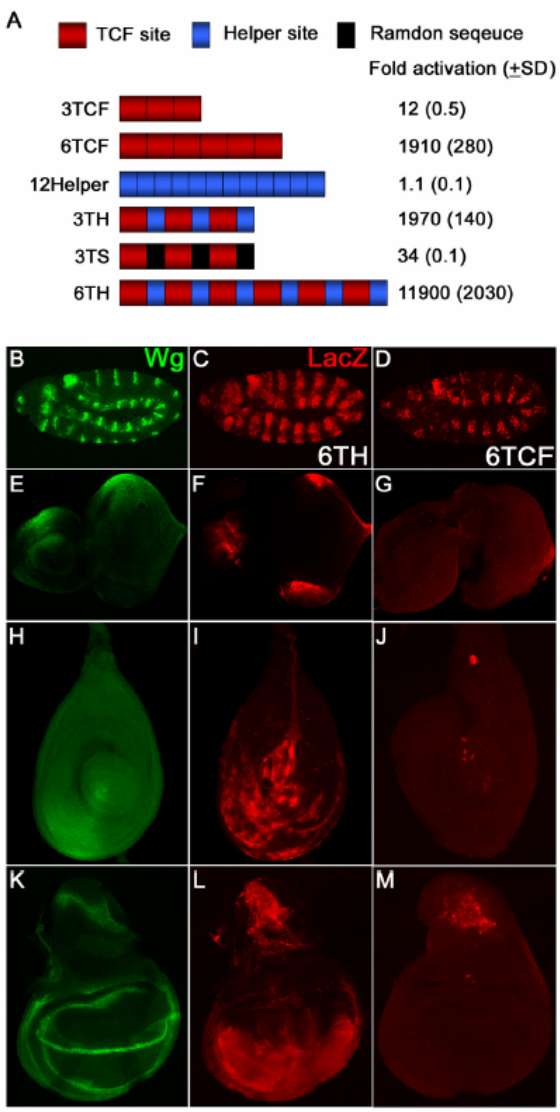


Figure 3.9. Helper sites have no activity by themselves but can augment TCF site-mediated transcriptional activation by Wg signaling. (A) TCF sites or Helper sites were multimerized and cloned upstream of a *hsp*70 core promoter/luciferase reporter and tested for activation by Arm*. TCF sites (3TCF and 6TCF) are responsive to Arm* but Helper sites alone (12Helper) are not. However, Helper sites adjacent to TCF sites greatly enhance activation by Arm* (3TCF vs. 3TH, 6TCF vs. 6TH). This effect is not observed when the Helper sites in 3TH are replaced by random sequence (3TS). (B-M) Confocal images stained for Wg (green) and lacZ (red) from early stage 11 embryos (B-D), late third instar eye-antennal imaginal discs (E-G), leg discs (H-J) and wing discs (K-M). Embryos and discs containing a six TCF-Helper site tamden (6TH) cloned upstream of a *hsp70* core promoter/lacZ reporter were double-stained for Wg and lacZ (B, C, E, F, H, I, K, L) while only the lacZ pattern is shown for the construct with six TCF sites (6TCF; D, G, J. M). As with cultured cells, the presence of Helper sites greatly increased the ability of the reporters to be expressed in a pattern consistent with activation by Wg signaling.

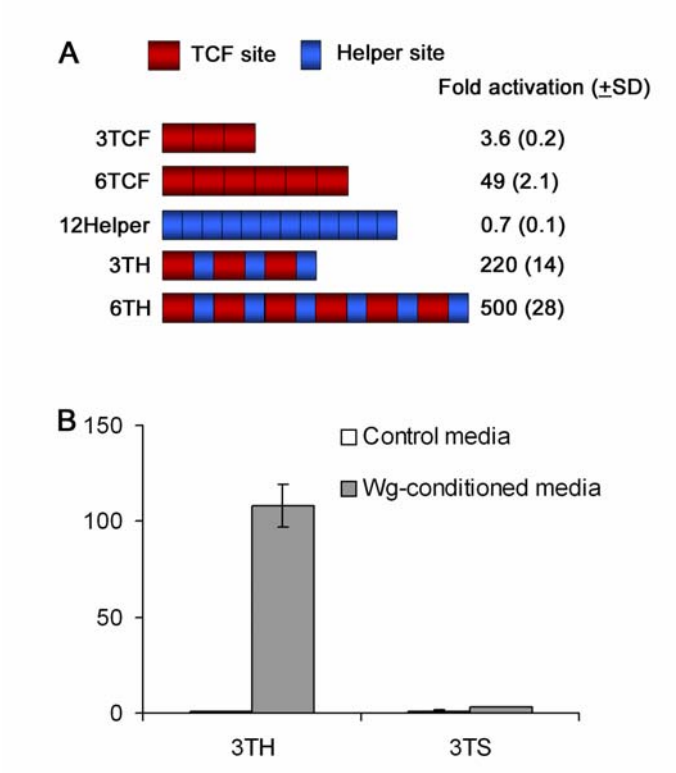
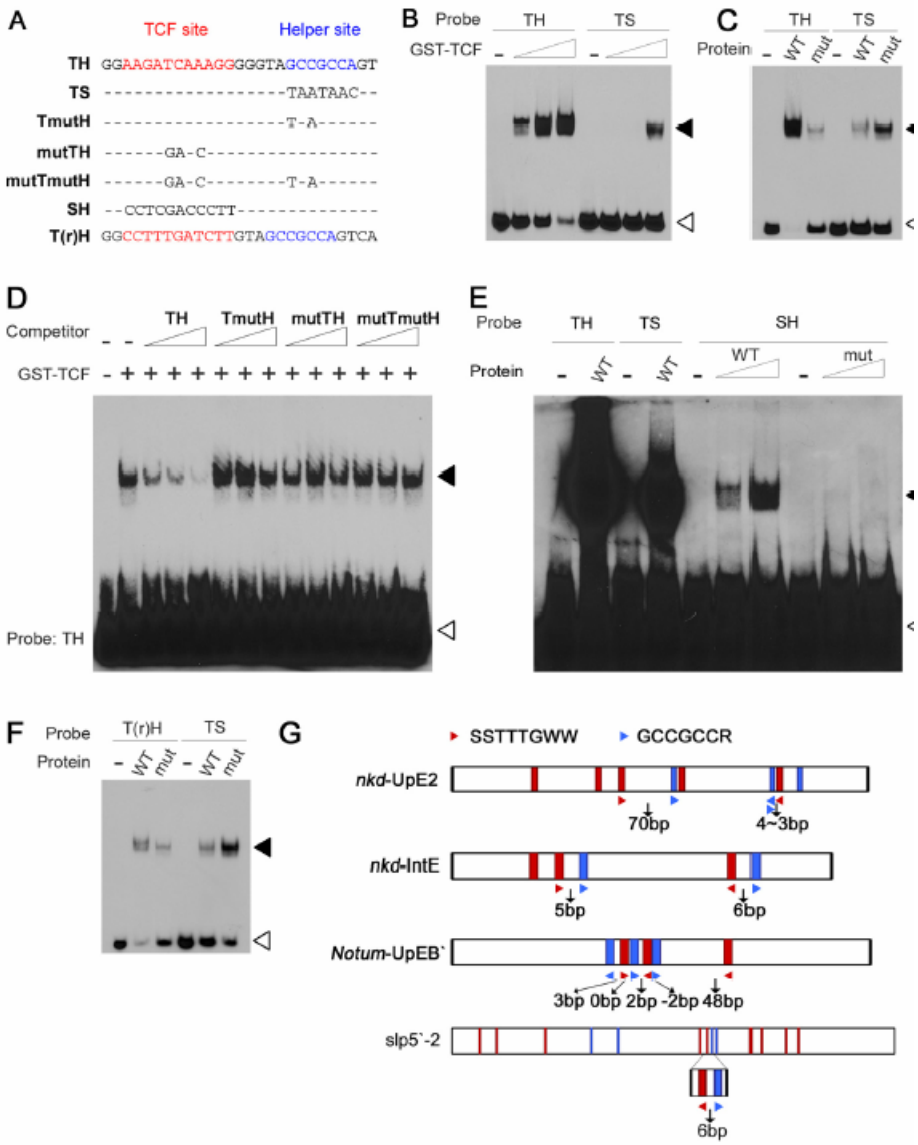


Figure 3.10. Helper sites augment TCF site activity. (A) Cartoons of synthetic WREs and summary of reporter assays in Clone8 cells are shown. Either TCF sites or Helper sites were multimerized and cloned upstream of a *hsp*70 core promoter/luciferase reporter. Similar to the results from Kc cells (Figure 4A), Arm* has no effect on reporters containing Helper sites alone (12Helper). However, Helper sites greatly enhance the activity of TCF sites to induce luciferase expression in response to Arm* (3TCF vs. 3TH, 6TCF vs. 6TH). (B) 3TH and 3TS reporters (see Figure 4A) were activated by Wg-CM in Kc cells. 3TH induces luciferase expression much stronger than 3TS.

Figure 3.11. Helper sites enhance binding of TCF's HMG domain to TCF sites through interaction with C-clamp. (A) The sequences of probes and competitors used are shown. EMSA was performed with GST-TCF fragments containing both the HMG domain and C-clamp. (B) Increasing concentrations of GST-TCF (0.1, 0.3 and 1 μM) were incubated with DNA probes (4nM). GST-TCF binds to the TH probe better than TS. White arrowheads indicate free probe and black arrowheads indicate the protein-DNA complexes. (C) Binding of TH and TS oligonucleotides (4nM) to WT GST-TCF or C-clamp mutant proteins (1μM; mutated residues indicated in Figure 6A). The C-clamp mutant displays weaker affinity for TH but a greater affinity for the TS probe. (D) Competition assays where mixtures containing GST-TCF (20nM) and TH probe (4nM) were incubated with unlabeled competitors at 10, 20 and 50 molar excess. The competitors containing mutations in either TCF sites or Helper sites don't compete with the labeled TH probe as efficiently as the TH competitor. (E) TH and TS probes (20nM) were incubated with wild type GST-TCF (1μM). SH probes (20nM) were incubated with WT or C-clamp mutant GST-TCF (1 and 5μM). WT protein binds to SH with low affinity, but this binding was not observed with the C-clamp mutant. White arrowheads indicate free probes and black arrowheads indicate protein-DNA complex. (F) Binding of T(r)H and TS oligonucleotides (4nM) to WT GST-TCF or C-clamp mutant proteins (1μM). As with the TH probe in panel C, the C-clamp mutant displays weaker affinity for T(r)H (i.e., note that the amount of unbound probe is much greater for the C-clamp mutant). (G) The spacing and orientation of functional Helper sites in relation to nearby TCF sites are variable. TCF sites were defined as eight core sequences (SSTTTGWW) and Helper sites were defined as seven consensus sequences (GCCGCCR). In the WREs shown here, the spacing between Helper site and nearby functional TCF sites varies from 0 to 70 bp. The direction of arrowheads indicates the orientation of each Helper site or each TCF site. Functional Helper sites are found in either orientation. The sequence of second Helper site in *nkd*-UpE2 is TGCCGGCA. Therefore, this helper site can be read out in both orientations: TGCCGGC (reverse complement) and GCCGGCA


A
TCF site
Helper site
TH GGAAGATCAAAGGGGGTAGCCGCCAGT
TS ----------TAATAAC--
TmutH ----------T-A------
mutTH ------GA-C----------
mutTmutH ------GA-C--------T-A------
SH --CCTCGACCCTT----------
T(r)H GGCCTTTGATCTTGTAGCCGCCAGTCA
B
Probe
TH
TS
GST-TCF
C
Protein
WT
mut
D
Competitor
TH
TmutH
mutTH
mutTmutH
GST-TCF
Probe: TH
E
Probe
TH
TS
SH
Protein
WT
mut
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Probe
T(r)H
TS
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G
SSTTTGWW
GCCGCCR
nkd-UpE2
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slp5`-2
6bp

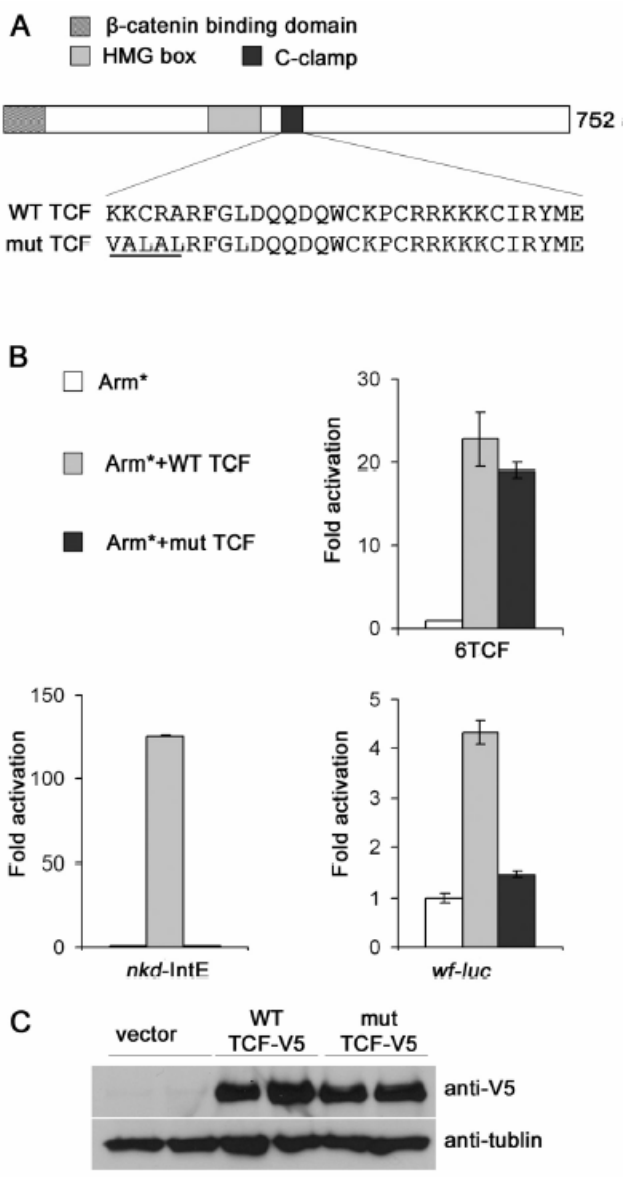

Figure 3.12. The C-clamp motif of TCF is required for the full activation of Helper site-dependent WREs. (A) Cartoon of *Drosophila* TCF indicating the position of the HMG and C-clamp domains. The underlined amino acid sequence of the C-clamp indicating the five residues altered in the mutant. (B) TCF rescue assays where endogenous TCF was depleted by treating dsRNA against TCF 3`UTR. Each WRE reporter was co-transfected with Arm* and V5-tagged TCF expression plasmids. The activity of a 6TCF/luciferase reporter was efficiently rescued both by wild type and C-clamp mutant TCF. However, the mutant didn't rescue the activity of the *nkd*-IntE/luciferase and *wf-luc* reporters. Luciferase activity in the presence of Arm* but without TCF expression was normalized to 1.0 for each reporter. (C) Western blots with a V5 antibody of whole-cell extracts from transfected cells demonstrating that wild type and mutant TCF were expressed at similar levels.

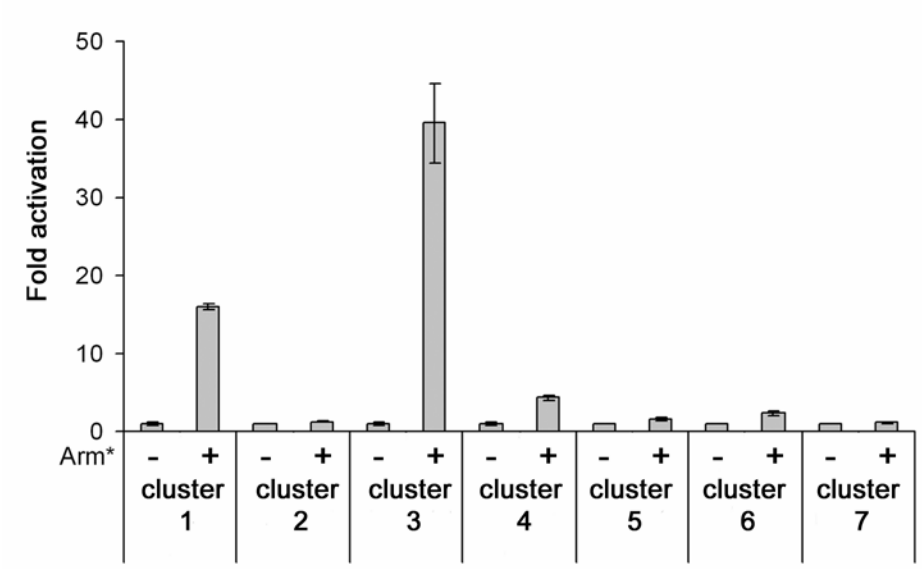

Figure 3.13. A genome-wide search for WREs containing TCF and Helper site clusters identified new WREs. Seven of the clusters identified were cloned upstream of a *hsp*70 core promoter/luciferase reporter. Each reporter was co-transfected with an Arm* expression plasmid in Kc cells and luciferase activity was measured. Two reporters containing cluster 1 or cluster 3 were significantly activated by Arm*.

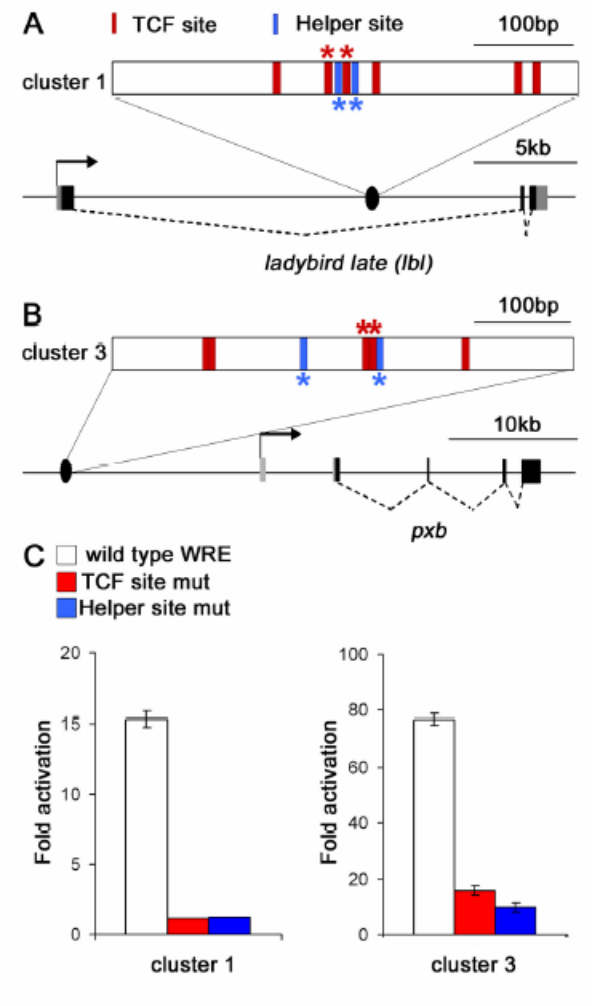

Figure 3.14. A genome-wide search for TCF-Helper site clusters identified new WREs. (A, B) Schematic diagram of the *lbl* (A) and *pxb* loci (B) showing the locations of the identified clusters. The gene structure for *pxb* was drawn based on a *pxb*-PB isoform. Red and blue stars indicate the location of mutated TCF sites and the Helper sites, respectively. (C) The fragments containing cluster 1 (478 bp) or cluster 3 (484 bp) were cloned upstream of a *hsp*70 core promoter/luciferase reporter. In Kc cells, both clusters activate luciferase expression when co-transfected with an Arm* expression plasmid. Mutation in the Helper site or nearby TCF sites significantly reduces the Arm*responsiveness of the reporters.

Table 3.1. Sequences of genomic fragments containing TCF clusters in *nkd* loci

Site	Sequence
Site 1 (*nkd*-UpE1)	GGCTGGGCTCGATGCAGATAAGAGGCATATACGCTTGTCAAGATTTGCTTTGATGTCT TTTTCATGTGCCCGACTTGGGTGCGGCCAGCTCCATGTCCAATGCTACATCACTGCAC AGGGGAAAATGCAAGGAAGCATGGAAAAATACATAATAAATGATAAAATTCTTGTATTT CAAAGATGGATATATGCCTTCATAATAAATTATTTTATACAATATTTAGTACAATATTCCA CTCCATTTTATATTCTTCTTATCTTAGCTGAGCAGTTTTTAAGAGATAGTTTTTGATATTC CTTTTTAGAACTCAATTGCTTTTCGTGCAGTGCACGCCTCTGCTTAATGCTGCTTAGCT CTGTCGCTTTGGTTCTCTAGTGTGGGTTGACAACAATTAAAAAGTGACGGGCAAGACA AATCGGCAGGAGGAGGAGGAGGAGAAAGAGCAGGAGGAAGGCGTGCGGATGCCAG GAGGTCTGCCAACTTAAGTAGCAGCAAATACACAAAAAGCAAATTCTCTTTGGAAGCAT TTCAATTGGCTTTTGTTTATGCGAGAGATTACCCCGTCCGGCGACCCCTTTGAATTTGC TTTGAAGTCGAGCATTTCCATGGGTCCGCCACGGTGTGAGTAAGTTTTATTTTTGGTAG CGAAGAGATTTTATTAGCAAAAGGGTTGGGAAAGCGCTTTGAAGTTCAAGTGTAGGCG CAGCAGCGGCCCCATTGATATATTTATTTCGCCGCACAATCGCTCGCTTTGTGGCCGA CGCAAATTGAAATATCGCATCAATTTGCCATGCCGCCGTCGCGCTTTGTAATAAAAGTA AATAATAATTTAATAAAAGGCAGCAAAAAACCACCGCAAACTACAAA
Site 2	AGGAGAATGCCGGAGCAGCGACACACAAAAATAGAACGGAAAATCGGGCATGGTGCT GAAGAAGAAAAACACAACGGCAGGGCAGATCAAAAGGAAAGCGACCCATGGAAAATA AATAGAATTTTTTTTGGAAGCAGATCGAAGAGGCGAAGGGATCGGGATAGGGATAGG GATCGCGATCGCGATCAATAGAACGTTTCGCTTTGGCTCCTGCTGCTGCTTAGAGATC GGCTCCTTAGTCATGGGTAGCATATCATGGACGTAAATAAACAAGGTCCGTGTTTCCA AAGTGGGATCCGATCCGAATTGGAAATGGACGACCGAATCGGACGACCTTTCTGCCA AACGAGCAACGTTGATGTCCGAATCGCTGAAGTAACATCAATCAATCAATGTTCCCTG GGTTCCTTTTTTCTGTCCACAAATTCATTGGAAATATTCTGGGGCCCCCATCCACCTCT TTACTTTTCTGGAGTCTTTTTTTTCATTGGTTTGTTTTGTTTCGCTTTGAAGCCCCAGAG AGAGATTTCCTCCTCATCGGCATCGGCTGGCAATTGGTCTGCGCCAATTAAATGCCAG GCCAACACTTTGAACCCTTTGCAGGACGACAAGCCCCGTACACTAATTGCTGGCAGAT GCAGGGGGAATTCAAAGGAAACCTCCAAAGGCACACCGAAGCATGCAACTTCAAAGA AAGCAGCCTCGAAAACACTACACAACAACAAACGGGAAGAGAGTCGTACAAAAAATGA AAATCAAGCGAATTGAAATGCCGACGCCGTTTAAAAACAACGTACGAACATTTCTTACC TGTT
Site 3 (*nkd*-IntE 1.2kb)	GCTCTCGGGCCACTTCTTGGAATTATACCGCACTTAGGTCGCCCTTCGTCCACCAGGC CATAACTTTGGCCTAAAAAAACAGAGCTCAAGCTTTCATTAGGGGGTCATAACTTTAAC AGAAACGTTACAACGAGATAAAGATACAAATTAGTGTAGAAAACGTAAAACCCATTAAA GTTGTAAATATGTTTCGATATATGAGTAACAAAATCAAAGAACAGTCCAAACTTTTATAT TTATTAATACAAATTGCTTTGCAAACAAAAAACCAGCTAGCACTTTCAAAAGGCGTAAA GGAAAACACGTTCTAAGCAAATATTTACGGAAACTCTGAAACTCTCTGTTGACGGTTTG TTCCAATAACTCATAACTCTGATGGAAGAACCGTGTTGGCTCTTCTTCTTTGCGAATAA TACATATATACGAGGGTCGGTACCTCTGACGTCTGATTGATAAGCGAACCCCCGCGCC TTATCTTAATAATATTTAATGCATGGCAAGGCGAAGCGTGGCGAGGCCGAAGCGAAAA GTGTTGTCACCAAGGGGGGAAAGGGTGAAAAATGCGGGCCCTGTTGGCTATGGCGG CTATAGGGGCCTATTGGGGGCCAGGGGTATAGTTTGTGTATAGTGAAAATGAAATTGA AAATAATACAAAACGCTTTGTCGGTCATTAGCTTTGAGTGGACGCCGCCACCGCCGCA CTTTGCCGCTTTCGAGTATTTTTTTTTGGGGATAGTCGCGTCATTTGTGCGCTCGGTTT GCTTTGGGTTCGGTTCGGTTCGGTTTGGTTTTGATTATGTCAAAGCGAAAAAGCCGCC AAACATATGTGCCTCCAACCACATGCCTGTCTGCCCGGCTGCTTTGAGGAAAGCGGTC TGGGAAAATTTTGGGGAAGACCCCAAAAAAGTGGGGAAACTCTGGATTTTATTTAGCG AAAGAGGAAGAAGTTCTTGAGAGAGCAATAGCACTATGAGCACCGCTGTATTCAGTAA TCATGCTTTGTAAAATATATTGTATAATACATTCGATTATGATTTATTCCTTTAACATCTT AAAATACTATACAATGCAGTACATACTTGGCTAGTTTGGCAATCTTTTTCCCTTATAACG ACTACGTGCCATGTCCACGCATATATGGAAATGATCAGTATTTAATGATTTTCCATTTTA TCTCACTGAAGCAAAGTGGCAGGC

Table 3.1. Sequences of genomic fragments containing TCF clusters in *nkd* loci (continued)

Site 4	AGATACAGATACAAACACAAGTGCAGATACAGATACAGCCAAGACAAAGGAGCTGGCA ATTGAGCGATGTTGTCGCATTGTTGCACATATTTAGCAGCCATTTTCAATTTCATTTGTG CCACAATTTAAAATGCAAAACACAGGCGAGGATGTCTGTGCCCTGGGAATAGGTAGCA TTGTATCTGGGATAGGATGGAATATCGGGGGACTTTGACAAATGCCGCCTCCACTTGG CCTAGGTGCTAATGGTTCGCTGGTGGTCGGCGGTCCGGAGTACTCGGACTTTCAGCT CCCCTCCGCGGCGGGCTTATTGAAATTGATGAGCGGCCAAGTACGTGGCAGCTTTGC TTAAATCTACCCCAATCCCCAACCCCCAGCCCCAATACCCAACCGTGAACCCCAACCA TGAGCCGCATCTCTGTGTTTCTGACGACCCTGGGCCTATAAGCGCAACTCAGTCTGAC TTTGACATTGATTGATTGCTCTGGCAGTGTAATTTATATGATTGTATTAGGGGAGTTCG CGGGGTCTTCACTTCTCCACACTTTGTTATTAATAGTTGCGCCGCCTCTGGTTGTTATT TCAACAACAAACAAATTCTGCTGAAAATTGTTTTCGTTTATAAAATATGCCCGGGCAATT CATTTTTAACGGTTCGCCATGCACTTTAGAGCTCTGAATCGGTGAATATTTTGCCACCC AAATCGAGTCCCAAATCCACACAAGAGTTGGGAGATTTTGGGGCGAGAGTTGGTGCAA AATTGATGGCAAGTGCTTACGGATAAAAAGGTAAACCAGACATACTCTTTGTCAAAGAA GATTAGGCATAAATTGAAGATTATTAAAAACAAGCTATTAGACGGGCTTAGAAAGATCA AATTAAAGGTTGCTATGAATTTTGATTGATTAACAAGCGC
Site 5	AGTAAAACCACCCATTCCTGGCTCCCACTCTCTGCTTCCCCTCGAAATAATTGCTACTG GTATTTAGCAAGCATTCCCTTTGATAAGATTTAGCCCCGTGAATGGTGCCGGTAGAAAT AAAAAACAACAACAAAAGAAAAACACAAAAAAAAAAAAATAAAAGTAGGTAACAAAAAG TGAGGCAGATTTCTTCGGCTGATAGGGAATAATTTTTTTAGGACCCGTTTGATTTTTGC CGTTCTTATTGCCCCGGCTCGATAAGTGCCATGCCAAATTGTGGTTGTAAATTGTGAA GTGTCAAAAAAATATTATAAAAAATAGACGTAGAGTATAAAACACAAATTGAACGGCAC TCAAGTCGGTTTGATCTAGTGATCGAATTTCGATCAGACGGATGATTAAGTCTCGGACA GTAATATCAAAATAACTTGCTTGTGAACGTCACAGTTTCTTTTATGAATTTAAATAAGAA TTTAAAATTAAAATTTAGTTTTACGTGATAGTTGCTCTTTTGTATTTTCTAAGAATTTTCTT TTAACTTAAAATGTACGGATTACAATAGAAGAAGTTAGCCACAATTAGGTTCGTGTTAA CTTTAATTTTTCCTCTTCTGGACTATTTCACTTAATTTTTTTCCCATCGCCGGATGACCA ATTTCATTTATGGTTGCTGGGTATATATGTACATAAGTATGTAGGCACTTTTTTTTGGCA TTTCTTGATCTTTATCACAAAGGGCCGAGCATTTGTTGTTTTCTCGCCACTCGCTTGAC TCGATGGTATGCGACTTCTCATTAATTGTTTGTTGCTTTGGTTATTGCCGGTCGTTCGC GTCAAAAACACTTAACTTCATTAACACTTACATGGCAGATACGAGATATCTGGCCGCAA CTA

Predicted good TCF sites in each fragment are shown in red.

Table 3.2. Primer sequences for dsRNA, WRE reporters, in situ inbridization probes and GST-TCF expression plasmid

dsRNA	TCF (3`UTR)	TTAATACGACTCACTATAGGGAGACTGATCGCCATGGATTTGTA GAA TTAATACGACTCACTATAGGGAGAGTTTTAGTGTGTATTGTCTGT TT (underlined sequences are T7 promoter sites)
WRE reporters	*hsp70* minimal promoter	ATCTCGAGCTCGAGATCTGAGCGCCGGAGT (XhoI) ATAAGCTTAAGCTTCCCAATTCCCTATTCAGAGTTCTC (HindIII)
	nkd-UpE2	AATAACGCGTCAGGAGGTCTGCCAACTTAAGTAG (MluI) AATTCCCGGGGGGCCGCTGTCGGCCAACTG (XmaI)
	nkd-IntE	GCCACGCGTATAGTTTGTGTATAGTT (MluI) CCCAGCCCGGGTTCCTCAAAGCAACC (XmaI)
	Notum-UpE1	CGATCGGTACCGGCGTTGGTAACC (KpnI) CGATGAAGATCTAGAGGACGGCGAGG (BglII)
	Notum-UpE2	CCTCTGGTACCTCATCGTCATCGTC (KpnI) GCGCTCAAGCTTAGATCTCACCGTA (BglII)
	Notum-UpE2`	CCTCTGGTACCTCATCGTCATCGTC (KpnI) CCAAAAGATCTACATTTTCTTGCGG (BglII)
	slp5``-2	GCGACGCGTAGGATCTCGAATCGCTAATC (MluI) GCGCCCGGGATGTGGATCTCTGGCAATCC (XmaI)
	Cluster 1	CCTGAACGCGTGACCTTCCCCAGCTC (MluI) GAGCGCTCGAGAATAAGCACTCGATG (XhoI)
	Cluster 2	CGCGAAGTACGCGTGCTCATCTC (MluI) CGCGCTCGAGATGCTTTTTGTCT (XhoI)
	Cluster 3	CATTCACGCGTGTCAGCCCAGTG (MluI) CGCTGATCTTCTCGAGAATTTGT (XhoI)
	Cluster 4	AACGGGCACGCGTTTAAAATTGGA (MluI) GATATTTGCTCGAGGCCATTTGA (XhoI)
	Cluster 5	ATAGCACGCGTGAGAGGCATTGG (MluI) GCCCCTCGAGGATGTATTTGATT (XhoI)
	Cluster 6	AAGCGAATTACGCGTAACAATCA (MluI) GCGCAGTTCTCGAGTTCCTTCAA (XhoI)
	Cluster 7	GGAACGCGTTCAATTTGATGCCAACAT (MluI) TCCAGATAAACATGCCCTCGAGTTA (XhoI) (Restriction enzyme sites are underlined.)
in situ hybridization probe	*nkd*	GAATTAATACGACTCACTATAGGGAGAGCTGCTGGTCAGCGAAC GTGACAATAA GAATTAATACGACTCACTATAGGGAGACAGACCCGTGGGCAACT TCTTCAGTTT (underlined sequences are T7 promoter sites)
GST-TCF	TCF fragment	AAGAATTCCCCCATATTAAGAAGCCA (EcoRI) GCTCTCGAGTTCGGCGGGCCCATT (XhoI) (Restriction enzyme sites are underlined.)

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CHAPTER IV

FURTHER STUDEIS OF C-CLAMP AND HELPER SITES IN WRES IN DROSOPHILA

ABSTRACT

Wnt/Wg signaling regulates the expression of target genes through TCF. TCF has a HMG domain that is known to bind specific sequences in Wnt/Wg response elements (WREs). Currently, it has been shown that *Drosophila* TCF and human TCF E isoforms contain an additional DNA binding domain, the C-clamp. In *Drosophila*, the C-clamp was suggested to physically and functionally interact with a DNA sequence motif called a TCF Helper site (Helper site). However, it remains unknown how often the Wg pathway utilizes the C-clamp and Helper sites to control target gene expression. Here, I describe ongoing experiments which will help to elucidate the biological importance of the interaction between the C-clamp and Helper sites in *Drosophila*.

INTRODUCTION

The Wnt/Wingless (Wnt/Wg) signaling pathway plays important roles in multiple developmental events and in adult tissue homeostasis, mostly by regulating gene expression through TCF/LEF proteins (TCF) (Logan and Nusse, 2004). In the absence of Wnt/Wg stimulation, TCF is thought to repress Wnt/Wg signaling targets (Figure 1.2A)

(Cavallo et al., 1998). Wnt/Wg signaling converts TCF from a repressor to an activator, inducing the transcription of target genes (Figure 1.2B) (Logan and Nusse, 2004). TCFs are known to bind a specific DNA sequence of STTTGW (S: G or C, W: A or T) through their HMG domains (Figure 1.5) (Parker et al., 2007). Consistently, mutations of TCF binding sites (TCF sites) in Wnt/Wg response elements (WREs) significantly reduce the responsiveness of WREs to Wnt/Wg signaling (eg. (Brannon et al., 1997; Chang et al., 2008a; Riese et al., 1997; Yamaguchi et al., 1999)).

The context in which TCF sites are located also seems to be important to determine the activity of WREs. It was shown that only subsets of TCF binding site clusters can recruit TCF and function as WREs (Figure 3.1). In addition, TCF sites sharing the same sequence in a WRE were shown to contribute to the activity of the WRE differently, suggesting that there are other cis-regulatory elements that affect the binding or activity of TCF (Chang et al., 2008a). Current analyses of WREs have found that functional TCF sites often have a neighboring sequence of 'GCCGCCR' (R: A or G) we have called TCF Helper sites (Helper sites) (Figure 3.4-3.8). Surprisingly, mutations of Helper sites in WREs significantly reduced the WRE activation by Wg signaling in a manner similar to mutations of TCF sites (Figure 3.4-3.8). These results suggest that Helper sites, as well as TCF sites, are necessary for WREs to activate transcription in response to Wg signaling.

The Helper site was suggested to interact with the C-clamp of *Drosopihla* TCF, thereby enhancing the binding of fly TCF to a consensus TCF binding site *in vitro* (Figure 3.11). Consistent with this result, dTCF containing mutations in the C-clamp motif failed to activate several WREs (Figure 3.12). The importance of the C-clamp in

the activation of WREs was also shown in human TCF-E isoforms that contain a C-clamp (Atcha et al., 2003; Atcha et al., 2007; Hecht and Stemmler, 2003). The *LEF-1* and *CDX-1* WREs are specifically activated by TCF-E isoforms, but not by other TCF proteins (Atcha et al., 2003; Hecht and Stemmler, 2003). Mutations or deletion of the C-clamp in human TCF-E prevented the TCF from activating *LEF-1* and *CDX-1* WREs (Atcha et al., 2003; Atcha et al., 2007; Hecht and Stemmler, 2003), suggesting that the C-clamp is required for TCF to activate some WREs. However, it remains to be determined whether *LEF-1* and *CDX-1* WREs have specific cis-regulatory elements such as Helper sites that interact wih the C-clamp in human TCF-E isoform.

Here, I would like to address some remaining questions concerning the interaction of Helper sites and C-clamp in flies. Is the reduced activation of WREs caused by mutations of Helper sites (Figure 3.4-3.8) due to the loss of TCF binding *in vivo*? Are Helper sites necessary for repressing the WRE activation in the absence of Wg signaling as well? Do the important developmental processes regulated by Wg signaling require the C-clamp of dTCF? The strategies to solve the questions and current results will be presented in this Chapter.

RESULTS AND DISCUSSION

The binding of TCF to a WRE is dependent on Helper sites

Several WREs of Wg target genes contain Helper sites near functional TCF sites (Figure 3.4-3.8 and 3.11). Similar to mutations of the TCF sites, mutations of the Helper sites greatly reduced the Wg responsiveness of the WREs (Figure 3.4-3.8). *In vitro*, it was shown that a Helper site can enhance the binding of fly TCF to a consensus TCF site

(Figure 3.11). These results suggest that in addition to TCF sites, Helper sites may be required for TCF to bind to WREs *in vivo*.

The possibility that Helper sites affect the binding of TCF to WREs *in vivo* was tested by measuring TCF binding on *Notum*-UpEB`, a WRE of *Notum*, through Chromatin immunoprecipitation (ChIP) assays in transgenic reporter flies (Figure 4.1). A *Notum*-UpEB`/lacZ reporter exhibits lacZ expression in a similar pattern to the Wg expression domain in a broad range of tissues (Figure 3.6 and 3.7). *LacZ* expression is observed at late stage 10 embryos, and continues through later embryogenesis (Figure 3.6 and data not shown). Mutations of either TCF sites or Helper sites significantly reduced the *LacZ* expression in the embryonic tissues where *Notum*-UpEB` is normally active (Figure 3.6 and 4.1A). Whether the reduction in the *LacZ* expression is due to the loss of TCF binding was tested by ChIP with anti-TCF antibody.

One potential problem of ChIP with transgenic reporter flies is that genomic locations where each *Notum*-UpEB` reporter is integrated could affect the TCF binding or the accessibility of anti-TCF antibody. In order to reduce the potential position effects, three independent insertion lines of each reporter construct were used. In addition, TCF enrichment on *Notum*-UpEB` relative to the adjacent lacZ reporter was used instead of absolute percentage of input (Figure 4.1B and 4.1D).

For ChIP, transgenic fly embryos containing *Notum*-UpEB`, *Notum*-UpEB`^{TCFmut} or *Notum*-UpEB`$^{Helpermut}$ reporters were collected 5 to 10 hours after egg laying, which approximately corresponds to embryonic stage 11 to 13 (Figure 4.1A). *Notum*-UpEB` occupancy by TCF was measured by quantitative PCR with transgene specific primers (Figure 4.1B). Consistent with being a WRE *in vivo*, TCF was bound to *Notum*-UpEB`,

and mutations of TCF sites (*Notum*-UpEB`$^{TCFmut}$) significantly reduced the TCF binding (Figure 4.1C). Mutations of Helper sites (*Notum*-UpEB`^{Helpermut}) also caused a dramatic reduction in TCF binding (Figure 4.1C). This result indicates that Helper sites play critical roles in recruitment of TCF to *Notum*-UpEB`, thereby contributing to the activation of the WRE by Wg signaling *in vivo* (Figure 4.1C).

The activation of a WRE in the *ladybird late* (*lbl*) locus is dependent on Helper sites

The genome-wide search for clusters of TCF sites and Helper sites identified two WREs (Cluster 1 and Cluster 3) that can respond to Wg signaling in Kc167 (Kc) cells, a *Drosophila* cell line (Figure 4.2A and 4.3A)(Chang et al., 2008b). The Wg responsiveness of these WREs was shown to be dependent on Helper sites as well as on TCF sites using a luciferase reporter assay (Figure 4.2A and 4.3A)(Chang et al., 2008b).

In order to know whether these WREs can respond to endogenous Wg signaling in a more physiological context, transgenic reporter flies were generated. Cluster 1 is located within the first intron of *lbl*, a gene known to be genetically regulated by Wg (Jagla et al., 1998). Cluster 1 drove the expression of the lacZ reporter in cells near, or within, the Wg expression domain at stage 16 embryos (Figure 4.2E). The lacZ expression along the body segments is somewhat reminiscent of the *lbl* expression in somatic muscles at the late stage embryos (Jagla et al., 1998), implying that Cluster 1 could be a *lbl*-WRE. In addition, the close location of lacZ-expressing cells to the Wg expression domain is consistent with the genetic regulation of *lbl* by Wg (Jagla et al., 1998). However, more careful analyses would be required to conclude that Cluster 1 is an authentic WRE of *lbl*. To test this more direct, localization of *lbl*- and/or lacZ-

expressing cells should be compared by immunostaining transgenic fly embryos for both LBL and LacZ simultaneously (Jagla et al., 1998). In addition, it should be determined whether the lacZ expression driven by Cluster 1 can be genetically regulated by Wg signaling. lacZ expression was also observed at earlier stage embryos but some known expression patterns of *lbl* such as ones in pericardial cells were missing (Jagla et al., 1997; Jagla et al., 1998; Junion et al., 2007).

The LacZ expression driven by Cluster 1 at late stage embryos was dependent on TCF sites and Helper sties. Mutation of the TCF sites in Cluster1 reduced the lacZ expression to below level of detection in the late stage embryos (Figure 4.2F). Similarly, mutation of the Helper sites caused a dramatic reduction in the lacZ expression (Figure 4.2G), implying that Cluster 1 could be a WRE. The analysis of Cluster 1 suggests that we could identify new potential WREs *in silico*.

TCF sites repress the activation of Cluster 3 in the absence of Wg signaling

Another TCF/Helper site cluster identified *in silico*, Cluster 3, is located between two genes of unknown functions, *cg5302* and *pxb* (11.5kb downstream of *cg5302* and 15.2kb upstream of *pxb*) (Figure 4.3A)(Chang et al., 2008b). The regulation of these two genes by Wg signaling has not been studied. However, *pxb* was shown to be expressed in the regions similar to Wg expression domain in the midline cells and late stage embryos (Inaki et al., 2002; Wheeler et al., 2006).

Cluster 3 drives the *LacZ* expression around the second constriction of embryonic midgut in a similar pattern to Wg and in the hidgut (Figure 4.3E). In stage 16 embryos, the midgut is divided into four chambers by three constrictions. At this stage, *wg* is

expressed where the second midgut constriction occurs (arrow in Figure 4.3B). Cluster 3 directed the lacZ expression to the similar region as the Wg expression domain in the midgut (arrow in Figure 4.3E). Strong lacZ expression was also observed in the hindgut (arrowhead in Figure 4.3E). The lacZ expression pattern in the midgut suggests a possibility that Cluster 3 could be activated by Wg signaling in this tissue. This possibility could be tested if the lacZ expression driven by Cluster 3 is reduced under the *wg* null mutant background. It is not known whether either endogenous *cg5302* or *pxb* is also expressed in the midgut (Inaki et al., 2002). In situ hybridization or analysis of enhancer traps will be required to determine if either gene is regulated by Cluster 3.

In Kc cells, mutations of either TCF sites or Helper sites in Cluster 3 resulted in a large reduction in Wg responsiveness. However, in the embryos, mutation of the TCF sites of the Cluster 3 reporter did not reduce the lacZ expression in the midgut and in the hindgut (Figure 4.3F). Rather these mutations caused strong ectopic expression in the entire midgut (Figure 4.3F). This result shows that the TCF sites mutated are required to repress the activation of Cluster 3 far from the Wg source in the midgut.

The derepression of Cluster 3/lacZ by mutation of TCF sites suggests the role of TCF in the repression of Cluster 3. TCF is thought to bind and repress WREs in the absence of Wg signaling (Figure 1.2, refer to Chapter I). Therefore, loss of TCF binding to WREs would relieve WREs from the TCF-mediated repression, causing the expression of target genes in the absence of Wg signaling. Whether TCF repress Cluster 3 outside of Wg expression domain in the midgut could be determined by depleting TCF proteins by RNAi in the visceral mesoderm or endoderm of fly embryos containing the Cluster 3/lacZ reporter.

Mutations of Helper sites in Cluster 3 also caused the derepression of lacZ expression in the midgut (Figure 4.3G). However, the ectopic expression was not as strong as the one caused by mutations of TCF sites (compare Figure 4.3G with Figure 4.3F). This could suggest that repression of WREs in the absence of Wg signaling does not require Helper sites. However, there is a caveat in the interpretation of this result. Helper sites consist of 7 nucleotides (GCCGCCR). In the previous analyses of Helper sites in WREs (Chang et al., 2008b), all 7 nucleotides in Helper sites were mutated to determine their contribution to the activation of WREs. However, for Helper sites in Cluster 1 and 3, only two positions were mutated (GCCGCCR; mutated nucleotides were underlined). These subtle mutations were enough to reduce the activator function of Helper sites in Cluster 3 (Figure 4.3A) but might not be sufficient to affect the repressor function of Helper sites. This possibility could be tested by generating Cluster 3/lacZ reporter flies containing mutations in all 7 nucleotides of the Helper site.

Generation of transgenic flies expressing dsRNA targeting TCF 3`UTR (P[*UAS-TCF*^{RNAi}])

Wg signaling controls various developmental processes by regulating gene expression through TCF (Logan and Nusse, 2004). Does the C-clamp of TCF contribute to all of these developmental roles of Wg signaling. To address this question, we have designed a TCF rescue experiment in flies. Fly TCF could be depleted through dsRNA targeting TCF 3`UTR in various tissues at different developmental times. The loss of TCF could be rescued by expressing wild type TCF or mutant TCF containing mutations

in the C-clamp. Since the TCF transgenes have a heterologous 3`UTR, it will not be targeted by dTCF dsRNA.

TCF dsRNA was designed to target the 3`UTR of all the TCF isoforms reported except pan-RI (Figure 4.4B and 4.4C). The expression of dsRNA is controlled by the UAS/Gal4 expression system (Figure 4.4A)(Lee and Carthew, 2003). Although there are ten different TCF mRNA isoforms reported (Figure 4.4C, more information can be found on Flybase website), TCF isoforms can be classified into four groups based on the primary amino acid sequences deduced from mRNA isoforms: RA(=RB=RC=RD=RE=RF=RG), RJ, RH and RI. The TCF encoded by the RA isoform is the best characterized (refered as dTCF-A in (van de Wetering et al., 1997)). The function of the other isoforms remains unclear.

Transformant lines containing a TCF dsRNA expressing transgene were generated (Figure 4.4A). It has already been tested whether dsRNA targeting the TCF 3`UTR can inhibit Wg signaling *in vivo* (Figure 3.12). Ectopic activation of Wg signaling in the developing eyes (eg. by GRM-Gal4>GMR-Arm* (GMR-Arm* in short)) causes a reduction in eye size of adult flies (Kennell et al., 2008; Parker et al., 2002). The depletion of endogenous TCF by expressing dsRNA targeting the ORF repressed the small eye phenotype caused by GMR-Arm* (data not shown). It will be tested whether TCF dsRNA targeting 3`UTR can also repress the eye phenotype caused by GMR-Arm*. This experiment is in progress.

Fly strains expressing TCF (P[*UAS-TCF*]) were generated

Fly strains which can express wild type or mutant TCF under the control of UAS were generated. Among TCF isoforms, the RA isoform, which is the same as the one used in the rescue assays done in Kc cells (Figure 3.12), was used. In order to distinguish the exogenous TCF from endogenous TCF, TCF transgenes were dual-tagged with V5 epitope and 6xHis (Figure 4.5A). Three types of mutations were introduced in the TCF gene to destroy the function of the C-clamp (Figure 4.5B). The expression level of each TCF protein could be determined by western blot analysis with anti-V5 or anti-His Antibodies. It will be tested if wild type TCF or mutant TCF can rescue the developmental defects caused by depletion of endogenous TCF.

EXPERIMENTAL PROCEDURE

Chromatin Immunoprecipitation (ChIP) and Real-time quantitative PCF (Q-PCR)

ChIP was performed as described (Parker et al., 2008) with the following modification; embryos containing *Notum*-UpEB` reporters (Chang et al., 2008b) were collected 5 to 10 h at 25 ºC or 10 to 20 hr at 18ºC after egg laying. In total, 70 ul of embryos were lysed into 400ul of SDS lysis buffer. Ten percent of the 400 ul lysate (the concentration of lysate was about 15ug/ul) was used for each pull-down. Rabbit anti-TCF antibodies generated in the Cadigan lab were used (Fang et al., 2006). Immunoprecipitates were analyzed using quantitative PCR. The following oligonucleotides were used: 5`-ACCAGTTTACCCGGTTAGTGC-3` and 5`-TTTATACTCCGGCGCTCCTC-3` for *Notum*-UpEB` transgene, and 5`-AACCAGCCATCGCCATCT-3` and 5`- CTGTAATTCCGCCGATACTGA-3` for *LacZ*.

Immunostaining and microscopy

Immunostaining were performed as described (Parker et al., 2002). Briefly, rabbit anti-LacZ (1:500) (Abcam Inc.) and mouse anti-Wg antisera (1:100) (Developmental Studies Hybridoma Bank at the University of Iowa) were used. Samples were examined by using Leica confocal miscroscope DM6000 B (Leica) and processed in Adobe Photoshop 7.0.

Fly strains

P[*UAS-TCF*^{RNAi}] strains were constructed as follows. 3`UTR of dTCF was amplified by PCR with following primers: 5`-CTGATCTAGATGGATTTGTAGAATTG-3` and 5`-GTTTTCTAGAGTATTGTCTGTTTATT-3` (XbaI site is underlined). The amplified PCR products were cloned into the pWIZ vector as described (Lee and Carthew, 2003). The P[*UAS-TCF*] strains were generated as follows. Amino acid coding regions of V5/His-tagged wild type TCF or C-clamp mut TCF were generated by digesting pAc TCF-V5 or pAc mutTCF-V5 (Chang et al., 2008b) with KpnI and StuI. The TCF fragments were cloned into pUAST digested with KpnI and XbaI (Brand and Perrimon, 1993). 5` overhang of XbaI in pUAST was treated with Klenow.

FIGURES

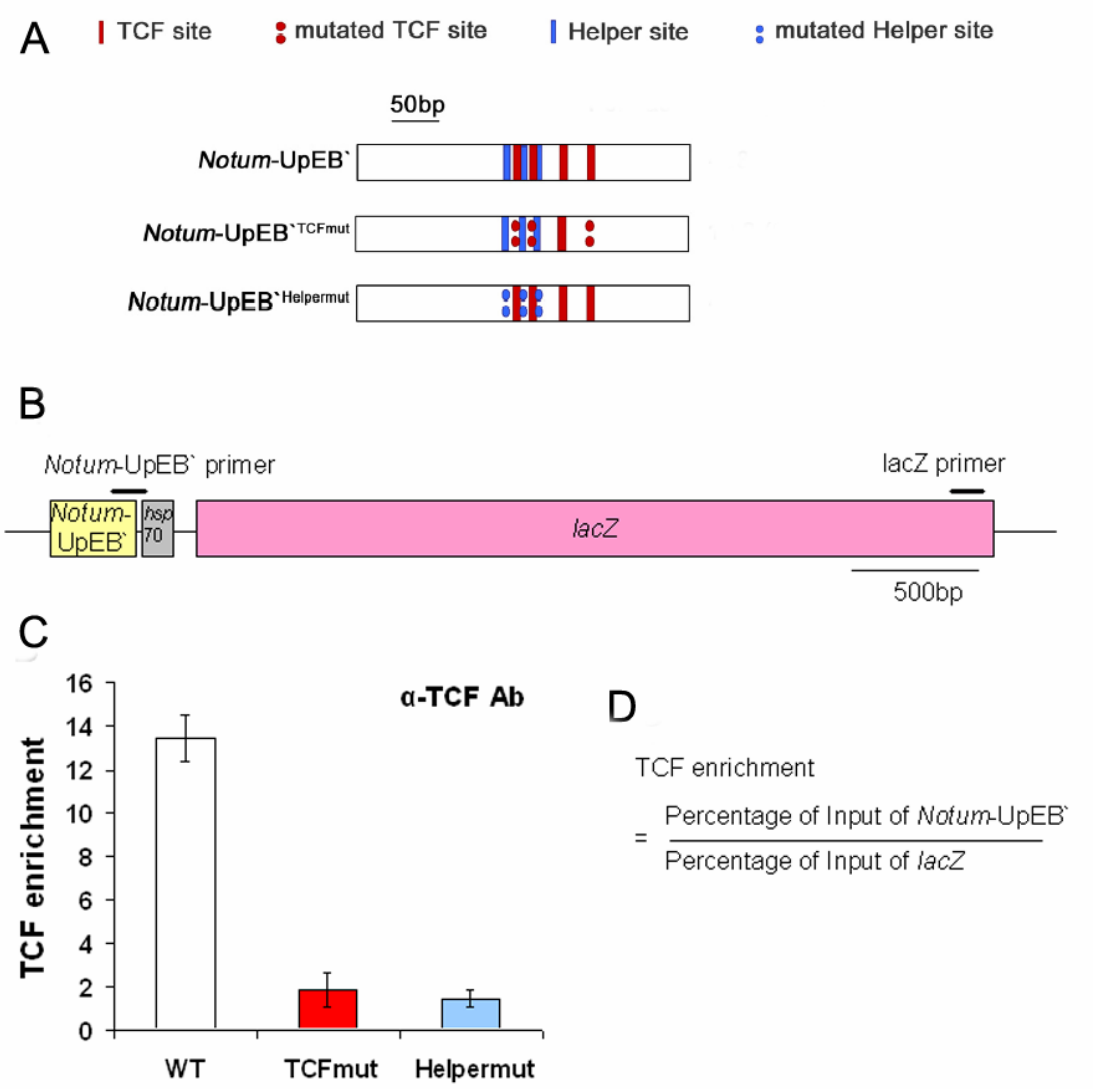

**Figure 4.1. Helper sites are necessary for TCF to bind to *Notum*-UpEB`.** (A) Taken from (Chang et al., 2008b). Cartoon of *Notum*-UpEB` showing predicted TCF sites, Helper sites and their mutations. (B) Cartoon depicting the *Notum*-UpEB` reporter integrated in to transgenic flies. The regions amplified in quantitative PCR are marked as black lines. The forward primer and reverse primer for *Notum*-UpEB` region are located within *Notum*-UpEB` and within the *hsp70* minimal promoter, respectively. Therefore, the primer set can amplify only a transgene, not endogenous *Notum*-UpEB`. (C) TCF enrichment in *Notum*-UpEB` was measured by TCF ChIP followed by quantitative PCR. Mutations in either TCF binding site (red bar) or Helper sites (blue bar) significantly reduced the TCF binding to *Notum*-UpEB`. Each bar represents the mean of three independent transgenic lines with the standard deviation indicated. (D) Description of how TCF enrichment was determined.

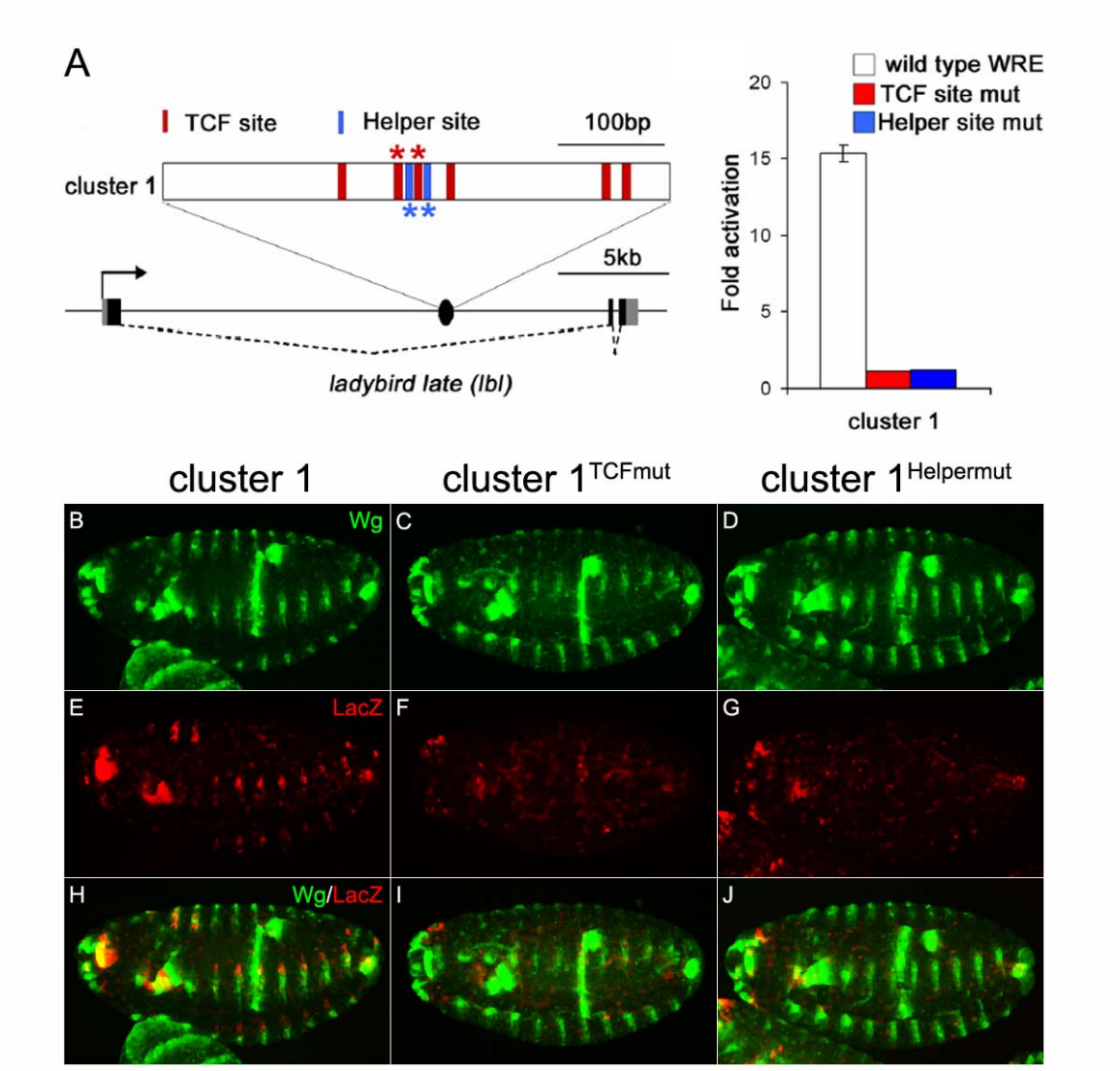

Figure 4.2. Cluster 1 contains Helper sites that are essential for Wg signaling responsiveness. (A) Taken from (Chang et al., 2008b). Schematic diagram of the *lbl* loci showing the location of Cluster 1 identified by *in silico* search for WREs (Chang et al., 2008b). Red and blue stars indicate the location of mutated TCF sites and the Helper sites, respectively. Cluster 1 activates luciferase expression when co-transfected with an Arm* expression plasmid. Mutation in the Helper site or TCF sites significantly reduces the Arm* responsiveness of the reporter. (C-J) Confocal images of stage 16–17 embryos of [Cluster 1-lacZ] flies immunostained for Wg (green) and lacZ (red). Cluster 1 activates LacZ expression in cells near Wg expressing cells (B,E,H). LacZ expression pattern in the anterior and posterior part of embryos partially overlaps with Wg expression domain (B,E,H). Mutation of the TCF (F) or Helper sites (G) significantly reduced LacZ expression. Two lines of wild type Cluster 1, three lines of Cluster 1^{TCFmut} and three lines of Cluster $1^{Helpermut}$ were examined.

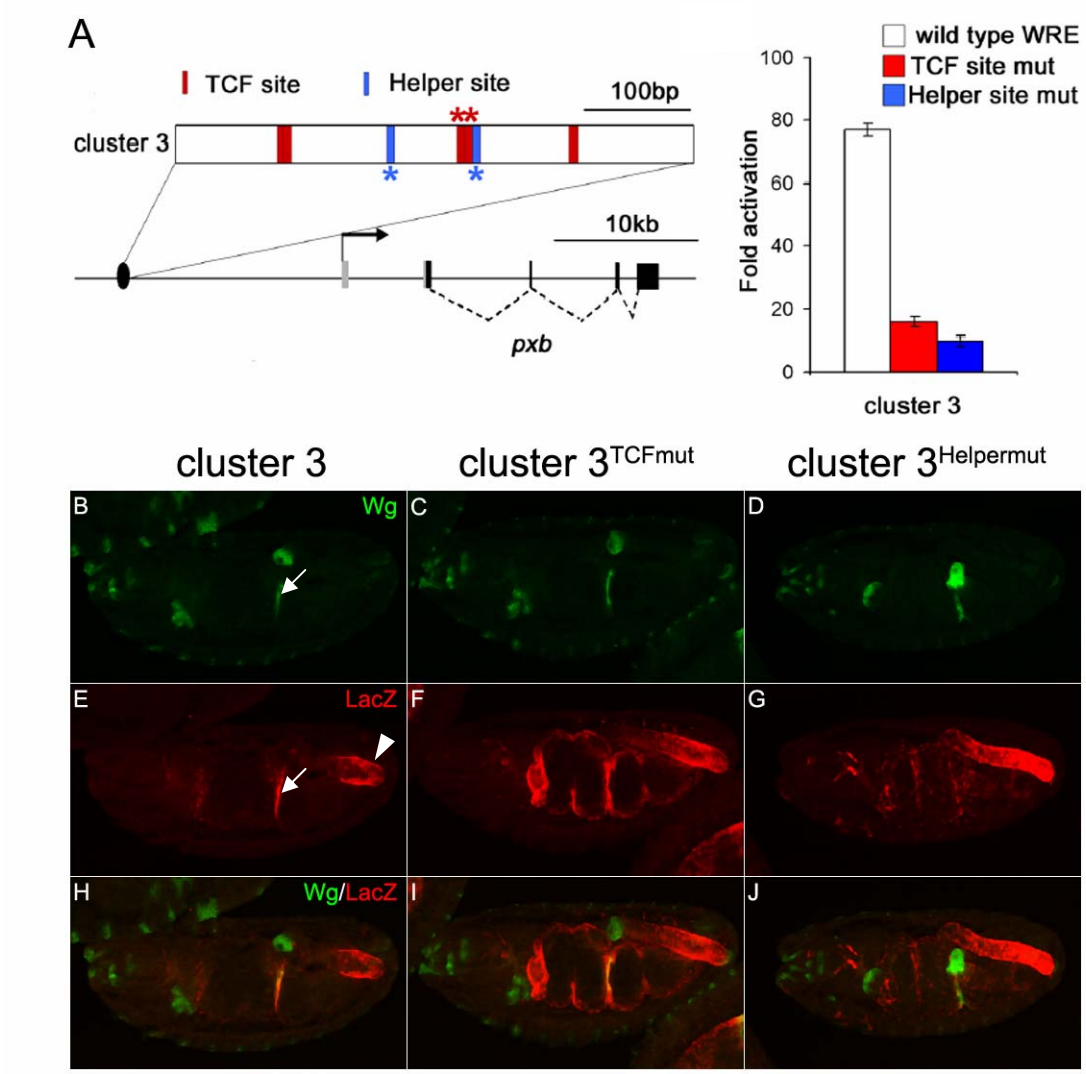

Figure 4.3. TCF sites repress the activation of Cluster 3 in the absence of Wg signaling. (A) Taken from (Chang et al., 2008b). Schematic diagram of the *pxb* gene showing the location of Cluster 3 identified by *in silico* search for WREs (Chang et al., 2008b). *cg5302* (not shown in this cartoon) is located 11.5 kb upstream of Cluster 3. Red and blue stars indicate the location of mutated TCF sites and the Helper sites, respectively. Cluster 3 (484 bp) activates luciferase expression in response to overexpression of Arm*. The activation of Cluster 3 is TCF sites- and Helper sites-dependent. (B-J) Confocal images of the stage 16–17 embryos of [Cluster 3-lacZ] flies immunostained for Wg (green) and lacZ (red). Cluster 3 activates LacZ expression around the second constriction of midgut in a similar pattern to Wg (B,E, arrows). LacZ expression is also observed in hindgut (E, arrowhead). Mutation of TCF sites dramatically derepressed the lacZ expression throughout the midgut (F). Mutation of Helper sites caused the weak derepression in lacZ expression in midgut (G). However, mutations of neither TCF sites, nor Helper sites, reduce the lacZ expression in the regions where Cluster 3 is normally active. Three lines of wild type Cluster 3, three lines of Cluster 3^{TCFmut} and two lines of Cluster $3^{Helpermut}$ were examined.

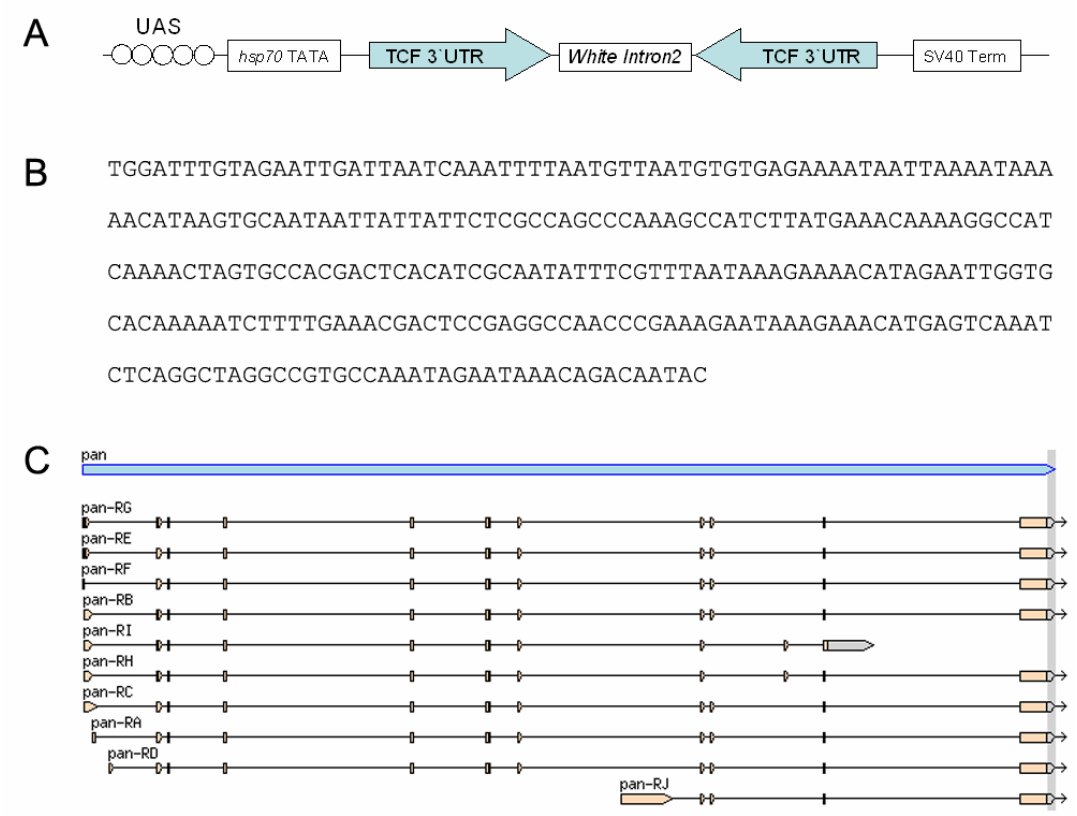

**Figure 4.4. P[*UAS-TCF^{RNAi}*] strains generate dsRNA against TCF 3`UTR.** (A) Schematic diagram of *UAS-TCF$^{RNAi}$* in pWIZ vector (Lee and Carthew, 2003). A Head to Head inverted repeat will generated a loopless hairpin RNA that can target the TCF 3`UTR. (B) The sequence of clone TCF 3`UTR is shown. (C) *pan* loci is shown. The *pan* gene is shown in blue. Structures of mRNA reported in fly base are shown. Pink boxes indicate the ORF. Light blue boxes show the UTR. *UAS-TCF^{RNAi}* can target all the mRNA isoforms except pan-RI.

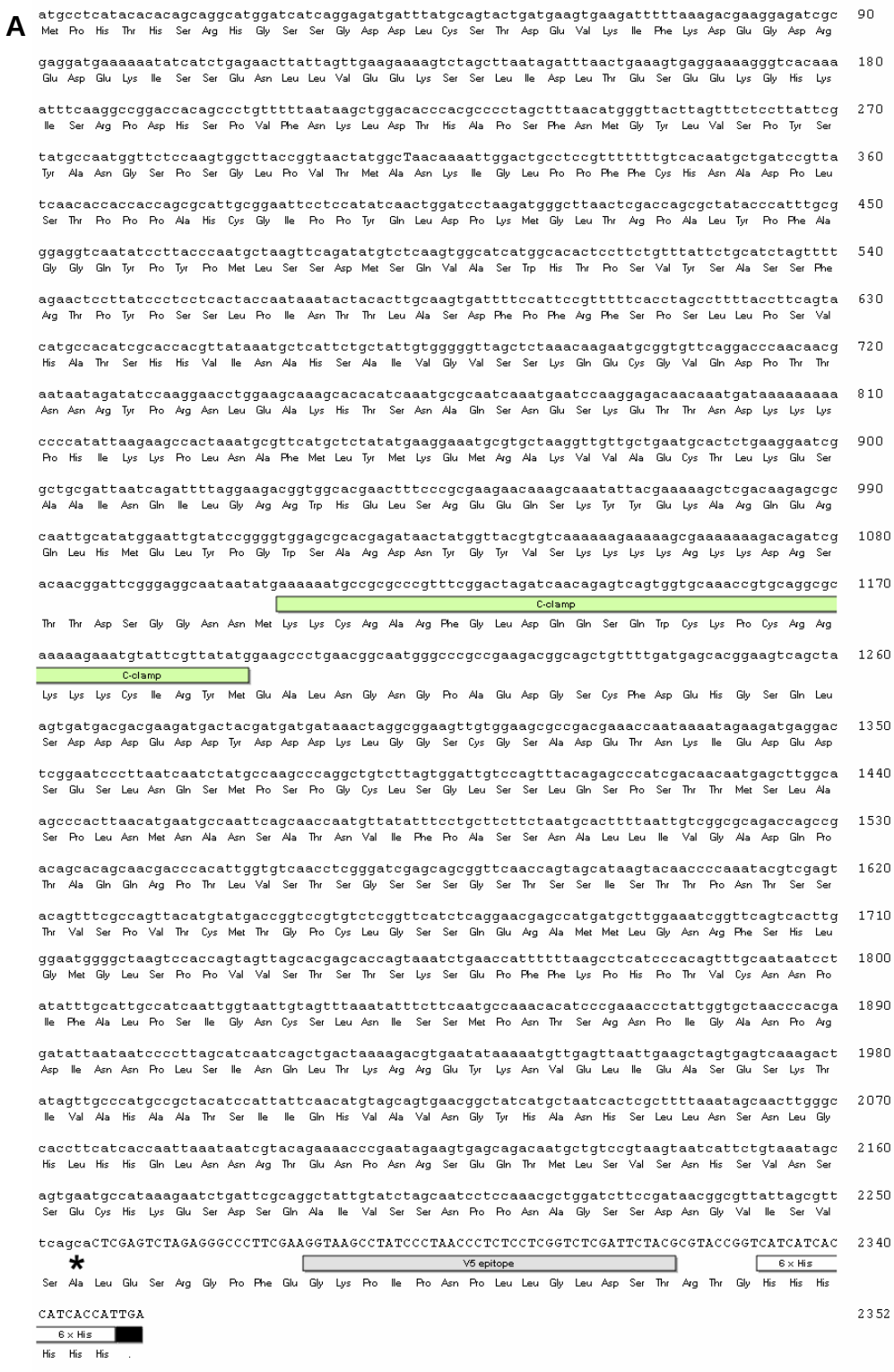

Figure 4.5. (will be continued on the next page)

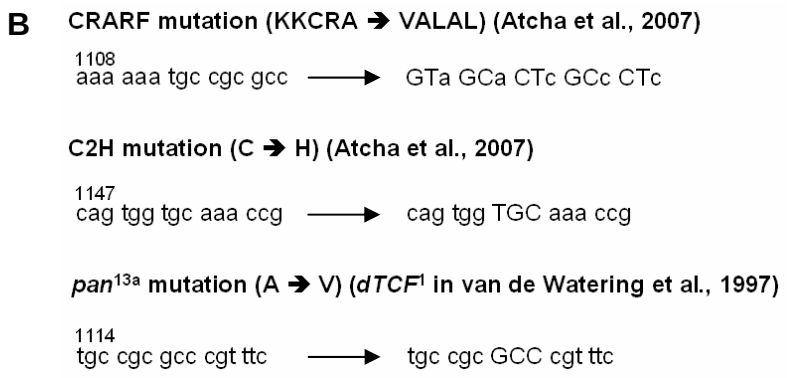

Figure 4.5. P[*UAS-TCF*] express V5/6xHis-tagged TCF proteins. (A) Nucleotide and amino acid sequences of V5/6xHis-tagged TCF are shown. Lowercase letters show nucleotide sequences from the ORF of *dTCF* (*Pan*). Star (*) indicates the amino acid replacing stop codon (Chang et al., 2008b). C-clamp, V5 epitope and 6xHis are indicated with a green box, a grey box and a white box, respectively. Black box indicates the stop codon. (B) Three types of mutations designed to destroy the C-clamp are shown. Nucleotide sequences of wild type TCF are shown on the left. The numbers above the nucleotides indicate the position of the first nucleotide in V5/6xHis-tagged TCF. Nucleotides mutated in each mutant are capitalized on the right. The detailed information on each mutation can be found in the references cited above.

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CHAPTER V

DISCUSSION AND FUTURE DIRECTIONS

My thesis research identified Helper sites, new cis-regulatory elements that are as essential as TCF binding sites for the activation of several WREs by Wg signaling *in vivo*. By interacting with the C-clamp of TCF, Helper sites enhance the binding of TCF to WREs. In this Chapter, remaining questions will be discussed and the potential experiments to address these questions will be suggested.

Organization of TCF binding sites and Helper sites in WREs

Analysis of six WREs revealed that there is no apparent pattern to the spacing and orientation of Helper sites in relation to nearby functional TCF binding sites (Figure 3.11). However, this does not necessarily to mean that TCF does not have a preference for a certain organization of its binding sites in WREs. For example, p53 binds to its target sites as a homo-tetramer. Each p53 dimer binds to a half-site that consist of a 10 bp motif. p53 tetramer binding sites in p53 responsive elements (p53 REs) contain two half-sites separated by a spacer that is usually composed of 0-21 bp (Riley et al., 2008). Close analysis of p53 REs, however, found that p53-activated genes prefer 0 bp-length spacing but p53-repressed genes tend to have longer spacers (Riley et al., 2008). Therefore, the examination of the hidden rules of the spacing and orientation between Helpere sites and

TCF binding sites would be important to determine a likely correlation between a certain WREs organization and its specific biological readout, as well as increasing our ability to locate WREs through *in silico* searches.

There are two general approaches to examining spacing and orientation requirement of Helper sites for WRE activation. The first approach is to analyze more WREs in a similar way to what was done in Chapter III. There are experimentally validated WREs, whose expression was shown to be Wg signaling-dependent *in vivo* (e.g. *Ultrabithorax* (*Ubx*), *even-skipped* (*eve*) loci and *nkd*-UpE1) (Chang et al., 2008a; Knirr and Frasch, 2001; Riese et al., 1997). In addition to increasing the sample size to analyze the organization of Helper sites in natural WREs (Figure 3.11), these experiments could help us to understand how broadly Helper sites are required in Wg signaling-mediated transcription and to fine-tune the consensus Helper site sequences (Figure 3.8).

The second approach is to determine the relative affinity of TCF to DNA oligos containing both TCF binding sites and Helper sites with different spacing and orientation through EMSA. Currently, Erik Anderson, a rotation student in Cadigan lab, found that the orientation of a helper site in relation to a TCF site can affect the binding affinity of TCF to a TCF/Helper site pair *in vitro* (Figure 5.3). The correlation between the affinities of TCF binding and WRE activity could be further-tested in natural WREs. For example, in the *nkd*-IntE, the second TCF site- Helper site pair strongly contributes to the *nkd*-IntE activity (Figure 3.3). By changing the organization of the second TCF-Helper site pair, the contribution of TCF binding affinity to WRE activity could be determined. This information could also enhance the efficiency of *in silico* identification of WREs.

The function of 'CGGTTTGCTT' motif in the *nkd*-IntE

Unbiased mutagenesis of *nkd*-IntE found three regions that are required for Wg signaling responsiveness in Kc cells (Figure 3.2). Two regions (Region A and Region C in Figure 3.2) contain TCF binding sites and Helper sites (Figure 3.3). The other region (Region B in Figure 3.2) consists of 10 bp sequences, (CGGTTTGCTT). Although the sequence resembles the TCF sites (SSTTTGWW, S: G/C, W: A/T), the mutations that are supposed to destroy the binding of TCF in this element didn't reduce the activation of the *nkd*-IntE, suggesting the element is not a TCF site (Figure 3.3). The result showing the absolute requirement of Region B (CGGTTTGCTT) to *nkd*-IntE activity in culture cells (Figure 3.2) raised the possibility that this 10 bp motif could be as critical as TCF binding sites or Helper sites *in vivo*.

Whether Region B is required for WRE activation by endogenous Wg signaling could be tested by generating transgenic *nkd*-IntE reporter flies containing mutations in Region B. If the mutation affects the activity of *nkd*-IntE in specific tissues, these would indicate that Region B contains a cis-regulatory element required for tissue specific transcriptional regulation by Wg signaling. Therefore, WREs of other Wg target genes regulated in the same tissue could be searched for a similar element. If Region B is required for Wg responsiveness in various tissues, the functional relationship between Region B and TCF, or TCF binding sites, could be examined with the same approaches as ones made for Helper sites (Chapter III). One interesting experiment would be to generate a synthetic WRE reporter containing TCF binding sites, Helper sites and Region B (Figure 3.9). A 6TH reporter containing TCF binding sites and Helper sites respond to Wg signaling in many tissues (Figure 3.9). However, the expression pattern did not

completely recapitulate the activation of endogenous Wg signaling (Figure 3.9). If the new synthetic WRE responds to Wg signaling in more tissues than 6TH, it could suggest that clusters of TCF binding sites, Helper sites and a motif in Region B constitute a minimal cis-regulatory element that is required for Wg signaling mediated transcription *in vivo*.

The role of Helper sites/C-clamp interaction in the target gene repression in the absence of Wg signaling

Current analysis of a WRE identified *in silico* (Cluster 3) suggested that Cluster 3 is repressed by TCF in the absence of Wg stimulation (Figure 3.14 and 4.3). In the midgut of *Drosophila* embryos, Cluster 3 activates the expression of lacZ in a very restricted region where *wg* is expressed (Figure 4.3). Mutations in TCF sites caused the strong de-repression throughout the entire midgut. Mutations of Helper sites in Cluster 3 also resulted in derepression, but weakly, as compared with mutations of TCF sites. Although further careful examination is required to make a solid conclusion, this result suggests that the interaction between Helper sites and the C-clamp might not be as important in repression as in activation of WREs.

A traditional model of Wnt/Wg signaling describes TCF as a static DNA binding protein that binds to WREs regardless of Wnt/Wg signaling (Figure 1.2). However, current studies have suggested that this is not always the case. *nkd*, *cg6234* and *notum* are genes directly regulated by Wg signaling (Fang et al., 2006; Parker et al., 2008). Depletion of TCF in *Drosophila* cell line causes the de-repression of the expression of these Wg target genes, suggesting the repression of those genes by TCF in the absence of

signaling (Fang et al., 2006; Parker et al., 2008). Interestingly, the stimulation of Wg signaling further recruited TCF to WREs of these Wg targets (Chang et al., 2008b; Fang et al., 2006; Parker et al., 2008). This increase was not due to enhanced expression or nuclear localization of TCF (Chang et al., 2008b). Consistent with these results, it was shown that β-catenin enhances the binding of LEF-1 on chromatinized DNA containing consensus TCF binding sites (Tutter et al., 2001).

TCF binding to WREs in the absence of Wg stimulation was also suggested to be regulated by chromatin remodelers, ISWI and ACF1 (Liu et al., 2008). The depletion of ISWI and ACF1 resulted in the reduction of TCF binding to WRE in the absence of signaling, suggesting ISWI and ACF1 promotes the binding of TCF to target sites.

These reports suggest a modified model of Wg signaling. In the absence of Wg signaling, TCF binds to WRE with the help of ISWI and ACF1. Since a small amount of TCF in WREs could keep the target genes silent, the Helper sites/C-clamp interaction that enhances the binding affinity might not critical. When Wg stimulates cells, the stabilized β-catenin enhances the TCF binding to target sites, and the Helper sites/C-clamp interaction further stabilize TCF-WRE interaction.

There is another WRE where TCF sites were shown to be required for the repression in the absence of Wg stimultation. In the *dpp* visceral mesoderm enhancer (*dpp* BE enhancer), mutation of the TCF binding sites caused the de-repression of the reporter gene outside of the endogenous expression domain (Yang et al., 2000). This enhancer contains a potential Helper site (GCCGCCA). By mutating Helper sites in the *dpp* BE enhancer, the role of Helper sites, or the Helper site/C-clamp interaction, could be further tested.

Inhibitory activity of C-clamp?

In vitro, TCF binds to the DNA oligo containing both a TCF site and a Helper site (TH probe) with High affinity (Figure 3.11B). TCF also was able to bind to the DNA oligo containing only a Helper site (SH probe) (Figure 3.11E). These TCF-DNA interactions require an intact C-clamp, suggesting that the C-clamp functions as a weak DNA binding domain that can interact with a Helper site in WREs.

Interestingly, the C-clamp also seems to have an opposing activity. Mutations of the C-clamp motif in TCF enhanced the binding of TCF to the DNA oligo containing only a TCF site (TS probe) (Figure 3.11C). A similar effect was observed in human TCF-4E which contains a C-clamp (Figure 5.1) (Hecht and Stemmler, 2003). Deletion of the C-clamp motif in TCF-4E significantly increased the binding of TCF-4E to a TCRα25 oligo containing a TCF site (Figure 5.1E). These results suggest a potential inhibitory activity of C-clamp in the function of HMG domain.

How does C-clamp play both positive and negative roles in TCF-DNA interaction? C-clamp may inhibit the HMG domain from binding to the many non-functional TCF sites found throughout the genome (Figure 5.2A). At the location where both a TCF site and a Helper site exist, C-clamp may interact with the Helper site, releasing the HMG domain from the inhibition (Figure 5.2B). Consequently, the HMG domain could interact with a TCF site, further stabilizing the weak interaction between the Helper site and the C-clamp (Figure 5.2B).

There could be several direct or indirect ways for the C-clamp to inhibit the activity of the HMG domain. Further structural and biochemical studies would be required to solve the mechanism of the C-clamp function clearly. One of the potential

mechanisms which are easily testable is the direct binding of the C-clamp to the HMG domain. The binding could physically occlude the critical amino acid residues of the HMG domain or allosterically change the structure of the HMG domain, affecting DNA binding ability. Differently tagged C-clamp and HMG domains could be generated *in vitro*. Their interaction could be examined through *in vitro* protein interaction assays. Alternatively, EMSA could be used to determine whether an excess amount of C-clamp inhibits the binding of the HMG domain to a consensus TCF binding sites.

Target recognition by other TCF proteins

dTCF and E isoforms of mammalian TCFs have been suggested to specifically bind their targets with high affinity through bipartite interaction of the HMG domain and C-clamp (Mikyung's 2008, (Atcha et al., 2007). However, most of the mammalian TCF isoforms do not have a C-clamp. For example, no isoform of *LEF-1* encodes a C-clamp. Instead, the major isoforms of *LEF-1* contain the "N"-tail which doesn't have any known functional motif (Hovanes et al., 2000). LEF-1 was shown to bind to some WREs including a TCRα25 enhancer and a *Siamois* promoter stronger than other TCF proteins (Hecht and Stemmler, 2003; Pukrop et al., 2001). The *Siamois* promoter was only activated by LEF-1 not by TCF-4E in human cell lines (Hecht and Stemmler, 2003). These results suggest that different TCF proteins interact with endogenous WREs by distinct mechanisms. How do other TCFs achieve the specific target recognition?

The first possible mechanism is that other TCF proteins also have an additional DNA binding domain such as a C-clamp. Although TCF proteins have highly conserved domains, including a β-catenin binding domain and a HMG domain, the other regions of

TCF proteins vary among TCF family members (Hovanes et al., 2000). The non-conserved regions could function in a similar way to C-clamp. However, this possibility is less likely, at least for LEF-1 proteins. In the report showing that TCF-1E can recognize extended sites in addition to known TCF binding sites, it was also examined whether LEF-1 has similar characteristics through CASTing analysis (Atcha et al., 2007). Unlike TCF-1E, LEF-1 did not show any preference in the sequences flanking the TCF binding sites (Atcha et al., 2007). This suggests that LEF-1 might achieve the specificity of target selection by a different mechanism from increasing the size of its recognition sites.

The second possible mechanism is that TCF binds to WREs together with other proteins. For example, some Hox proteins such as Ubx are known to bind to target sites cooperatively with co-factors such as Exd (Peifer and Wieschaus, 1990). Similarity, TCFs that don't have a C-clamp might interact with other DNA binding proteins at WREs, thereby enhancing the affinity or specificity of the interaction between TCF and WREs.

The third possible mechanism is that the HMG domain of different TCF has different binding properties. The HMG domain among TCF family members are highly conserved, (Arce et al., 2006), and can bind to similar sequences in vitro (Figure 1.5.). However, it doesn't necessarily mean that they bind to identical sites with the same affinity *in vivo*. Different from *Drosophila*, vertebrate have several TCF genes. The HMG domains among vertebrate TCF are not exactly identical. Therefore, the HMG domain of different TCF that don't have C-clamp might bind to similar but distinct target sites with higher affinity than the HMG domain of TCF-E isoforms.

There could be several ways to determine the target selection mechanism by other TCF proteins such as LEF-1. LEF-1 can activate the *Siamois* WRE, but not *Cdx-1* WRE, although it can bind to TCF binding sites in the *Cdx-1* WRE *in vitro* (Atcha et al., 2003; Hecht and Stemmler, 2003). The functional motif in LEF-1 that is responsible for specificity could be found by generating a series of mutant LEF-1 proteins as well. Candidate regions, such as N-tail, could be deleted or mutated, and the functional importance of mutated regions could be tested by reporter assays with the *Siamois* WRE (Atcha et al., 2003; Atcha et al., 2007; Hecht and Stemmler, 2003). As a control experiment, the ability of mutants to activate a reporter containing high affinity TCF sites could be tested.

Conversely, the cis-regulatory elements that are required for the activation of *Siamois* WRE by LEF-1 could be searched. Helper sites that interact with the C-clamp of fly TCF were often found near functional TCF binding sites (Figure 3.11). The cis-regulatory elements required for the activation by LEF-1 might exist close to the functional TCF sites in *Siamois* WRE as well. To test this possibility, the TCF binding sites and its flanking sequences in *Siamois* WRE could be swapped with ones of *Cdx-1* WRE, or mutated. Once the cis-regulatory region is found, the motif in LEF-1 that functionally interact with the cis-regulatory elements could be searched as described above.

ACKNOWLEGMENTS

Figure 5.3. was drawn based on the experimental results performed by Erik Anderson.

FIGURES

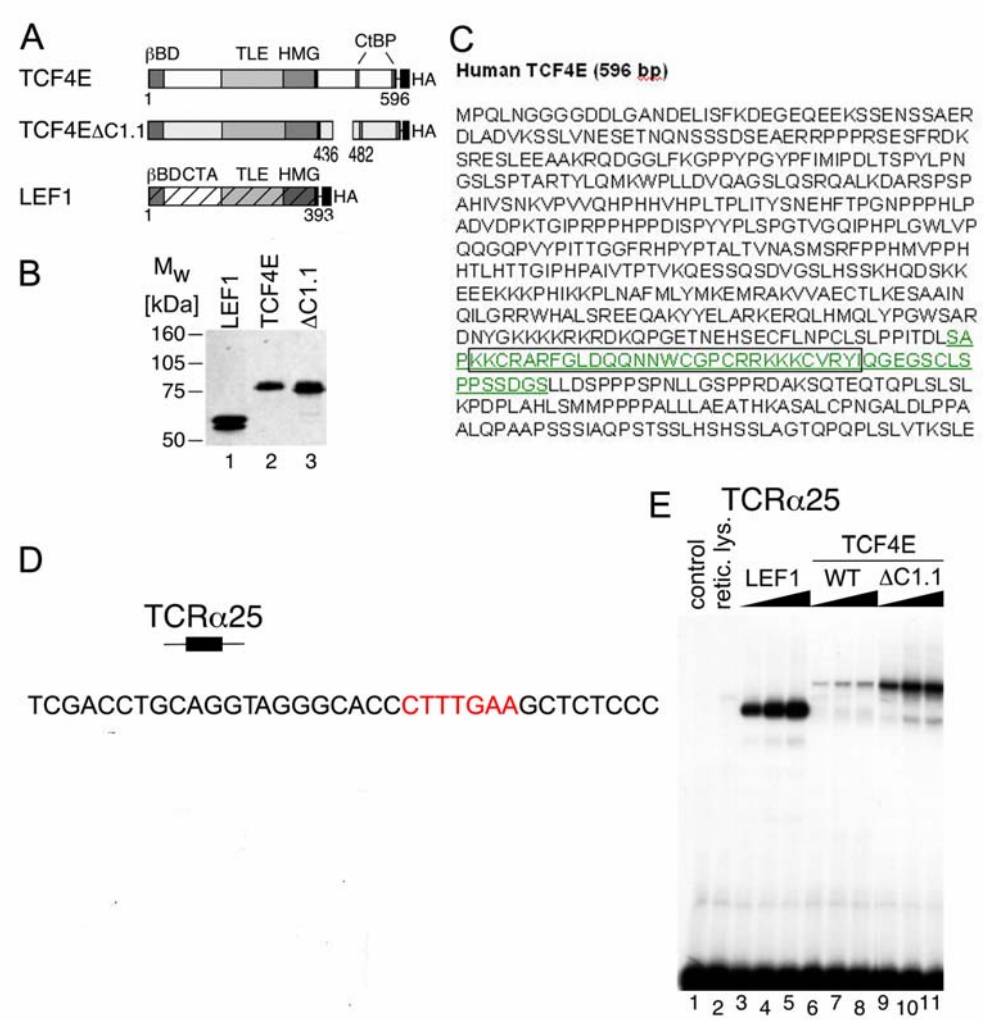

Figure 5.1. TCF4E C terminus inhibits TCF4E from binding to a TCF site with high affinity *in vitro*. Taken from (Hecht and Stemmler, 2003). (A) Cartoons of TCF/LEF proteins used in EMSA. Binding sites for β-catenin (βBD), Grg/TLE (TLE), and CtBP (CtBP) are shown. LEF contains the context-dependent transactivation domain (CTA). (B) Western blot analyses of *in vitro* translated TCF/LEF proteins used for EMSA with an anti-HA antibody. (C) The sequence of full-length TCF4E is shown. The region deleted in TCF4EΔC1.1 is underlined in green. The C-clamp motif in TCF4E is boxed. (C) Cartoon and sequence of the TCRα25 DNA oligo used in EMSA. A TCF site is shown in red. (D) Comparison of the binding affinity of different TCF/LEF proteins. LEF1 binds to the TCRα25 DNA probe much better than TCF4E (compare lanes 3-5 with lanes 6-8). Deleting C-clamp in TCF4E (TCF4EΔC1.1) significantly increase the binding affinity of TCF4E (compare lanes 6-8 with lanes 9-11), suggesting the inhibitory activity of the C-clamp in the interaction between TCF4E and TCF site.

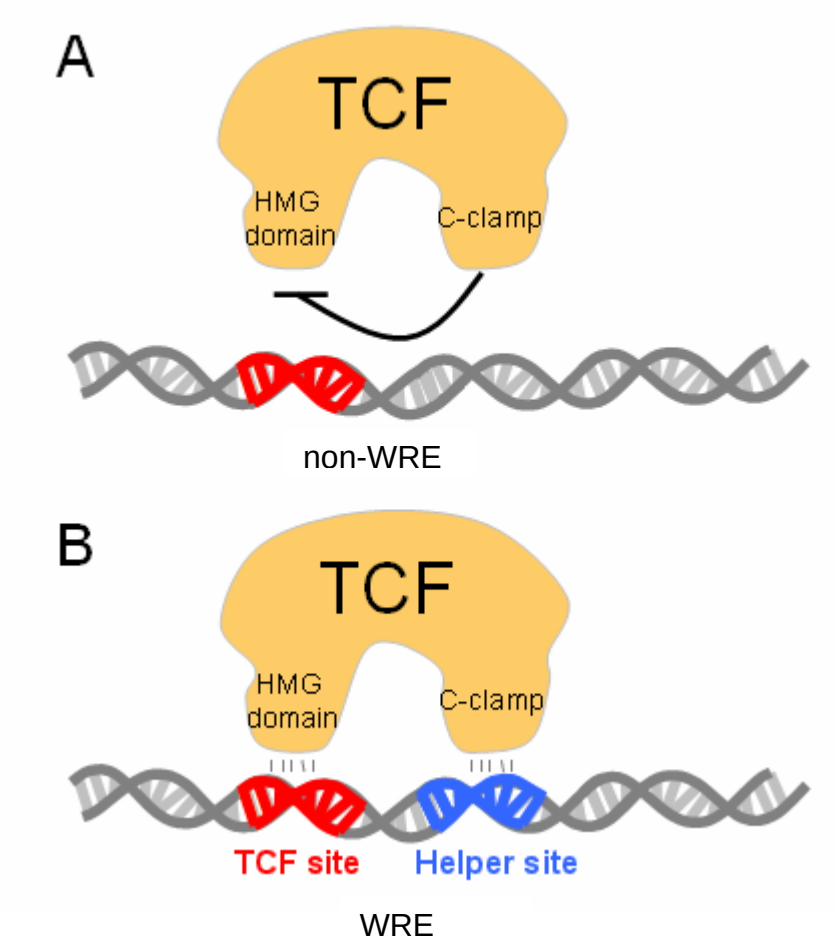

Figure 5.2. Models for the function of C-clamp in the absence (A) or presence (B) of a Helper site. (A) In the absence of Helper sites in DNA, the C-clamp interacts with the HMG domain to prevent HMG domain from binding to TCF sites. (B) In the presence of Helper sites, the C-clamp interacts with Helper sites rather than with the HMG domain, allowing the HMG domain to bind to TCF sites. The cooperative interaction of the HMG domain/TCF sites and C-clamp/Helper sites stabilize the TCF binding to WREs.

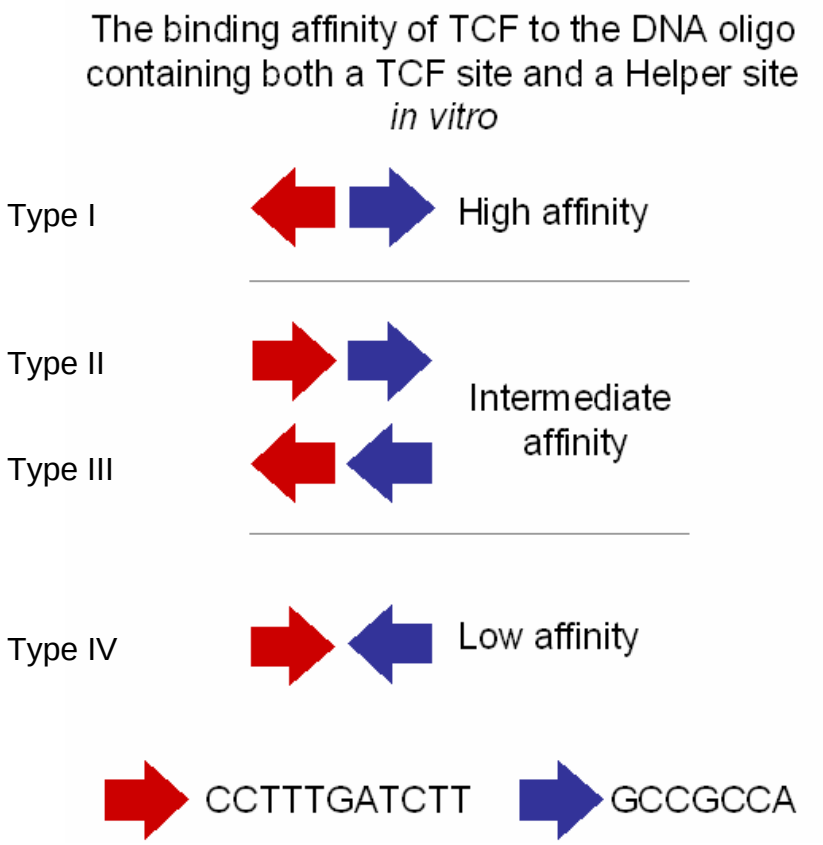

Figure 5.3. The orientation of a Helper site in relation to a TCF site affects the binding affinity of TCF *in vitro*. Summary of the *in vitro* protein-DNA interaction assay, EMSA, results. The binding of GST-TCF protein (Figure 3.11) to the DNA oligo containing a TCF-Helper site pair was tested *in vitro*.

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