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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

GENERAL INTRODUCTION

Wnts comprise a large family of proteins that are found from mammals to cnidarians (Cadigan and Nusse, 1997 and Guder et al., 2006). Wnts function via binding to cellular receptors and activate multiple downstream signaling cascades. It has been demonstrated that Wnt signaling is repeatedly used during the entire lifespan to control numerous cellular processes (Cadigan and Nusse, 1997; Guder et al., 2006; Lee et al., 2006; Marikawa et al., 2006; Walston and Hardin, 2006). In addition, misregulation of Wnt activity has been implicated in many human diseases and developmental defects (Logan and Nusse, 2004; Barker and Clevers, 2007; Polakis, 2007).

In order to control cell fates and behaviors, Wnt signaling regulates the transcription of its downstream target genes. For instance, Wnt signaling can induce *c-Myc* transcription in human colorectal cancer cells (He et al., 1998). *c-Myc*, which is frequently amplified in multiple cancers, might be important to induce breast tumors in mice when it is constitutively expressed in the mammary gland (Cowling et al., 2007). Thus, it is conceivable that oncogenicity of the Wnt signal in human colon cells might be in part executed through activation of a proto-oncogene, *c-Myc*. Consistent with this idea, deletion of *c-Myc* is shown to rescue the phenotypes caused by the high level of Wnt signaling in mice intestines (Sansom et al., 2007), indicating the importance of gene regulation in Wnt signal transduction.

Wnt signaling can regulate multiple genes in a cell/tissue-specific manner. Numerous Wnt genes have been discovered, mainly via genetic and biochemical analyses (a list of Wnt targets can be found from the Wnt homepage, http://www.stanford.edu/~rnusse/pathways/targets.html). For the last 5 years, technologies such as microarrays, comparative genomics and chromatin immunoprecipitation assays on a genome wide scale have allowed researchers to reveal a large set of Wnt target genes from many different contexts (Vlad et al., 2008). Consistent with the many functions of the Wnt pathway, targetomes (a full list of target genes) are quite different depending on the cell type and time points. Comparison of the different targetomes, which have been identified by multiple microarray analyses revealed that less than 5% of all target candidates were found in all studies (Vlad et al., 2008). This may indicate that pleiotropic activities of Wnts in many different tissues are largely achieved by regulating its target genes in a cell/tissue-specific manner.

The two main topics of this thesis are the mechanisms underlying (1) Wnt-mediated transcriptional activation and (2) the regulation of Wnt activity in a gene and cell specific way. In this general introduction, the current model of transcriptional regulation by Wnt signaling is discussed. In addition, I list some of the known Wnt targets and give a few examples of how Wnt activity is regulated.

Wnts have pleiotropic action

Studies in animal model systems, such as the cnidarian *Hydra magnipapillata,* the nematode *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster*, the zebrafish *Danio rerio*, the South African clawed frog *Xenopus laevis*, the mouse *Mus musculus*,

and multiple cell lines originated from normal or cancerous human tissues showed that Wnts play essential roles in many different cellular processes including development, organogenesis, adult tissue homeostasis and diseases. The roles of Wnts in early development and organogenesis are described in more detail.

Wnt signaling and body patterning

The Wnt pathway has been reported to regulate body patterning during embryogenesis throughout the animal kingdom. In cnidarians, the simplest metazoa with two germ layers, Wnts are suggested to initiate the patterning of the oral–aboral axis in both ecto- and endoderms (Guder et al., 2006). In four-cell stage *C. elegans* embryos, *mom-2* (a nematode Wnt) activity is important for the polarized cell division which later determines endoderm and mesoderm specification (reviewed in Walston and Hardin, 2006). In *Xenopus*, maternal Wnt11 in oocytes is essential for dorsal-ventral axis specification in embryos (Tao et al., 2005).

Studies done in two popular models, fruit flies and mice have provided some insight of downstream target gene regulation of Wnts in a developmental context. In *Drosophila*, loss of function alleles of *wingless* (*wg*), a fly Wnt gene results in embryonic lethality via disrupting the embryonic body plan (Sanson, 2001; Bejsovec, 2006). This is in part because the activity of *wg* is required for determination of the borders of each segment primordium, or parasegment. At this developmental stage, Wg is expressed in a single row of cells marking the posterior border of each parasegment and maintains expression of a homeodomain gene, *engrailed* (*en)* in adjoining cells to specify the anterior edge of parasegments (reviewed in Sanson, 2001; Bejsovec, 2006).

In mice, *Wnt3a* is required for the specification of trunk and tail paraxial somatic mesoderm fates (Takada et al. 1994). One target that is directly activated by Wnt signaling in this context is *Brachyury (T)*, a DNA-binding transcription factor (Yamaguchi et al., 1999). Mutations in *T* lead to a loss of trunk and tail mesoderm which is similar to the phenotype of *Wnt3a* mutation (Yamaguchi et al., 1999 and the reference therein). Thus it is conceivable that the activity of Wnt3a in mesoderm specification is achieved, at least in part, through the activity of *T* (Yamaguchi et al., 1999).

Wnt signaling and organogenesis

Wnt signaling is required for organogenesis from embryonic stages to adulthood. Mammalian organs that require Wnt activity for the development and the repair include heart, kidney, bone, small intestine and reproductive system (Clevers, 2006; Bernard ad Harley, 2007; Merkel et al., 2007; Tzahor, 2007).

In flies, each adult appendage, such as wing, leg, eye and antenna, is newly developed from a single layer of epithelial cells termed the imaginal disc (Demerec, 1994). The imaginal discs originate from embryonic epithelium and only increase their size via cell proliferation until late third instar stages. During metamorphosis, cells in the imaginal discs go through extensive developmental processes under the control of multiple signaling pathways including the Wnt signal.

Here the roles of Wnts in development of mouse kidney and fly appendages are explained in more detail.

(a) Wnt signaling and mouse kidney development

In mice, embryonic kidney development begins when a group of cells called the ureteric bud (UB) invades pre-specified mesenchyme cells known as the metanephric mesenchyme (MM). This UB continues to branch within the MM and eventually forms the collecting duct system and extra-renal ureter. A subset of mesenchymal cells, shortly after invasion of the UB, undergoes a mesenchymal to epithelial transition to form the renal vesicles (RVs). These RVs are where the majority of the nephron is originated from (reviewed in Merkel et al. 2007)

wnt9b$^{-/-}$ mice die within 24 h after birth with severe defects in kidney (Carrol et al., 2005). The mutant kidney has significantly less branches derived from the UB. Conversely, Wnt9b treatment can lead to survival and branching of the isolated UB in culture medium, suggesting the Wnt signal as a sufficient and necessary component of branch morphogenesis (Park et al., 2007). While multiple targets of Wnt9b including another Wnt, Wnt4 have been identified in this context (Park et al., 2007), *T* which is activated by Wnts in the early mesodermal mouse tissues is not expressed in the kidney (Kidney Development Database, http://golgi.ana.ed.ac.uk/tranfack.html), indicating that *T* is a tissue-specific Wnt target.

(b) Wg/Wnt signaling and fruit fly wing development

The gene *wingless (wg)* was named after the wing to notum transformation phenotype caused by a hypomorphic mutation of the *wg* gene (wg^{1}) (reviewed in Besjovec, 2006). In the late third instar stage, Wg expression is very prominent in a narrow stripe across the dorsal/ventral (D/V) boundary of the wing imaginal disc (Baker,

1988; Williams et al., 1993; Couso et al., 1994). In cells adjacent to the Wg-expressing D/V boundary, the Wg signal controls formation of sensory bristles, the peripheral sense organs of the wing margin via, in part, activating proneuronal transcription factors such as *achaete (ac) and senseless (sens)* (Phillips and Whittle, 1993; Couso et al., 1994; Nolo et al., 2000). In addition, other transcription factors genes such as *Distal-less (Dll)* and *vestigial (vg)* are expressed in a wider region flanking the D/V stripe to achieve proper wing development, consistent with Wg being a morphogen (Neumann and Cohen, 1997; Baena-Lopez and Garcia-Bellido, 2006).

While cell fate specification along the D/V boundary is in part controlled by Wg activity, the determination of anterior-posterior (A/P) axis in the wing requires other genes such as *en* (Dahmann and Basler, 2000). *en*, encodes a transcriptional repressor that is evenly expressed in the posterior half of the developing wing and specifies the posterior compartment of the wing, in part, via repressing expression of Hedgehog (Hg) signaling targets (Dahmann and Basler, 2000). Interestingly, unlike in fly embryonic epidermis where *en* expression is regulated by Wg signaling, *en* expression in the wing imaginal discs appears to be largely independent of Wg activity based on its unrelated expression pattern to the *wg* pattern, indicating that *en* is regulated by Wg signaling in a tissue-specific manner.

In summary, Wnt signaling displays manifold activities in numerous distinct temporal and special contexts. Several layers of evidence such as *en* in fruit flies and *T* in mice have strongly suggested that dynamic regulation of multiple targets is essential for Wnt signaling to achieve its pleiotropic function.

In the following section, three different branches of downstream cascades that are activated by Wnts are described.

Wnts can activate multiple downstream cascades

Consistent with their numerous roles in biology, Wnts can activate at least three separate downstream cascades, each of which can exhibit different transcription outputs. The downstream cascades regulated by Wnts can be mainly grouped as the β-catenin dependent canonical pathway and the β-catenin independent noncanonical pathways (Fig. 1.1).

The Wnt/β-catenin pathway

Activity of the canonical pathway, often called the Wnt/β-catenin pathway mainly relies on the change of β-catenin protein level in cells (reviewed in Parker et al., 2007). In the absence of signaling, a cytoplasmic protein β-catenin is constitutively produced but also constitutively degraded resulting in low, steady-state levels in the cell. When cells receive a Wnt signal, degradation of β-catenin is inhibited, resulting in the increase of the protein in the cytoplasm. This stabilized β-catenin moves into the nucleus where it binds to a sequence-specific DNA binding protein, TCF/LEF (T-cell factor/Lymphoid enhancer factor) on the Wnt target loci to alter gene transcription.

This Wnt/β-catenin pathway is required for many known Wnt roles in normal development and disease conditions. For instance, two previously mentioned Wnt targets, *en* in fruit flies and *T* in mice are activated by a TCF- and β-catenin-dependent

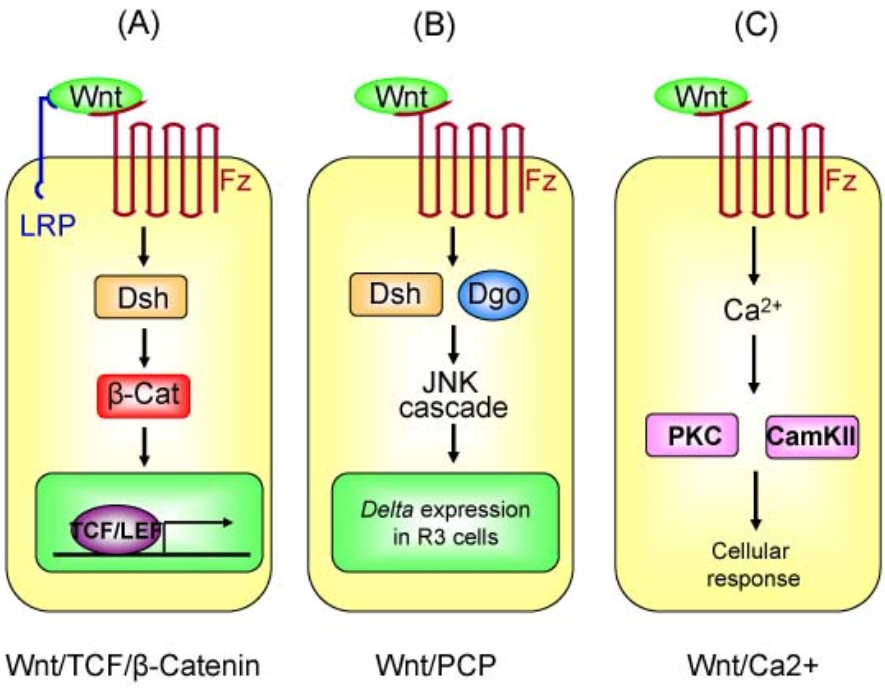

Fig. 1.1. Simplified diagrams of the three known Wnt signaling pathways. (A) Canonical Wnt signaling centers on the regulation of a cytoplasmic protein, β-catenin. The Wnt signal transduced through membrane receptors, Fz and LRP, can increase the level of β-catenin in a Dvl-dependent manner. β-catenin translocates into the nucleus where it binds to a sequence-specific DNA binding protein, TCF/LEF1, to alter the gene transcription. (B) In the Wnt/PCP pathway (known as the Wnt/JNK pathway in vertebrates), Fz also acts as a receptor to transduce the signal to Dsh. Dsh in a complex with Dgo, activates downstream cascades such as the JNK pathway. One of the known Wnt/PCP signaling targets is the *Delta* gene encoding a ligand in the Notch pathway from photoreceptor R3 cells in Drosophila. (C) The Wnt/Ca^{2+} pathway signals through the Fz receptors and employs Ca^{2+} as a cytoplasmic effecter. Ca2+ released from the endoplasmic reticulum upon Wnt stimulation can activate protein kinase C(PKC) and calcium/calmodulin-regulated kinase (CamKII). (adopted and modified from Lee et al., 2006)

manner. As the Wnt/β-catenin pathway is the main interest of this thesis, more details of this canonical pathway will be discussed in the following section.

The Wnt/Planar Cell Polarity pathway

The Wnt/Planar Cell Polarity (PCP) pathway is also conserved in metazoa and allows cells to form structures in response to positional information (Jenny and Mlodzik, 2006; Fig. 1.1B). Examples of cellular processes requiring the Wnt/PCP pathway include the polarized cell divisions during worm development, compound eye development in fruit flies, and the proper alignment of bristles on insects, scales on fish, feathers on birds, and hairs on mice.

The molecular mechanisms of the Wnt/PCP pathway have been best studied in *Drosophila* (Jenny and Mlodzik, 2006). The Wnt/PCP pathway shares some components used in the canonical Wnt/β-catenin pathway such as a seven-pass transmembrane receptor, Frizzled (Fz) and a cytoplasmic effecter Dishevelled (Dsh, Dvl in vertebrates). The compound eye of fruit flies is composed of hundreds of unit structures called ommatidium. Each ommatidium contains 8 photoreceptor cells (R1 to R8) and these 8 cells are coordinately aligned with anterior/posterior and dorsal/ventral axes to achieve proper imaging process (Carthew, 2007). In the absence of Wnt/PCP signaling caused by loss-of-function mutation in *fz*, the precise alignment of photoreceptors is lost, even though individual ommatidium structures form proper (Zheng et al., 1995; Jenny et al., 2003, 2005).

During larval eye development, Wnt/PCP signaling specifies the fate of R3 and R4 photoreceptors. In R3 cells where Wnt/PCP activity is required, Fz seems to act as a

receptor and transduce the signal into Dsh (Zheng et al., 1995). Dsh, which can interact with Fz via its PDZ domain, associates with Diego (Dgo; Jenny et al., 2005) to activate alternative downstream components including the small GTPases (Rho and Rac; Fanto et al., 2000), the Ste20-like kinase, Misshapen (Msn; Paricio et al., 1999) and a JNK/MAPKinases (Weber et al., 2000). A Wnt/PCP target gene in R3 cells is *Delta (Dl)*, encoding a ligand of the Notch signaling pathway. Delta specifically exposed on the surface of R3 cells in turn signals to its receptor Notch (N) present on the neighboring R4 cells, leading to the R4 fate specification (Fanto et al., 19998).

In R4 cells, Wnt/PCP activity is suppressed by the four-pass transmembrane protein Van Gogh/Strabismus (Vang/Stbm) and the cytoplasmic proteins Prickle (Pk). Pk, which can associate with Vang, may antagonize Wnt/PCP signaling activity in part by interacting with Dsh, which excludes Dgo from the Dsh complex (Taylor et al., 1998; Jenny et al., 2005).

While the identity of ligands in fly PCP signaling is still unclear, there is strong evidence suggesting Wnts act as ligands for this pathway in worms and vertebrates. In *C. elegans* early embryos, a worm Wnt, *mom-2* is required for the polarized cell division which requires *mom-5* (*Frizzled*) and *dsh-2* (Thorpe et al., 1997; Walston et al., 2004). In zebrafish and frog embryos, Wnt5a and Wnt11 are required to activate Wnt/PCP signaling during organogenesis and gastrulation, respectively (Rauch et al., 1997; Smith et al., 2000).

The Wnt/Ca^{2+} pathway

The Wnt/Ca^{2+} pathway also requires a Fz receptor but signals through a different cytoplasmic effecter, intracellular Ca^{2+} (Fig. 1.1C) (Kohn and Moon, 2005; Slusarski and Pelegri, 2007). Ca^{2+} is released from the endoplasmic reticulum upon Wnt stimulation and can activate calcium/calmodulin-regulated kinase (CamKII) and protein kinase C (PKC).

In zebrafish embryos, depletion of Wnt5 reduces calcium fluxes and ectopic expression of Wnt5a, Wnt11 or rat Fz2 increases the frequency of intracellular Ca^{2+} release with a G-protein activity-dependent manner (Slusarski et al., 1997a, 1997b). Consistent with Ca^{2+} being a secondary messenger of Wnt signaling, activity of CamKII and PKC in *Xenopus* embryos expressing ectopic Wnt5a or rat Fz-2 increases judged via *in vitro* activity assays (Sheldahl et al., 1999). In zebrafish, loss-of-function mutation of *wnt5* results in hyperdorsalization and this defect can be partially rescued by constitutively active CamKII (Westfall et al., 2003), collectively suggesting the Ca^{2+} pathway as one of physiological downstream cascades of the Wnt pathway.

TCF/β-catenin is specific for the canonical Wnt pathway

While some components are shared by the three branches of Wnt signaling (e.g. Fz receptors by all three cascades, Dsh by the Wnt/β-catenin and Wnt/PCP cascades), the TCF/β-catenin complex, which is essential for transcription regulation by so called the canonical Wnt pathway appears to be specific to the canonical Wnt pathway. Consistent with this notion, in a four-cell-stage *C. elegans* embryo, Wnt signaling polarizes an endoderm precursor called EMS. The polarization of this cell orients its mitotic spindle

and this process is controlled by the Wnt/PCP pathway (Schlesinger et al., 1999). Reduction in *mom-2 (wnt), mom-5* (*Frizzled*) and *dsh-2* via RNA interference disrupts spindle orientation (Thorpe et al., 1997; Walston et al., 2004) however, depletion of *pop-1 (TCF)* from embryos yields largely normal spindle formation (Schlesinger et al., 1999). This suggests that TCF and probably its binding protein β-catenin are not required for the Wnt/PCP pathway.

In the case of the Wnt/ Ca^{2+} pathway, β-catenin also appears to be unnecessary. When Weeraratna and coworkers (2002) induced the cellular invasion of certain melanoma cells *in vitro* by transfecting *wnt5a*, there was a dramatic increase in activated PKC. This Wnt5a-induced PKC activation was reduced when an antibody of Fz was added to cells to inhibit Fz activity. Interestingly, however, there was no noticeable increase of β-catenin in the cytoplasm or in the nucleus, suggesting that signal transduction by the Wnt/Ca^{2+} pathway is independent of β-catenin.

In this thesis, gene regulation by the Wnt/β-catenin pathway is the main focus. Extensive studies of the Wnt/β-catenin pathway for years have revealed several target genes in the various contexts. But many questions about the mechanisms underlying the transcription of each gene and its tissue-specific regulation remain unsolved. In the following section, our current knowledge of the Wnt/β-catenin pathway is summarized.

The canonical Wnt/β-catenin pathway

Signal reception at the cell surface

(a) Main Wnt receptors, Fz and LRP

The current model for canonical Wnt signaling is that Wnt ligands activate signaling by binding two transmembrane proteins, Frizzled (Fz) and Low-density-lipoprotein-related protein, (LRP), resulting in formation of a hetero-trimeric complex (Fig. 1.2).

Fz is a seven-pass transmembrane protein with an extracellular cysteine-rich domain (CRD) and a cytoplasmic tail containing a conserved motif (KTXXXW) (Cadigan and Nusse, 1997). Partial or total loss of fly *fzs* is reminiscent to loss of Wnt signaling (Chen and Struhl, 1999), indicating their importance in the pathway. *In vitro* binding assay and structural analysis revealed that Fz can bind to Wnts via its CRD domain and also interact with a cytoplasmic protein, Dsh via its conserved motif (KTXXXW) (Wu and Nusse, 2002; Wong et al., 2003). This interaction has been correlated with transduction of Wnt signal into the cytosol (Yanagawa et al., 1995). Consistent with this, when overexpressed, Fz can promote the membrane localization and the phosphorylation of Dsh, which are tightly correlated with Wnt signal activity (Yanagawa and Nusse et al., 1995; Lee et al., 1999; Umbhauer et al., 2000; Takada et al., 2005). In addition, *Xenopus* Fz with mutations on KTXXXW motif can no longer induce *siamois*, a Wnt/TCF/β-catenin target, further supporting the importance of Fz and Dsh interaction (Umbhauer et al., 2000).

Another receptor protein, LRP is a single transmembrane protein which has large extracellular and short cytoplasmic domains (Liu and Cadigan, 2006). Like Fz receptors,

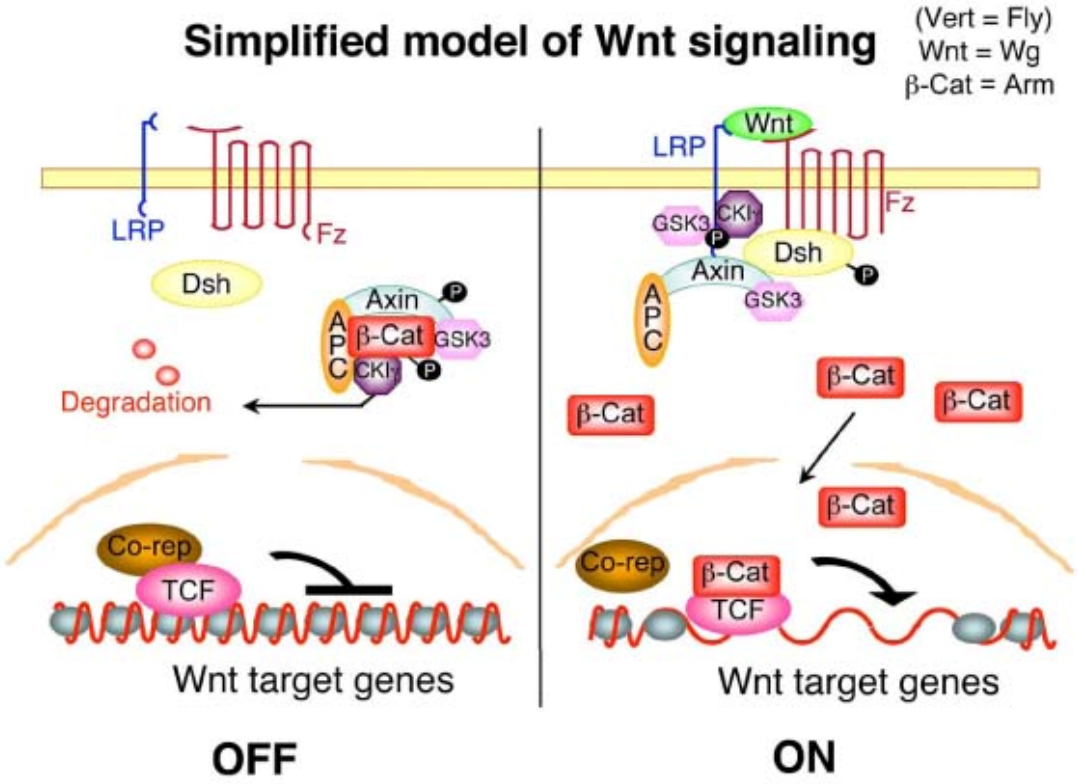

Fig. 1.2. Schematic model of the canonical Wnt pathway. (A) In the absence of Wnt ligands, a protein complex containing APC, Axin, CKIγ and GSK3 phosphorylates β-catenin (Arm in flies), targeting it for the proteosome-mediated degradation. In the nucleus, TCF is bound to transcriptional co-repressors, repressing transcription of target genes. (B) Once bound by Wnt, the Fz/LRP receptor complex activates the canonical signaling pathway. Fz can promote Wnt-mediated Dsh phosphorylation and recruit it to the membrane. Wnts are thought to induce the phosphorylation of LRP by GSK3β and casein kinase Iγ (CK1γ). The combinatorial action of Fz/Dsh and phospho-LRP recruits Axin to the membrane which leads to dissociation or inhibition of the destruction complex. Then, hypophosphorylated β-catenin becomes stabilized in the cytoplasm and translocates into the nucleus.

LRP is also essential for Wnt signaling and can bind to Wnt via its extracellular domain (Tamai et al., 2000; Cong et al., 2004). Loss-of-function mutations of the fly *LRP*, *arrow (arr)* and mouse *LRP5/6* phenocopies the one by loss of *wnt* or *β-catenin* (Liu et al., 1999; Huelsken et al., 2000; Wehrli et al., 2000). However, LRP receptors may utilize Axin instead of Dsh as its major cytoplasmic target. Axin is a component of the protein complex that destabilizes β-catenin. Phosphorylated LRP upon Wnt stimulation can bind to Axin and relocate it from the cytosol to the cell membrane (Tamai et al., 2004; Davidson et al., 2005). This membrane-recruited Axin is targeted for degradation (Willert et al., 1999; Tolwinski et al., 2003), promoting β-catenin stabilization and downstream signaling processes.

The following notions support the idea that Fz and LRP may act cooperatively: (i) extracellular domains of Fz and LRP can form a complex in a Wnt-dependent manner (Tamai et al., 2000); (ii) forced interaction between Fz and LRP can promote LRP phosphorylation and the canonical signaling (Cong et al., 2004; Zeng et al., 2008).

(b) Context-dependent Wnt receptors, Ryk and Ror2

Recent studies suggest two putative receptor tyrosine kinases, Ryk and Ror2 also act as canonical Wnt signaling receptors in some contexts. Ryk which can interact with Wnt and Dsh can mediate the canonical Wnt pathway in mouse neurites (Hsieh et al., 1999; Patthy, 2000; Lu et al., 2004). Ror2, which can interact with Fz, can enhance the ability of Wnt3a to activate a TCF/β-catenin-dependent reporter in lung epithelial cells (Oishi et al, 2003; Li et al., 2008). While their contribution to the Wnt pathway may not be as great as Fz and LRP (Halford et al., 2000; Inoue et al., 2004), Ryk and Ror2 may

be used as Wnt receptors in some tissues and allow Wnts to display unique transcription output.

(c) Wnt signaling regulators at the cell surface

The signal reception at the cell surface can be regulated by several mechanisms. Two Wnt binding factors, Wnt-inhibitory factor-1 (WIF-1; Hsieh et al., 1999) and Cerberus (Piccolo et al., 1999) can inhibit the pathway. Secreted Frizzled-related proteins (sFRPs), which can also bind Wnts, can promote and inhibit the pathway depending on its concentration (Uren et al, 2000). Dickkopf (Dkk) inhibits the canonical pathway by binding to LRP (Ginka et al., 1998). Others like Norrin (Xu et al., 2004), R-spondin (Nam et al., 2006) and WISE can promote the canonical Wnt signaling in part through their association with receptors. While it is not secreted, heparin sulfate proteoglycans on the cell surface has also been implicated in Wnt signaling (Lin, 2004).

Cytoplasmic events

(a) Destabilization of β-catenin in the absence of Wnt signaling

β-catenin is one of the central components of the canonical Wnt/TCF/β-catenin pathway. While β-catenin, which also acts as a cell adhesion molecule interacting with E-cadherin (Kemler, 1993), is constitutively expressed, it is also constitutively degraded by the destruction complex (Fig. 1.2) (reviewed in Clevers, 2006; Parker et al., 2007). The destruction complex is composed of two scaffold proteins, Axin and Adenomatous Polyposis Coli (APC), and two serine/threonine kinases, glycogen synthase kinase 3 (GSK3) and casein kinase 1 (CK1). β-catenin is anchored to the destruction complex via

interacting with Axin and APC and undergoes sequential phosphorylation. CK1 first phosphorylates β-catenin at Ser 45 (Amit et al., 2002) as a priming site then GSK3 phosphorylates the other three amino acids, Thr 41, Ser 37 and Ser 33 at N-terminus (Liu et al., 2002; Yanagawa et al., 2002). Phosphorylation of β-catenin is followed by ubiquitylation by an E3-ubiquitin ligase, β-TrCP, leading β-catenin to proteosome-mediated degradation (Latres et al., 1999; Liu et al., 1999; Winston et al., 1999). This leads to a low cytoplasmic level of β-catenin in the absence of the Wnt signal (Yanagawa et al., 2002).

(b) Stabilization of β-catenin upon Wnt signal reception

Upon reception of the signal, the complex of Fz and LRP inhibits the activity of the destruction complex leading to the stabilization of β-catenin (Fig. 1.2; Clevers, 2006; Liu and Cadigan, 2006). Upon Wnt stimulation, Axin is recruited to the cell membrane via, at least, two different mechanisms. One way of Axin recruitment needs Fz and Dsh. Fz receptors on the cell membrane can promote the phosphorylation of Dsh by CK1ε and Par1 and recruit the protein to the cell membrane (Umbhauer et al., 2000; Takeda et al., 2005). Dsh can associate with Axin via its DIX (Dishevelled and Axin) domain. Thus Axin may be recruited to the cell membrane in a Dsh and Fz-dependent manner (Zeng et al., 2008). Another way to recruit Axin to the cell membrane is dependent on LRP receptors. LRP can associate with Axin via its C-terminal domain. The binding between LRP with Axin is regulated by Wnt signaling via phosphorylation of a LRP C-terminal fragment by GSK3 and CKIγ (Davidson et al., 2005; Zeng et al., 2005).

Two recent studies suggest that these two branches work cooperatively and dynamically (Bilic et al., 2007; Zeng et al., 2008). They showed that phosphorylation of LRP by Wnt3a is dependent on Fz, Dsh and Axin. They also showed that the interaction of Axin with GSK3 is required for LRP6 phosphorylation. Collectively, it is conceivable that Axin may be delivered to the membrane along with other destruction complex members including GSK3 and CK1 via its association with Dsh. Then GSK3 and CK1 may phosphorylate LRP, reinforcing Axin recruitment to the cell membrane.

How this membrane relocation of Axin inhibits the activity of the destruction complex is unclear, but two mechanisms have been suggested. Axin is shown to be degraded in Wnt-stimulated cells. Thus, removal of a scaffold Axin may lead to the dissociation of the destruction complex. Another is Wnt-dependent dephosphorylation of Axin as this modification appears to reduce the interaction between Axin and β-catenin (Willert et al., 1999).

Upon Wnt stimulation, the stabilized β-catenin translocates into the nucleus. The mechanisms for nuclear localization of β-catenin are unclear as it does not seem to have any known NLS (nuclear localization signal). This may rule out the possibility that the classical importin-α/β system is involved in β-catenin nuclear import (Suh and Gumbiner, 2003; Yokoya et al., 1999).

Nuclear events when Wnt signaling is off

(a) TCF as a transcriptional repressor

The canonical Wnt signaling can directly activate target genes (Riese et al., 1997; Lee and Frashch, 2000; Yang et al., 2000 Knirr and Frasch, 2001). In the absence of the

signaling, these activating genes should be silenced or kept low. The best known mechanism for this repression is TCF/Grg-mediated repression (Fig. 1.3).

TCF proteins bind to Wnt targets via their high mobility group (HMG) domain. 4 TCF proteins (Lef1 and TCF1, 3 and 4) from the vertebrate genome and 1 TCF from flies (called Pangolin or Pan) and worms (called Pop-1) are distinguished by HMG domains (Clevers and van de Wetering, 1997; Laudet et al., 1993). The consensus sequences that the HMG domain preferentially binds was first discovered by *in vitro* protein-DNA binding assays. These *in vitro* studies determined SSTTTGWW (S= G or C, W= A or C) as high affinity binding sites to the HMG domain. Later, several Wnt targets from the fly genome provided more sequences that are functional TCF binding sites (Brannon et al., 1997; Galceran et al., 1999; He et al., 1998; Knirr and Frasch, 2001; Lee and Frasch, 2000; Riese et al., 1997; Tetsu and McCormick, 1999; Yamaguchi et al., 1999). Through *in vivo* mutagenesis approaches done with fly Wnt targets, sequences like ACTGTGAA, which are not the stronger interacting sequences, have been shown to be functionally important (Knirr and Frasch, 2001; Lee and Frasch, 2000), providing .

The best evidence supporting the role of TCF in repression of Wnt targets came from *in vivo* mutagenesis assays done in context of reporter genes. When an 812 bp genomic fragment from 5' upstream of *decapentapleigic* (*dpp*) was cloned into a reporter plasmid and integrated into the fly genome, it drove a reporter gene expression in embryonic visceral mesoderm (VM) where *dpp* is normally expressed (Yang et al., 2000). Mutation of one or both of two TCF sites found in this 812 bp fragment caused significant expansion of reporter expression outside of VM but did not reduce reporter activity in VM (Yang et al., 2000). Such an expansion of reporter expression

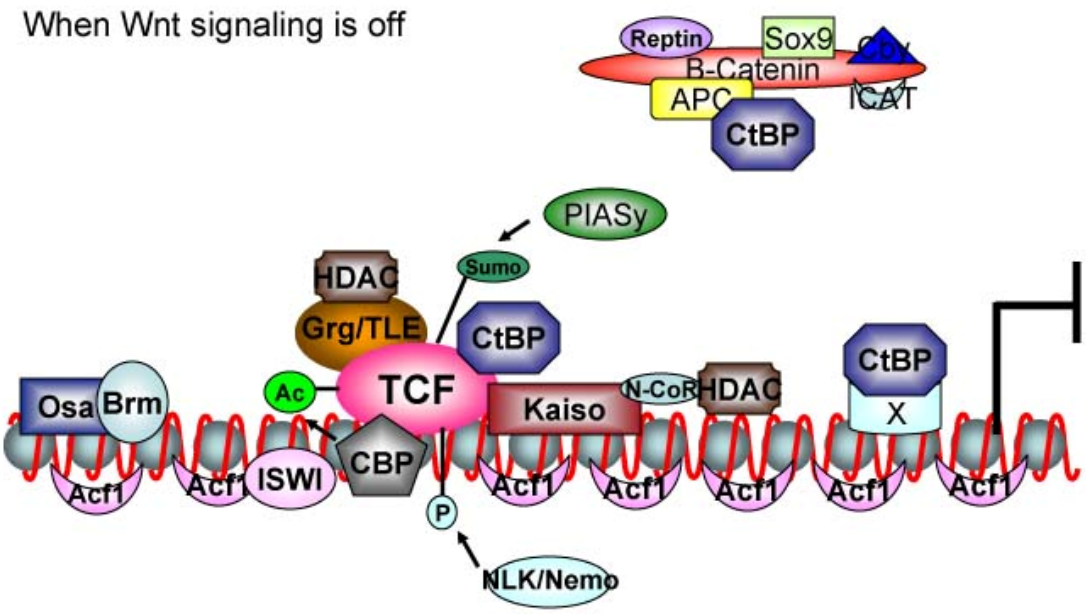

Fig. 1.3. Summary of nuclear factors that can repress the transcription of Wnt targets in the absence of the signal. Depending on the mode of action, nuclear factors implicated in the gene suppression shown here can be grouped. Components that can interact with TCF and bring other transcription repressors to WREs are Grg/TLE, CtBP, and Kaiso. Factors that can bind to β-catenin and suppress the interaction between β-catenin and TCF are seen on top with β-catenin. These factors may act as TCF/β-catenin buffers that prevent a very low level of β-catenin from activating Wnt targets in the absence of signaling. The third group contains factors that can modify TCF. Phosphorylation by NLK/Nemo, acetylation by CBP and sumoylation by PIASy of TCF can suppress the TCF-dependent transcription. The last group has factors implicated in chromatin remodeling such as Osa, Brm, ISWI and Acf1. HDAC can be recruited to Wnt target loci by several factors, including Grg/TLE and Kaiso. CtBP can be recruited to DNA via two distinct mechanisms. For the pool of CtBPs that are recruited to the Wnt target loci in a TCF-independent manner, the docking protein is still unknown and is shown as an X in this and the cartoon in Fig 1.4. (adopted and modified from Parker et al., 2007)

caused by mutation in TCF sites is also observed from Wnt Response Elements (WREs) of other Wnt targets including *even-skipped* (*eve*) and *Ultrabithorax* (*Ubx*) in *Drosophila* and *siamois* in *Xenopus* (Brannon et al., 1997).

Strong genetic evidence supporting the role of TCF in gene repression came from studies of vertebrate TCFs. *TCF3* deletion in mice causes a typical neuronal defect that is often caused by elevated Wnt signaling activity (Merrill et al., 2004). Consistently, zebrafish lacking active *TCF3* have undeveloped eyes and brains which are similar to phenotypes caused by elevated Wnt signaling (Kim et al., 2000; Mukhopadhyay et al., 2001; Niehrs et al., 2001).

While it is clear that TCF can act as a repressor of some Wnt targets, not all Wnt targets require negative regulation by TCF. For instance, unlike derepressable genes *Dpp, Ubx, eve*, and *siamois* as mentioned above, mutation of TCF sites in *sloppy paired 1* (*slp1*) in flies or in *Xnr3, T* and *Delta-like* in vertebrates abolishes their expression but no obvious depression is detected (Mckendry et al., 1997; Yamaguchi et al., 1999; Lee and Frasch, 2000; Galceran et al., 2004).

(b) Groucho-related proteins (Grgs) or Transducin-Like Enhancer of Split (TLE)

Several factors that can repress the transcription in a TCF-dependent or independent manner have been discovered. The best characterized co-repressor is a group of proteins called Transducin-Like Enhancer of Split (TLE) or Groucho-related proteins (Grgs) (Fig. 1.3). There is one Grg/TLE gene in flies (*grocho, gro*) and 5 Grg/TLE genes are present in vertebrates (Grg1, 3, 4, 5 and TL2) (Chen and Courey, 2000).

Flies with mutations in the *gro* gene suppress a *wg* phenotype (Cavallo et al., 1998). Conversely, misexpression of TLEs can suppress TCF/β-catenin mediated transcription in cultured cells and during secondary axis specification in *Xenopus* embryos (Brantjes et al., 2001; Roose et al., 1998).

Grg/TLE proteins can associate with TCF proteins via its glutamine (Q)-rich and glycine/praline (GP)-rich domains in the N-terminal region. Fly Gro proteins have been shown to associate with is Rpd3, a fly HDAC (Chen and Courey, 2000; Chen et al., 1999) and mammalian Grg-4 can interact with HDAC1 (Brantjes et al., 2001). In cell cultures and fly development, the activity of Rpd3 is important for Gro-mediated gene repression. β-catenin-mediated transcription can be repressed by Grg-4 in mammalian cells, but not by Grg-5, which cannot associate with HDAC1 (Brantjes et al., 2001).

Taken together, the current model is that Grg/TLE recruits HDACs to TCFs in the absence of Wnt signaling to repress transcription of Wnt targets. The chromatin modification by HDACs may lead to the compact status of chromatin which is often associated with transcriptional repression. Alternatively, Grg/TLE proteins may act as transcriptional co-repressors by competing with β-catenin for binding to TCF (Daniels and Weis, 2005).

(c) C-terminal binding protein (CtBP)

A second co-repressor is C-terminal binding protein (CtBP). Genetic evidence suggesting CtBP as a transcriptional repressor in the Wnt pathway has been collected from various model systems. Overexpression of CtBP can antagonize β-catenin-mediated transcription in human HEK-293 cells and repress expression of Wnt targets in

flies (Valenta et al., 2003; Hamada and Bienz, 2004; Fang et al., 2006; Sierra et al., 2006). Consistently, loss of two mouse *CtBP* genes in embryonic fibroblasts increases the TCF/β-catenin reporter activity (Hamada and Bienz, 2004), and reduction in CtBP by RNAi in fly cells causes derepression of a Wnt target, *naked cuticle* (*nkd*) (Fang et al., 2006).

One of suggested mechanisms underlying CtBP-mediated repression is dependent on TCF. *Xenopus* and human CtBP are shown to interact with TCF (Brannon et al., 1999; Valenta et al., 2004) (Fig. 1.3). This CtBP-binding region of TCF3 is required for the repression activity of TCF3 in amphibian embryonic axis formation (Brannon et al., 1999), although the interaction between CtBP and TCF has not been seen in other organisms (Hamada and Benz, 2004). Thus, the first model suggests that CtBP is recruited to the promoter via TCF to repress transcription.

Interestingly, however, a study of fly CtBP provided evidence showing that CtBP recruitment to the Wnt target locus does not require TCF (Fang et al. 2006; Fig 1.3). Moreover, double depletion of CtBP and TCF exhibited synergistic effects in derepression of Wnt targets compared to single depletion (Fang et al., 2006). This synergism is consistent with CtBP working independent of TCF. Taking together, it is likely that CtBP may be recruited to the Wnt target genes in multiple ways.

The mechanism by which CtBP co-represses Wnt target genes is largely unclear. One mechanism recently proposed is that displacing or degrading of nuclear β-catenins on the promoter by the activity of CtBP/APC complex (Hamada and Bienz, 2004; Sierra et al, 2006). However, another model shows repression by CtBP in the absence of

signaling when β-catenin is not nuclear. Thus, there should be further studies to discover the mechanisms of CtBP function.

(d) ICAT and Chibby

The attempts to identify β-catenin-interacting proteins found two small proteins, ICAT (Tago et al., 2000) and Chibby (Cby, Takemaru et al., 2003; Li et al., 2007 (Fig. 1.3) Structural and biochemical assays showed that these two proteins can bind to C-terminal region of β-catenin, which presumably inhibits the formation of TCF/ β-catenin complex. Genetic evidence also consistently shows their roles in transcriptional repression (Tago et al., 2000; Tamkemaru et al., 2003; Li et al., 2007). The physiological roles of ICAT and Cby have been suggested as buffering proteins that can inhibit association between TCFs and β-catenin in the absence of Wnt signaling (Tamkemaru et al., 2003)

(e) Kaiso and Reptin/TIP49b

There are also at least two more nuclear factors that may inhibit TCF/β-catenin transcriptional activity, in part, via recruiting other cofactors. The BTB/POZ zinc finger protein member Kaiso can bind to DNA directly and associate with TCF proteins (Fig. 1.3). It can repress the transcription of Wnt targets in *Xenopus* embryogenesis, presumably via recruiting nuclear corepressor N-CoR which can bring HDACs to the Wnt target gene locus (Park et al., 2005).

Another antagonist is a DNA-stimulated ATPase, Reptin/TIP49b (Fig. 1.3). Reptin/TIP49b and its counteracting partner Pontin/TIP49a are known to act

antagonistically in canonical Wnt signaling in several contexts via their interaction with β-catenin (Bauer et al., 2000; Rottbauer et al., 2002). While the mechanism of Reptin action is not clear, the possible role in chromatin remodeling can be speculated based on the fact that both Reptin and Pontin can associate with several chromatin remodeling complexes (Cai et al., 2003; Ikura et al., 2000; Jin et al., 2005).

(f) CBP/p300, NLK/Nemo, and PIASy

Another layer of transcriptional repression can occur at the level of TCF. At least three types of posttranslational modification of TCFs are known to influence transcription (Fig. 1.3).

Two related acetyltansferases, CBP and p300 are shown to interact with fly and human TCFs (Waltzer and Bienz, 1998; Li et al., 2007). In flies, acetyl transferase domain of CBP can acetylate TCF *in vitro* in its N-terminal region. This modification reduces the ability of TCF to associate with Arm, consistent with reduced Wnt activity by CBP overexpression seen in flies.

Alternatively, MAP kinase-related protein kinase NLK/Nemo can phosphorylate TCFs in worms, flies and mammals (Ishitani et al., 1999 and 2003; Zeng and Verheyen, 2004). In the case of phosphorylation, the binding affinity of TCF to β-catenin appears to be unchanged but its association to DNA is weakened (Ishitani et al., 1999 and 2003, Rocheleau et al., 1999; Shin et al., 1999; Smit et al., 2004).

Finally the Protein Inhibitor of Activated Stat (PIAS) family, PIASy, which can sumoylate LEF1, can also inhibit LEF1-mediated transcriptional activation when overexpressed (Sachdev et al., 2001). While the data from the overexpression is

informative, it should be mentioned that PIASy knockout mice do not show any obvious Wnt-related phenotypes, weakening the possibility that PIASy is a physiological Wnt repressor (Roth et al., 2004).

(g) Sox

Besides TCFs, several Sox family members can bind DNA via their HMG domains (Fig. 1.3). Some of Sox proteins such as *Xenopus* Sox 3, Sox 17 and mouse Sox9 can also interact with β-catenin (Zorn et al., 1999; Zhang et al., 2003; Akiyama et a., 2002 and 2004) and appear to act as inhibitors of Wnt signaling.

(h) Chromatin remodelers

Genes implicated in chromatin remodeling are also important for silencing Wnt targets. Activation of the pathway results in rapid and significant increase in histone acetylation at several Wnt response elements (WREs) (Feng et al., 2003; Parker et al., 2008). As histone acetylation is tightly correlated with transcriptional activation, it may need to be suppressed in the absence of signaling to keep the chromatin for a more closed status. Consistent with this idea, several factors described above are already linked to other proteins implicated in chromatin remodeling. Grg/TLE, Kaiso and Reptin & Pontin complex are known to associate with HDACs.

Moreover, there are several pieces of evidence supporting that activity of the ATP-dependent chromatin remodeling complex is essential for repressing Wnt target gene expression (Liu et al., submitted). ISWI and ACF1, members of the ISWI family of ATP-dependent chromatin remodelers are required for the repression of Wnt target genes

in *Drosophila*. Depletion of *iswi* or *acf1* or in combination caused depression or further activation of several Wnt targets in cultured fly cells and in fly tissues. These two proteins are shown to be recruited to the Wnt target locus and antagonize histone acetylation.

While the directness of the function is unclear, another ATP-dependent chromatin remodeler, SWI/SNF complex has been shown to be important in Wnt-directed repression. In *Drosophila*, Osa, a subunit of the fly SWI/SNF complex can inhibit the expression of Wnt target when overexpressed (Collins and Treisman, 2000). In addition, the core ATPase composing the SWI/SNF complex, Brahma also can cause derepression of Wnt targets (Collis and Treisman, 2000) when its ATPase domain was deleted.

Nuclear events when Wnt signaling is on

(a) TCF as a transcriptional activator

Once stabilized in the cytoplasm upon Wnt signaling, β-catenin translocates into the nucleus and convert TCFs from a transcriptional repressor to an activator (Fig. 1.4). The role of TCF as a transcriptional activator is well supported by several pieces of evidence collected from genetic studies and reporter assays in a broad range of animals. Loss-of-function mutations in *Drosophila* TCF homolog have phenotypes consistent with a strong reduction in Wnt signaling (Brunner et al., 1997; van der Watering et al., 1997). Consistently, homozygous mutations in *TCF4* in the mice intestinal epithelia cause the loss of intestinal polyps due to loss of stem cells in the crypts (Korinek et al., 1998). Wnt/β-catenin signaling is thought to promote the maintenance of these stem cells

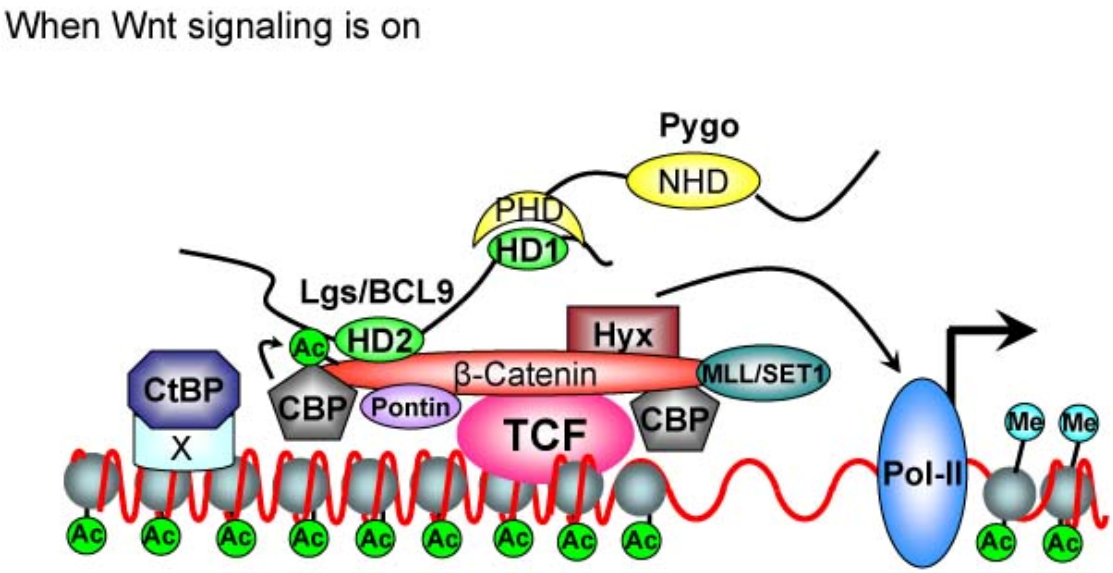

Fig. 1.4. Summary of nuclear factors that can activate the transcription of Wnt targets when the signaling is on. The recruitment of β-catenin causes at least two mechanisms to activate transcription: Removal of the Grg/TLE repressor and recruitment of other co-factors like the Lgs/Pygo complex. CBP with HAT activity is required for the acetylation of β-catenin and histones 3 and 4. CtBP, with the help of an unknown docking protein, can activate transcription in a gene-specific manner. Pontin, which is a predicted helicase, may also play a role in chromatin remodeling via its association with TRRAP/TIP60 HAT complexes. MLL/SET1 is a part of the histone methyl transferase complex. Consistent with the presence of multiple chromatin remodeling factors, a significant increase of histones H3 and 4 acetylation and H3K4 trimethylation are frequently seen on the Wnt target loci upon stimulation. (adopted and modified from Parker et al., 2007)

(Pinto and Clevers, 2005). In addition, disruption of TCF binding sites in several known WREs abolished or significantly impaired the activity of WREs.

These notions strongly suggest that TCF plays a positive role in the Wnt pathway. However, TCF appears not to have intrinsic transcription activation ability. When expressed alone, TCFs have no effect on reporter genes containing multiple TCF binding sites. However, co-expression of β-catenin results in a dramatic activation of TCF reporters (Korswagen et al., 2000; Molenaar et al., 1996; van de Wetering et al., 1997). This genetic interaction, along with the physical interaction between TCF and β-catenin (Graham et al., 2000), suggest that β-catenin converts TCF from a repressor to an activator.

(b) β-catenin

Recruitment of β-catenin to WREs is thought to activate the transcription via at least two mechanisms. First, β-catenin binding to TCF can displace the transcriptional repressor Grg/TLE from TCF. It has been shown that β-catenin and Grg/TLE bind competitively to TCF *in vitro* (Daniels and Weis, 2005) and recruitment of β-catenin to the *c-Myc* WRE is correlated with a removal of TLE1 (Sierra et al., 2006). As mentioned previously, HDACs are thought to be recruited to TCF in part through association with Grg/TLE (Brantjes et al., 2001; Chen and Courey, 2000). HDACs are removed from Wnt target loci when β-catenin replaces Grg/TLE (Kioussi et al., 2002; Sierra et al., 2006). Alternatively, β-catenin has also been suggested to associate with HDAC and inhibit its enzymatic activity (Billin et al., 2000). In any case, it is likely that reduction in

HDAC activity may occur upon the recruitment of β-catenin to WREs, leading to be opening-up of chromatin which can enhance the accessibility of other transcription factors.

Second, β-catenin has transcriptional activation domains in its N and C-termini. This has been shown by fusing β-catenin with a DNA-binding domain (e.g. the yeast transcription factor Gal4-DNA binding domain). When targeted to a promoter of a Gal4 reporter plasmid in this way, the N or C-terminal domains of mammalian, fly and worm β-catenin can significantly activate the Gal4 reporter expression (Hoffmans et al., 2005; Hsu et al., 1998; Natarajan et al., 2001). In this context, reporter transcription is independent on TCF, therefore it cannot be explained by removal of a transcription repressor from TCF. Instead, recruiting other transcription co-activators is likely to be the mechanism as described in more detail below.

(c) Legless/BCL-9

Genetic screens and biochemical approaches to find β-catenin-binding partners have discovered multiple factors that can associate with the N or C-terminal domains of β-catenin.

A necessary co-activator that can bind to N-terminal region of β-catenin is Legless (Lgs). *lgs* was first identified as a Wnt component in fly embryos. loss of *lgs* causes a segment polarity phenotype that is very reminiscent to the one of *wg* mutants (Kramps et al., 2002). Subsequently two human homologs of Lgs, BCL9 and BCL9-2 were found based on the fairly low degree of similarity in protein sequences (Kramps et al., 2002).

Evolutionary conservation of function was reinforced when the human BCL9 gene was shown to rescue *lgs* mutant flies (Kramps et al., 2002). A later study by Brembeck and others (2004) showed that mammalian BCL9-2 can interact with β-catenin and enhance the activation of TCF/β-catenin reporter in human HEK 293 cells. Reduction in BCL9-2 by morpholino injection causes patterning defects in *Xenopus* embryos that are very similar to those caused by reduced Wnt8-directed canonical signaling (Brembeck et al., 2004).

While there are no recognizable domains present in Lgs/BCL9 family proteins, three unique domains, HD1, HD2 and HD3 are found in all Lgs/BCL9 proteins. In the fly Lgs, HD2 binds to the N-terminal region of Arm and HD1 binds to the PHD domain of Pygopus (Pygo) (Kramps et al., 2002). This suggests that Lgs/BCL9 recruits Pygo, another essential transactivation factor, to the N terminus of β-catenin. This hypothesis was strongly supported when a modified Pygo protein that has Lgs HD2 domain but lacks its PHD domain was shown to rescue both *lgs* and *pygo* mutants (Kramps et al., 2002). This result strongly suggests that the main role of Lgs in Wnt-directed transcription is recruiting Pygo to WREs.

(d) Pygopus

pygo was also first identified in *Drosophila* as a binding partner of Lgs (Kramps et al., 2002) and as an essential Wnt component from three independent genetic screens (Belenkaya et al., 2002; Parker et al., 2002; Thompson et al., 2002). Studies done in flies and of two vertebrate Pygos in human cells and *Xenopus* embryos consistently support

Pygo's positive roles in canonical Wnt signaling (Belenkaya et al., 2002; Kramps et al., 2002; Parker et al., 2002; Thompson et al., 2002; Lake and Kao, 2003).

Pygo proteins contain two previously recognized domains and one unique domain. A nuclear localization sequences (NLS) and a conserved Lgs-interacting plant homeodomain (PHD domain) are located at the N and C-terminal regions of Pygo, respectively. In addition, Pygo has a unique N-terminal homology domain (NHD, conserved in vertebrate and fly *pygo* genes) that is essential for Pygo transactivation activity (Kramps et al., 2002; Parker et al., 2002; Thompson, 2004; Townsley et al., 2004; Parker and Cadigan unpublished)

Pygo proteins appear to play at least two roles to activate transcription. First, as a nuclear protein, Pygo can act as a nuclear anchor of Lgs and β-catenin (Parker et al., 2002; Thompson et al., 2004; Townsley et al., 2004). While Lgs normally locates in the nucleus in the wild type *Drosophila* tissues, it is no longer exclusively nuclear when *pygo* is deleted (Townsley et al., 2004). In addition, the level of nuclear Arm was also reduced in homozygous *pygo* mutant cells with activated Wnt signaling (Parker et al., 2002; Townsley et al., 2004). In mammalian cells, reduction in *BCL9-2* by RNA interference caused translocation of β-catenin from the nucleus to the plasma membrane (Brembeck et al., 2004). These data suggest that Pygo is important for keeping the level of β-catenin high in the nucleus. In agreement with this idea, the mutant phenotypes of *lgs* and *pygo* mutant flies were partially suppressed by expression of nucleus-targeted Arm (Townsley et al., 2004).

Second, Pygo proteins contain an intrinsic transactivation domain. Like β-catenin, Pygo can activate the Gal4 reporter transcription when fused to the Gal4 DNA binding

domain (Belenkaya et al., 2002; Thompson, 2004; Townsley et al., 2004). Importantly, this activity requires the NHD domain of Pygo, but is independent on Arm or Lgs (Stadeli and Basler, 2005). Consistently, a fusion protein of Pygo-TCF can rescue a *pygo* mutant in *Drosophila* in a Lgs and Arm-independent manner (Thompson, 2004). This suggests that the transactivation activity of Pygo is sufficient to activate Wnt target genes. This was further supported by the following observation (Hoffmans and Basler, 2004 and 2005). A single point mutation in the NHD domain of Pygo that does not affect Lgs binding and nuclear localization of Pygo severely reduces its activity to activate Wnt signaling. In conclusion, several pieces of evidence suggest that Lgs and Pygo are recruited to the N-terminal domain of β-catenin to activate transcription. Consistent with this, transcriptional activation by N terminal domain of Arm requires Lgs and Pygo but one by C-terminus of Arm does not (Stadeli and Basler, 2005).

(e) CBP/p300

Other cofactors that can interact with β-catenin are CBP and p300, two highly related acetyltransferases. CBP and p300 were found to bind directly to β-catenin, both in the N-terminal (Miyagishi et al., 2000; Sun et al., 2000) and C-terminal half of the protein (Daniels and Weis, 2002; Hecht et al., 2000; Miyagishi et al., 2000; Takemaru and Moon, 2000; Li et al., 2007). Consistently, CBP was shown to be recruited to the WRE of a Wnt target in fly cells in an Arm-dependent manner (Li et al., 2007; Parker et al., 2008). While CBP/p300 can suppress Wnt-directed transcription by acetylating TCFs, they can also promote transcription. Overexpression of CBP/p300 can enhance the ability of β-catenin to activate several Wnt reporter genes in cultured cells (Hecht et

al., 2000; Miyagishi et al., 2000; Sun et al., 2000; Li et al., 2007). Conversely, in flies reduction in CBP via null mutants or RNAi interference significantly reduced the expression of multiple Wnt targets (Li et al., 2007), indicating the positive role of CBP in the Wnt signaling. CBP and p300 have several domains including a histone acetyltransferase (HAT) domain (Hecht et al., 2000) and this HAT domain is necessary for CBP/p300 to promote transcription (Levy et al., 2004; Li et al., 2007).

Mechanistically, CBP and p300 can directly interact with N-terminal half of β-catenin and acetylate at least two lysines at 49 and 345 (Wolf et al., 2002; Levy et al., 2004). Substitution of lysine at 345 to other amino acids in β-catenin significantly reduced the interaction between TCF and β-catenin and suppressed the reporter activation by β-catenin (Levy et al., 2004).

In addition, CBP and p300 can acetylate the N-terminal tail of Histones 3 and 4 *in vitro* (Ogryzko et a., 1996). Histone acetylation is strongly correlated with transcriptional activation (Grant and Berger, 1999). Consistently, our lab showed that RNAi knockdown of *CBP* blocks Wnt-induced H3/H4 acetylation and suppresses the activation of Wnt targets in the fly cells (Parker et al, 2008). However, CBP may adopt another mechanism to induce transcription as HAT-defective CBP can still promote activation of some TCF/β-catenin reporters (Hecht et al., 2000).

In summary, the action of CBP/p300 in Wnt signaling is complex. CBP/p300 can modify β-catenin and Histones via its HAT activity to induce transcription. Despite the complex and contradictory mechanisms of CBP, it is clearly an important factor in Wnt transcriptional regulation.

(f) Hyrax

Powerful genetic screens done in flies identified a gene encoding a component of the Polymerase-Associated Factor 1 (PAF1) complex, Hyrax (Hyx), as a positive Wnt signaling component (Mosimann et al., 2006). Hyx is conserved from yeast to humans, and its human ortholog Parafibromin, encoded by the Hyperparathyroidism-Jaw Tumor syndrome (HTP-JT) tumor suppressor gene, can rescue *hyx* mutant flies (Mosimann et al., 2006). GST-pull down assays showed that Parafibromin can directly interact with the C-terminal region of β-catenin and can make a protein complex with BCL9 and hPygo2 (Mosimann et al., 2006). These data provide some insight into how Wnt signaling components communicate with the basic transcriptional machinery to enhance transcription, along with previously defined interactions between Wnt-specific transcriptional factors (e.g. CBP, and Pontin) and other basic transcription factors such as TBP and TFIIB.

(g) CtBP

CtBP, which is required for silencing Wnt targets in the absence of the signal, can also activate Wnt targets in a gene-specific manner (Fang et al., 2006). Depletion of CtBP resulted in significant reduction in some Wnt targets such as *CG6234* but not others such as *nkd* in fly cultured cells and tissues (Fang et al., 2006). CtBP is recruited to the WRE of *CG6234* in a Wnt-dependent manner, suggesting a direct role in Wnt signaling (Fang et al., 2006). While an interaction between CtBP and Arm has not been detected, CtBP can promote the transcription via the N terminal half of Arm but not by

the C terminal half (Fang et al., 2006). This may indicate that CtBP associates with Arm via other adaptors to mediate Wnt transcriptional activation.

(h) Pontin/TIP49a

As previously mentioned, the putative ATP-dependent DNA helicase Pontin/TIP49a can directly bind to the N-terminal half of β-catenin and can promote transcription (Bauer et al., 1998, 2000). Depletion of Pontin by RNA interference, or expression of a dominant negative Pontin (TIP49DN, which lacks helicase activity) inhibits expression of TCF/β-catenin targets in mammalian cells. Conversely, overexpression of Pontin can activate Wnt reporter genes (Bauer et al., 2000; Feng et al., 2003). TIP49 has previously been found as a member of the complex with chromatin remodeling factors such as TRRAP, several distinct HAT complexes, and BAF53 (Wood et al., 2000; Park et al., 2003; Feng et al., 2003). Therefore Pontin has been also suggested to play a role in the regulation of chromatin architecture. Consistent with this, reduction of Pontin activity by misexpressing TIP49DN decreased histone H4 acetylation on the promoter of a known TCF/β-catenin target (Feng et al., 2003).

(i) MLL/SET1

An attempt to identify C-terminal binding partners of β-catenin revealed numerous subunits of chromatin remodeling complexes, including histone methyl transferase containing mixed-lineage-leukemia (MLL1/MLL2)/SET1 complexes (Sierra et al., 2006). SET1 complexes mediate trimethylation of K4 on the N-terminal tail of Histone 3, an event considered critical for transcription initiation. Consistent with SET1 complexes

playing a role in Wnt target gene activation, the elevation of H3K4 trimethylation was detected from the locus of Wnt target *c-Myc* in human colon cancer cells (Sierra et al., 2006) and *nkd* in fly hemocyte cells (Parker et al., 2008). More importantly, reduction in MLL2, a member of a MLL/SET1 complex by RNAi interference markedly impaired *c-Myc* transcription by Wnt signaling (Sierra et al., 2006).

These data from many factors that are involved in regulation of chromatin (e.g. CBP/p300, Pontin/TIP49a and MLL/SET1) collectively suggest two things: (1) regulation of chromatin architecture is of paramount importance for Wnt signaling (2) various sorts of modifications including acetylation, ubiquitination and methylation are necessary for Wnt-directed transcriptional activation.

Target genes of the Wnt/β-catenin pathway

The canonical Wnt signaling ultimately activates transcriptional programs to change cell behaviors and fates. To induce such changes in cells, Wnt signaling may need to regulate not few but many genes at once in a certain context. Moreover, the manifold roles of Wnts in many different physiological and disease conditions suggest that Wnt targets are activated in a context dependent manner. In fact several genome scale studies monitoring transcription profiles show that elevation of Wnt signaling can cause the massive change in gene expression in a highly dynamic manner in different cellular environments (Willert et al., 2002; van der Wetering et al., 2002; Morkel et al., 2003; Weidinger et al., 2005). Thus a comprehensive understanding of Wnt-mediated transcription and its dynamics is important. However, many questions remain to be solved. For example, what are the target genes of the canonical Wnt signaling? Does

each gene get activated via the same mechanism? How does Wnt signaling regulate the target genes in a tissue-specific manner? In the following section, some known examples of Wnt targets and their regulation mechanisms will be summarized.

Tissue-specific Wnt targets

The majority of Wnt target genes appear to respond in a cell type specific manner. As genes that are directly regulated by the pathway are the main interest of this thesis, only those will be discussed further. Some examples of tissue or cell type specific direct Wnt target genes includes the homeobox gene *even-skipped (eve)*, which is directly regulated by Wnt signaling in fly pericardial progenitor cells, but it is not in the embryonic epidermis where Wnt signaling is also active (Knirr and Frasch, 2001). Wnt signaling in the midgut of fly embryos directly activates another homeobox gene, *Ultrabithorax (Ubx)* (Riese et al., 1997). Interestingly, Wnt signaling can also repress *Ubx* when the signaling level is high (Hoppler and Bienz, 1995). In *Xenopus*, canonical Wnt signaling is active on the entire dorsal side of the early embryo. However, the direct Wnt target, *siamois* is expressed only in the dorso-vegetal region of embryos (Crease et al., 1998). In mice, the DNA-binding transcription factor, *Brachyury (T)*, is directly induced by Wnt signaling in trunk and tail mesoderms (Yamaguchi et al., 1999), but not in the kidney where Wnt signaling is active.

Some mechanisms of tissue-specific responses to Wnt signaling

There are examples of mechanisms by which Wnts are thought to elicit tissue specific responses. In studies of several genes including *eve*, it is clear that for some

tissue-specific expression, Wnt activity is necessary but not sufficient and other signaling-dependent and/or cell-type specific transcription factors are also required to activate transcription. A 361 bp genomic fragment cloned from the *eve* locus can recapitulate the *eve* pattern in pericardial precursors when tested in flies (Knirr and Frasch, 2001). Site-directed mutagenesis of this *eve* enhancer revealed multiple DNA sequences necessary for the activity of the *eve* enhancer including binding sites of TCF, Med and Tin. Med is a Dpp pathway-specific transcription factor, and Tin is a transcription activator induced by the Dpp pathway. These data strongly suggest that *eve* requires the combinatorial action of Dpp/BMP and Wnt to be activated in pericardial cells, which is consistent with genetic data (Knirr and Frasch, 2001; Han et al., 2002). Thus it is conceivable that cells with Wnt signaling and not Dpp/BMP signaling may not be able to activate *eve*, which may explain the lack of *eve* expression in fly embryonic epidermis at least partially as *dpp* and *wg* are not coincidentally expressed here (Schwyter et al, 1995).

A further complexity comes from the notion that genes with a tissue-specific expression pattern commonly utilize multiple tissue-specific regulatory sequences. For example, a direct target of Wnt, *dpp* is expressed in a complex and highly dynamic pattern throughout development in many different tissues. At least four non-overlapping regulatory sequences of *dpp* expression have been found from the *dpp* locus. The expression of *dpp* in the wing and other adult appendages like the leg and the eye is controlled by enhancer elements located in the 3' of the transcription unit (Blackman et al., 1991) whereas *dpp* expression in the wing veins and embryonic tissues are regulated by separate sequences in the 5' of the gene (Yang et al., 2000; Sotillos and de Celis,

2006). Additionally, about 400 bp of DNA sequences in the 2nd intron of the *dpp* gene can drive reporter expression in the posterior spiracle during embryogenesis (Takaesu et al., 2002 and 2007). Among these multiple enhancers of *dpp*, functionally important TCF sites were found in the two embryonic enhancers (Yang et al., 2000; Takaesu et al., 2007) but not in the wing vein enhancer of *dpp*. Conclusively speaking, many Wnt targets may respond to Wnt signaling in a tissue-specific manner by having several tissue-specific enhancers and only some of these WREs may require Wnt activity. The combinatorial action of several inputs from signaling and local factors adds more complexity to tissue-specific regulation of genes.

It has also been suggested that factors such as TCF, β-catenin, and Gro may be involved in regulating tissue specific responses of Wnt signaling. For example, repression of *Ubx* in the fly embryonic midgut requires transcription repressors Brinker (Brk) and Teashirt (Tsh) (Saller et al., 2002). Presence of Brk and Tsh overcomes the activator TCF/Arm, resulting in low *Ubx* transcription (Saller et al., 2002; Waltzer et al., 2001). Other examples are *lines* and *split ends* that were identified from genetic screen done in *Drosophila*. The nuclear protein Lines was shown to be necessary in Wnt signaling transduction only in the dorsal epidermis of the embryo (Hatini et al., 2000). Split ends (Spen) is another nuclear protein required for Wnt transduction in larval tissues but not in embryos (Lin et al., 2003). While the mechanisms are unclear, Lines and Spen may somehow regulate a subset of Wnt targets in the nucleus.

The third example of tissue-specific regulation by Wnts is that TCF isoforms can recognize different sequences. In vertebrate TCFs, alternative splicing produces two isoforms of TCF-1 and TCF-4 that contain a C-terminal E tail domain. TCF-1 and TCF-

4 can activate targets like *LEF1* and *CDX1,* which cannot be activated by the E-tail lacking LEF1 (Atcha et al., 2003 and 2007). Interestingly the abundance of these isoforms can be also variable upon cell types, for instance, TCF-1 with E-tail is highly expressed in colorectal cell lines, compared to normal cells (Atcha et al., 2003).

The last example of tissue-specific gene regulation is the confinement of target expression via a negative feedback circuit. A variety of negative regulators of the pathway, such as *Axin2 (*Jho et al., 2002; Leung et al., 2002; Lustig et a., 2002), *sFRP* (Lescher et al., 1998), *Dkk* (Chamorro et al., 2004; Gonzalez-Sancho et al, 2004; Niida et al., 2004), *nkd* (Zeng et al., 2000), and *notum* (Gerlitz and Basler, 2002) are activated by the Wnt pathway and suppress Wnt signal transduction. In *Drosophila* embryos and developing wings, *nkd* and *notum* suppress Wnt activity by inhibiting Dsh, or controlling the signal reception at the cell surface. Loss of function mutations of *nkd* or *notum* lead to upregulation of Wnt targets in cells which normally do not activate such targets (e. g. *en* in embryos and *Distal-less(Dll)* and *neuralized (neur)* in the wing) (Zeng et al., 2000; Gerlitz and Basler, 2002).

Ubiquitously activated Wnt targets

While the majority of Wnt targets respond to Wnt signaling in a cell/tissue-specific manner, a few genes such as inducible feedback antagonists show a very high frequency in their response. Examples include *Axin2 (*Jho et al., 2002; Leung et al., 2002; Lustig et a., 2002), *nkd* (Zeng et al., 2000), and *notum* (Gerlitz and Basler, 2002). *Aixn2* is induced in multiple human cancer cells that have a high level of Wnt signaling (Jho et al., 2002; Leung et al., 2002; Lustig et a., 2002; Yan et al., 2001). More strikingly, *Axin2*

mRNA distribution displayed in whole mouse embryos shows a pattern that highly overlaps with the sites of Wnt signaling (Jho et al., 2002).

This is also the case for *nkd* in *Drosophila*. An EF hand protein, Nkd acts directly thorough Dsh to limit the effect of the Wnt signal during embryogenesis (Rousset et al., 2001, 2002; Wharton et al., 2001). Consistent with this, loss of *nkd* in *Drosophila* embryos results in patterning defects that is also seen in excess Wg signal activity (Zeng et al., 2000). Overexpression of *nkd* suppresses the activity of Wnt signaling in *Xenopus* (Zeng et al., 2000; Yan et al., 2001) and Wnt-mediated transcription in mammalian cells (Yan et al., 2001; Wharton et al., 2001). In *Drosophila*, the *nkd* expression pattern highly overlaps with the *wg* pattern and its expression is dependent on the pathway in every tissue examined during fly development (Zeng et al, 2000; Wharton et al., 2001). In addition, Wg signaling is sufficient to express *nkd* ectopically (Zeng et al., 2000), suggesting Wg as a main signal input for *nkd* transcription. A similar story exists for the vertebrate *nkd1*. The expression of *nkd1* in zebrafish, chicken and mice is reminiscent of the pattern of Wnt activity in several tissues, and human *nkd1* is elevated in multiple cancer cell lines where Wnt signaling activity is increased. Also, *nkd* expression vertebrates has been shown to be dependent on Wnt signaling (Zeng et al., 2000; Yan et al., 2001a, b; Wharton et al., 2001; Koch et al., 2005; Schmidt et al., 2006; Van Raay et al., 2007; Zhang et al., 2007).

Notum, a secreted protein, is thought to limit Wnt activity by regulating extracellular Wnt coreceptors Dally and Dally-like during larval development (Gerlitz and Basler 2002). The pattern of *notum* expression throughout multiple developmental stages in *Drosophila* revealed a pattern that is very similar to the *wg* pattern (Gerlitz and

Basler 2002). Wg signaling is necessary and sufficient to activate *notum* as seen in *nkd* (Gerlitz and Basler 2002).

Interestingly, multiple signaling pathways also induce their negative feedback inhibitors in many different cell types. Examples are *patched (ptc)* in Hg signaling, *daughters against dad (dad)* in Dpp/BMP/TGF-β signaling, and *argos* in EGF/RAS signaling. Like *nkd* and *notum*, each signaling is shown to be sufficient and necessary for activating its feedback antagonist (Alexandre et al., 1996; Golembo et al., 1996; Tsuneizumi et al., 1997; Zeng et al., 2000; Gerlitz and Basler, 2002).

Data from multiple examples suggest that the requirement for the transcriptional activation of such frequent responding genes can be relatively simple. For instance, *nkd* transcription could be activated mainly by Wnt signaling without any combinatorial action from other signaling or local factors. Consistent with this idea, the direct regulation of *Axin2* and *nkd* by Wnt signaling has been shown via the identification of their WREs. Both WREs of *Axin2* and *nkd* can respond to Wnt signaling and contain functional TCF sequences that are bound by TCF proteins (Jho et al., 2002; Feng et al., 2006; Li et al., 2007).

To learn more about the mechanism underlying transcription of genes like *nkd*, one can analyze regulatory sequences required for *nkd* expression. For most of genes with a dynamic pattern of expression, multiple tissue-specific enhancers may be necessary to employ distinct transcriptional mechanisms in certain tissue types. However, *nkd* may not need multiple enhancers if Wnt signaling controls its transcription in a simplistic manner in every cell that responds to Wnts.

Previously, Jho and other (2002) showed that 5.6 kb DNA fragment cloned from the *Axin2* locus can express a reporter, EGFP in most, if not all, tissues where endogenous *Axin2* is expressed. However, it has not been tested whether this 5.6 kb fragment has more than one WRE. It may contain a single WRE that is active in every *Axin2* positive cells. It is also equally possible that this 5.6 kb fragment contains multiple WREs as seen in the fly *wg* gene which contains multiple tissue-specific enhancers within a 6 kb fragment (Costas et al., 2003; Pereira et al., 2006). Thus further analysis of the 5.6 kb *Axin2* enhancer in mammals and/or characterization of *nkd* or *notum* enhancers in *Drosophila* need to be done.

Rationale

The Wnt pathway controls cell behaviors and fates by regulating the transcription of target genes. In order to have better understanding of Wnt-mediated transcriptional activation, a direct Wnt target in *Drosophila*, *naked cuticle* (*nkd*) was studied. *nkd* is an essential gene for fly biology due to its function as a negative feedback antagonist of Wnts. Most Wnt targets are regulated by the pathway only in certain cells. This cell-specific response to Wnts is achieved by utilizing multiple cell-specific regulatory sequences. In cntrast, *nkd* is ubiquitously activated by the Wnt pathway regardless of the cell type. This seemingly simple mode of activation raises the possibility that *nkd* may have a single WRE (Wingless Response Element) which is active in all *nkd*-expressing cells. To test this hypothesis, putative *nkd* WREs were identified from fly cultured cells and the *in vivo* activity of WREs were tested in multiple fly tissues.

Wnt signaling plays essential roles in a broad range of physiological and disease contexts and such pleiotropy of Wnts is mainly achieved by regulating specific target genes in a context-dependent manner. Previously a fly nuclear protein, Split ends (Spen) has been shown to promote Wnt signaling in a cell- and gene-specific manner. One interesting notion from the previous study is that a predicted dominant negative form of Spen expression can cause more severe defects in Wnt signal transduction than strong (probably genetic null) alleles of *spen*. This suggests that Spen have redundant partners in fly genome. This prediction leaded to the discovery of the fly gene, *spenito* which is the most closely related gene to *spen*. In this thesis, the functional redundancy of two

Spen proteins in Wnt signal transduction and the mechanisms underlying their function in the pathway were explored.

CHAPTER II

IDENTIFICATION AND CHARACTERIZATION OF WINGLESS RESPONSE ELEMENTS FROM A DIRECT TARGET GENE, *NAKED CUTICLE (NKD)* IN *DROSOPHILA*

THIS CHAPTER IS IN PREPARATION FOR SUBMISSION FOR PUBLICATION.

Abstract

Secreted proteins of the Wg/Wnt family act through a highly conserved signaling cascade to regulate transcription of target genes. In the current model, TCF, a sequence specific DNA-binding protein, is bound to regulatory *cis*-elements in the target gene locus and controls transcription, through interacting with other cofactors. In order to learn more about the Wg-directed transcriptional regulation, we studied *naked cuticle* (*nkd*), a direct target gene of the Wg pathway in *Drosophila*. From cultured fly cells, chromatin immunoprecipitation (ChIP) with TCF antibody found 2 genomic fragments from the *nkd* locus that are bound by TCF proteins. They are located in the upstream region and the intron of the *nkd* locus and named UpE and IntE, respectively. Their ability to respond to Wg signaling was test via reporter assays where a reporter expression is controlled by UpE or IntE upon Wg stimulation. Both fragments can activate the reporter gene expression upon Wg signaling in several fly cell lines. Consistent with being directly regulated by Wg signaling, each WRE loses Wg-

responsiveness when putative TCF sites were mutated. The *nkd*-WREs identified from cultured cells are also active in a variety of fly tissues in partially overlapping patterns, which are consistent with activation by Wg signaling. The sum of the WREs covers the entire pattern of *nkd* expression in larval tissues but the pattern in embryos is incomplete. When deleted from the endogenous *nkd* locus, loss of UpE significantly reduces the *nkd* expression whereas loss of IntE shows minimal effects. In conclusion, our data suggest that a frequent Wg responder, *nkd* requires multiple tissue-specific WREs to fully respond to the signaling and these multiple *nkd*-WREs act redundantly.

Introduction

The proper development and function of cells and organisms highly rely on the dynamic regulation of gene expression. The spatial and temporal expressions of certain genes are tightly controlled via regulatory sequences, or enhancers, lying in vast noncoding regions of the genomes (Costas et al., 2004). In order to achieve the complex pattern of expression, genes commonly use several distinct enhancers, each of which works in different tissues. One of many genes having multiple tissue-specific enhancers is *wingless (wg)*, a fly homolog of vertebrate Wnts. The Wg/Wnt functions as a ligand molecule in the signaling pathway, which is essential for the embryonic development and adult tissue homeostasis (reviewed in Cadigan and Nusse, 1997; Clevers, 2006). In *Drosophila*, the proper development of embryos and adult appendages depends on the accurate expression of Wg. During the embryogenesis, *wg* is expressed in various primordial cells to determine the segmentation and control organogenesis (reviewed in Cadigan and Nusse, 1997). In fly larvae, *wg* is expressed in multiple precursor tissues

called imaginal discs and controls the development of adult wings, legs and eyes and antennae.

To achieve its complex expression, *wg* appears to utilize at least 6 enhancers, each of which is active in different tissues. For the embryonic expression of *wg*, sequences immediately upstream of the *wg* transcription start site appear to be responsible as they can drive the reporter gene expression similar to the *wg* pattern in transgenic fly embryos (Lessing and Nusse, 1998). In larval stages, *wg* needs the genomic region further upstream of the locus to be expressed in the developing wing (Neumann and Cohen, 1996a). When this 5' wing enhancer is deleted in the spd^{fg} allele, *wg* expression in the part of the wing imaginal discs is significantly reduced (Neumann and Cohen, 1996b). Conversely, this wing enhancer can drive a reporter gene expression in a pattern very similar to the *wg* pattern from the wing pouch (Neumann and Cohen, 1996a). Similar reporter assays in transgenic flies revealed at least four more enhancers, two from the 3rd intron sequences (pupal abdomen and genitalia enhancers) and two from the 3' region of the *wg* locus (ventral imaginal discs and eye imaginal discs enhancers), respectively (Costas et al., 2004; Pereira et al., 2006).

Why does *wg* utilize multiple enhancers, each of which is active in different tissues? Enhancers contain multiple *cis*-regulatory sequences that are recognized by specific transcription factors. If transcription mechanisms that activate *wg* are different in various tissues, transcription factors and the binding sequences can be different as well. Consistent with this idea, requirement of signal inputs for *wg* expression differs upon cell types. Initial expression of *wg* in embryos is regulated by the classes of gap and pair-rule genes until gastrulation. From gastrulation to stage 10, *wg* expression is dependent on

Hh (Hedgehog) signaling, but, from stage 11, it becomes independent of Hh activity but requires its own signaling activity (reviewed in Sanson, 2001). Contrarily, Wg signaling inhibits its own expression in cells located in wing margin cells (Ruliferson et al., 1996). In addition, two putative DNA-binding, transcription factors, *nubbin (nub)* and *pannier (pnr)* are required for *wg* expression only in certain cells (Ng et al., 1995; Pereira et al., 2006). Taking together, one can conclude that multiple tissue-specific enhancers may be necessary for many genes to achieve their dynamic expression pattern.

However, some genes such as inducible feedback antagonists may be different. Multiple signaling pathways induce genes that antagonize their own activity to achieve a proper response (e. g. *naked cuticle (nkd), notum/wingful* (*wf*) in the Wg/Wnt pathway (Zeng et al., 2000; Gerlitz and Basler, 2002); *patched (ptc)* in the Hh pathway (Alexandre et al., 1996); *Dad (daughters against dpp)* in the Decapentaplegic (Dpp)/BMP pathway (Tsuneizumi et al., 1997); and *argos* in the EGF/Ras pathway (Golembo et al., 1996)). A unique thing of genes mentioned above is that they seem to be activated by their respective signaling regardless of the cell type. In addition, each signaling was shown to be necessary and sufficient to turn on its feedback inhibitors (Alexandre et al., 1996; Golembo et al., 1996; Tsuneizumi et al., 1997; Zeng et al., 2000; Gerlitz and Basler, 2002). Given their seemingly simple mode of activation, inducible feedback inhibitors may be controlled by a universal enhancer that can respond to the target pathway in most, if not all, of cells where the signaling is active. To test this possibility, we studied *nkd*, a Wg-inducible feedback inhibitor in detail using *Drosophila* as a model system.

In *Drosophila, naked cuticle* (*nkd*) encodes an EF hand protein that limits the effect of the Wg signal during embryogenesis (Zeng et al., 2000, Rousset et al., 2001, 2002; Waldrop et al., 2006; Chan et al., 2007). Nkd can interact with a positive Wg signaling component, Dishevelled (Dsh) via its EF domain and suppress the activity of Wg signal transduction (Rousset et al., 2001, 2002). Consistently, vertebrate *nkd* orthologs (*nkd1* and *nkd2*) are shown to interact with Dsh and can suppress Wnt activity (Yan et al., 2001a, b; Wharton et al., 2001; Van Raay et al., 2007). Besides Dsh, PR2A and PR130, two Protein Phosphatase type 2A (PP2A) regulatory subunits, can directly bind human Nkd1 and modulate the ability of hNkd1 to function as a Wnt inhibitor (Creyghton et al., 2005, 2006). In addition to its role in Wnt signaling, mammalian Nkd2, but not Nkd1, is required for the delivery of TGF-α (Transforming Growth Factor α) to the cell surface when both hNkd2 and TGF-α are overexpressed (Li et al., 2004).

In *Drosophila*, the *nkd* expression pattern is almost identical to the *wg* pattern except that *nkd* is more broadly expressed due to *wg* acting as a morphogen (Zeng et al, 2000; Wharton et al., 2001). Consistent with *nkd* being a target of Wg signaling, its expression is dependent on the pathway in every tissue examined during fly development (Zeng et al., 2000; Wharton et al., 2001). Similar cases exist in vertebrates. The expression of *nkd1* in zebrafish (Van Raay et al., 2007), chicken (Schmidt et al., 2006), and mice (Wharton et al., 2001) is reminiscent to the pattern of Wnts in several tissues, and human *nkd1* is elevated in multiple cancer cell lines where Wnt signaling activity is increased (Yan et al., 2001a, b; Koch et al., 2005). Also, *nkd1* expression from zebrafish to human is shown to be dependent on Wnt signaling (Yan et al., 2001a, b; Wharton et al., 2001; Koch et al., 2005; Schmidt et al., 2006; Van Raay et al., 2007; Zhang et al., 2007).

In addition to all this, our lab has provided strong evidence suggesting that *nkd* is a direct target of Wg signaling in fly Kc cells (Fang et al., 2006; Li et al., 2007; Parker et al., 2008). Taken together, the Wg signal appears to turn on *nkd* in most of tissues, presumably by a direct manner.

In the Wg-mediated transcription, a sequence-specific DNA binding protein, TCF are thought to be present on the target gene locus and exhibits dual roles (reviewed in Parker et al., 2007). In the absence of signaling, TCF keeps the target expression off and when the signaling is on, it promotes the transcription. This TCF-bound region is defined as a Wingless Response Element (WRE). From fly Kc cells, we found two genomic regions that are bound by TCFs from the *nkd* locus via Chromatin immunoprecipitation (ChIP) assays. These two fragments, one from the upstream and another from the intron of the *nkd* gene, are named UpE and IntE, respectively. When tested in flies, *nkd*-WRE reporters are expressed in patterns that are reminiscent of *nkd* transcript distribution. While *nkd*-WREs show the activity in partially overlapping manner, they show distinct activity in certain tissues, suggesting them as tissue-specific WREs. When deleted from the endogenous *nkd* locus, loss of UpE significantly reduces the *nkd* expression whereas loss of IntE shows minimal effects. In conclusion, our data suggest that a ubiquitously activated Wg target, *nkd* requires multiple tissue-specific WREs to fully respond to the signaling and these multiple *nkd*-WREs act redundantly.

Materials and Methods

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-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 (Fang et al., 2006; Li et al., 2007; Chang J. L, 2008). Briefly, 1 x 10^6 Kc, S2R+ or Clone 8 cells were seeded in 12-well plates and 10 ug of *Axin* or control dsRNA were added to each well. When *Axin* dsRNA was combined with another RNA duplex, 10 ug 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 R Nusse from Stanford University. Wg-CM was collected from dense cultures (typically 7-10 million cells/ml) lacking hygromycin. Usually 200 ul of unconcentrated Wg-CM was added to 600 ul cell suspensions containing 1-3 million cells. As a control, media collected from S2 cells was used. Cells were treated for 5 hours 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 syntheses were performed with Superscript III reverse

transcriptase (Invitrogen) according to the manufacturer's protocols. Additional details of the qRT-PCR and the primer sequences used are described (Fang et al, 2006).

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 x10^6 cells and 5-10 ul of TCF antibody were used per ChIP and all DNA samples precipitated were quantified with 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 *Mlu*I/*Xma*I 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' (*Xho*I site is underlined) and 5'ATAAGCTTAAGCTTCCCAATTCCCTATTCAGAGTTCTC3' (*Hin*dIII site is underlined) primers. The specific primers to amplify the UpE and IntE genomic fragments are the following: UpE, 5'TCCTACGCGTGGCTGGGCTCGAT GCAGATAA3' and 5'AATTCCCGGGGG GCCGCTGTCGGCCAACTG3'; UpE1, 5'TCCTACGCGTGGCTGGGCTCGATGCAGA TAA3' and 5'GGTGCCCGGG TTTGTAGTTTGCGGTGGT3'; UpE2, 5'AATTACGCGTCAGGAGGTCTGCCA ACTTAAGTAG3' and 5'AATTCCCGGGGGGCCGCTGTCGGCCAAC TG3'; IntE

(869 bp) 5'TTAGACGCGTGCTCTCGGGCCAC3' and 5'CCAGCCCGGGTTCCTC AAAGCAACC3'; IntE (255 bp) 5'GCCACGCGTATAGTTTGTGTATAGTT3' and 5'CCCAGCCCGGGTTCCTCAAAGCAACC3'. The *Mlu*I (ACGCGT) and *Xma*I sites (CCCGGG) are underlined. Deletions of the WREs (UpE #1, #2, #3, and IntE 558bp) were constructed 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 G or C to T (or vice versa). For UpE1 and UpE2 all eight nucleotides of the TCF binding site (SSTTTGWW) were substituted while the 2^{nd}, 5^{th} and 6^{th} 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 them into the fly genome by P-element transgenesis (Bestgene Inc.). The combined *nkd* WRE has a 1084 bp UpE fragment upstream of an 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 co-transfected 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. 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 quantitated 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 same amount of reporter and *pAcLacZ* and incubated for 2 days before the cells were treated with Wg-CM for 24 h before harvesting for the reporter assays.

***Drosophila* genetics**

Fly stocks were maintained on standard medium at 25°C unless otherwise indicated. The P[*Engrailed-Gal4*] (En-Gal4) and P[*Decapentapleigic-Gal4*] (*Dpp-Gal4*) are as described (Li et al., 2007). The dominant negative *TCF* transgene (P[$UAS\text{-}TCF^{DN}$]) and constitutively active arm (P[arm^{SC10}]) were obtained from was from M. Peifer (Pai, 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*/$nkd^{l(3)4869}$ 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 (Δ*UpE*) 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 Exelisis stock center, Harvard Medical School) were outcrossed to *w1118* 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 (Fig. 2.9) and molecular mapping with PCR confirmed the deletion. The Δ*UpE* allele is homozygous semi-lethal but Δ*UpE*/ *Df(3L)ED4782* transheterozygotes are viable and fertile. This indicates that the semi-lethality of homozygous Δ*UpE* 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 (Robertson et al., 1988). The *P*[*KG0529*] line was outcrossed to *w1118* 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 (Parker et al., 2002; Lin et al., 2004), 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’GAATTAATACGACTCACTATAGGGAGAGCTGCTGGTCAGCGAACGTGAC AATAA3’ and 5’GAATTAATACGACTCACTATAGGGAGACAGACCCGTGGGC AACTTCTTCAGTTT3’. 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,* Δ*UpE/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 ul of RNAase-free water and 2 ug of total RNA was used to synthesize cDNA using 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.

Results

The *nkd* locus contains two regions, UpE and IntE 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* (Fig. 2.1). Consistent with being a target of canonical Wg signaling through a TCF-Arm complex, knockdown of either *TCF* or *arm* significantly reduced this *nkd* induction (Fig. 2.1). When the *nkd* transcription unit and surrounding DNA (see cartoon in Fig. 2.2) 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 a major WRE in mediating Wg-dependent activation of *nkd* expression in Kc cells.

Occasionally, more modest TCF binding was also observed around 10 kB upstream of the *nkd* transcriptional start site (TSS) in Wg-CM treated Kc cells (D.S. Parker, unpublished observations). TCF binding to this upstream element (UpE) is much more pronounced when cells were treated with Axin RNAi, reaching levels seen at IntE (Fig. 2.2). Cells depleted for Axin and TCF lose TCF binding at both locations, demonstrating the specificity of the TCF antisera (Fig. 2.2). 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 (Fig. 2.2).

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

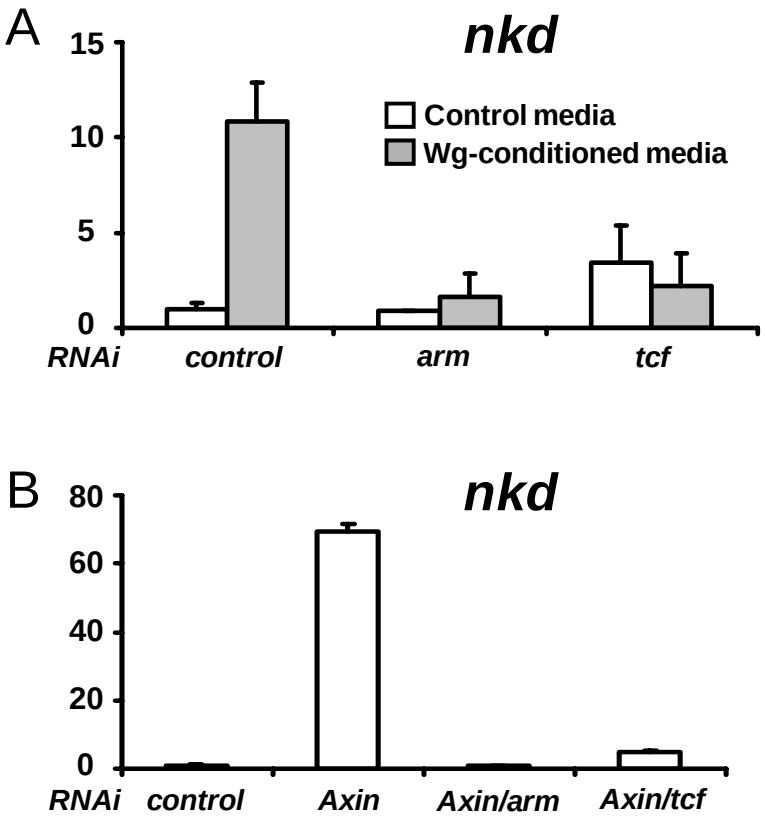

Fig. 2.1. *nkd* expression is highly increased upon Wg stimulation in fly Kc cells. The Wg pathway was activated with Wg conditioned media for 5 hours (A) or by RNAi depletion of *Axin* for 6 days (B). Both treatments resulted in elevated *nkd* expression, which greatly reduced by depletion with *arm* or *TCF*. Each bar is the mean of triplicates from cultures at each condition, with the standard deviation indicated. The data shown is a representative example from more than three separate experiments.

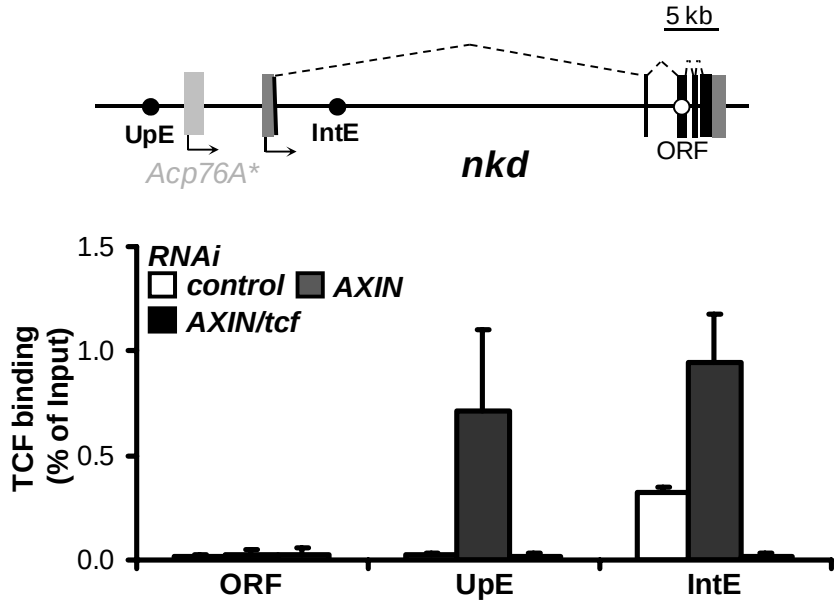

Fig. 2.2. TCF is recruited to two regions in or near the *nkd* locus.
(*upper*) Schematic diagram of the *nkd* locus showing the location of three regions (UpE, IntE and ORF) assayed for TCF occupancy. *Acp76A* is not expressed at detectable levels in Kc cells. (*lower*) Binding of TCF in Kc cells with the indicated RNAi treatments. In the absence of Wg 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 the ChIP signal is specific for TCF. Each bar is the mean of duplicate immunoprecipitations measurements with the standard error indicated. The data shown is a representative example from more than three separate experiments.

reporters can be assayed for Wg responsiveness by cotransfection with a stabilized form of Arm (Arm*) (Freeman, 2001; Fang et al., 2006; Parker et al., 2008). This UpE fragment was activated almost 1000-fold by co-expression with Arm* (Fig. 2.3A), indicating that it possesses a very high level of WRE activity.

To further localize the WRE activity in UpE, it was split into three parts and tested in the reporter assay. None of the smaller fragments (#1, #2 or #3) had high WRE activity (Fig. 2.3A). However, regions containing the 5' (UpE1) or 3' (UpE2) two thirds of UpE were dramatically activated by Arm* (Fig. 2.3A). Dissection of the IntE region gave a different result. In this case, all of the WRE activity of the original IntE fragment was contained in the 3' most 255 bp (Fig. 2.3B).

UpE1, UpE2 and IntE all contain multiple predicted TCF sites (Fig. 2.3) that are conserved among the sequenced *Drosophila* species, including the distantly related *D. virilis* (Fig. 2.4B, C). Mutation of the five individual TCF binding sites in UpE2 demonstrated that three were required for full WRE activity (Fig. 2.5A). In IntE (255bp), all three predicted TCF sites were bound by the HMG domain of TCF in a DNaseI protection assay (data not shown). All three of these sites contributed to WRE activity (Fig. 2.5B). Simultaneous mutation of three TCF sites in UpE1 resulted in a 16-fold reduction in Wg responsiveness (Fig. 2.3A). More emphatically, mutation of two TCF sites in UpE2 or all three sites in IntE completely abolished activity of these WREs (Fig. 2.3A, B). Together with the ChIP data, the mutagenesis results demonstrate that these WREs are directly activated by TCF-Arm in Kc cells.

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)

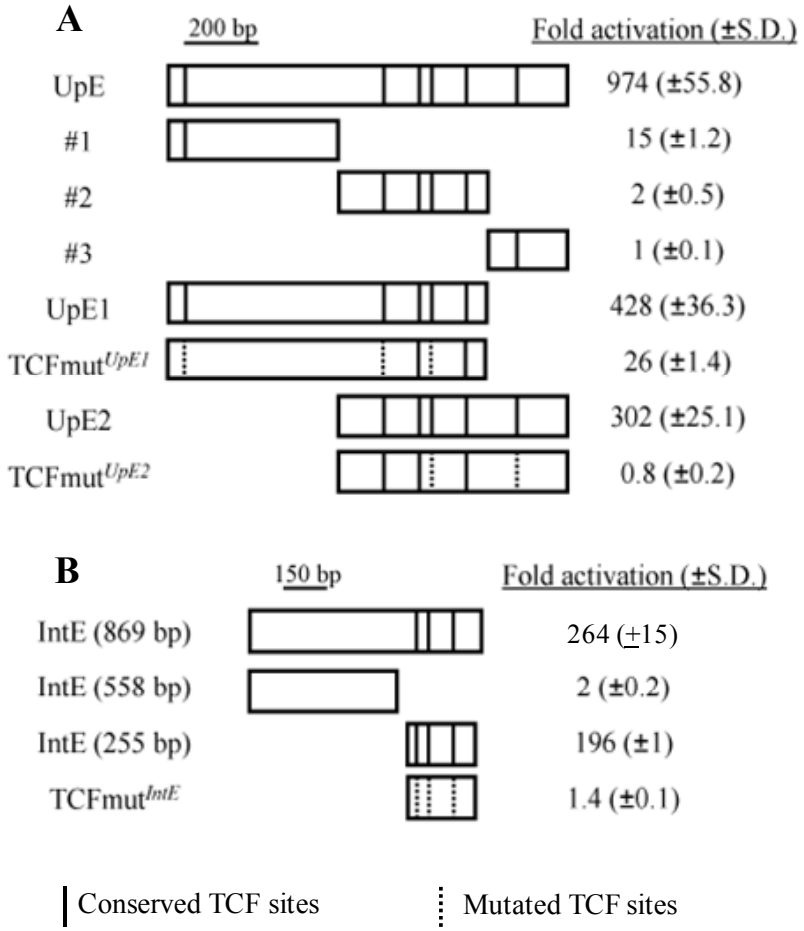

Fig. 2.3. Dissection of the UpE and IntE regions reveals WREs that contain functional TCF binding sites. (A) When cloned upstream of a *hsp70* core promoter/luciferase reporter, UpE activated luciferase expression when co-expressed with an Arm* expression plasmid. UpE was divided into three fragments (#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. Each result is the mean of triplicate transfections, with the standard deviation indicated in parenthesis. The data shown is a representative example from more than three separate experiments.

Table 2.1. Wg-responsiveness of *nkd*-WREs in multiple fly cell lines

	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)

(van de Wetering et al., 1997). Despite the lack of obvious distinction in quality or conservation of these TCF sites, the functional contribution of individual site to WRE activity is quite different (Fig. 2.5). This suggests that more information than the recognized TCF site influences it functional importance.

UpE1, UpE2 and IntE are highly responsive to Arm* in Kc cells (Fig. 2.3), 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, the activation of these WREs is not only seen with Arm* co-expression, as all the WREs are also highly activation by Wg-CM treatment (Table 2.1).

The TCF binding and reporter gene data suggests that the UpE region is a WRE for the *nkd* locus but it is also possible that it activates 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). Because of its proximity to UpE and its high level of responsiveness to the pathway, *nkd* is the likely target of Wg-mediated transcriptional activation by UpE.

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)	

Fig. 2.4. 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 functional TCF sites in reporter context (Fig. 2.2). "(rev)" indicates the site is on the reverse strand. (B, C) The alignment of TCF sites and flanking sequences between *D. melanogaster* and *D. virilis*. We found there is a high correlation between conservation in sequences and functionality of TCF sites. While 3 non-conserved TCF sites in UpE or IntE did not show any functional contribution 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, T3 and T5 sites in UpE show no functionality in the reporter assay context.

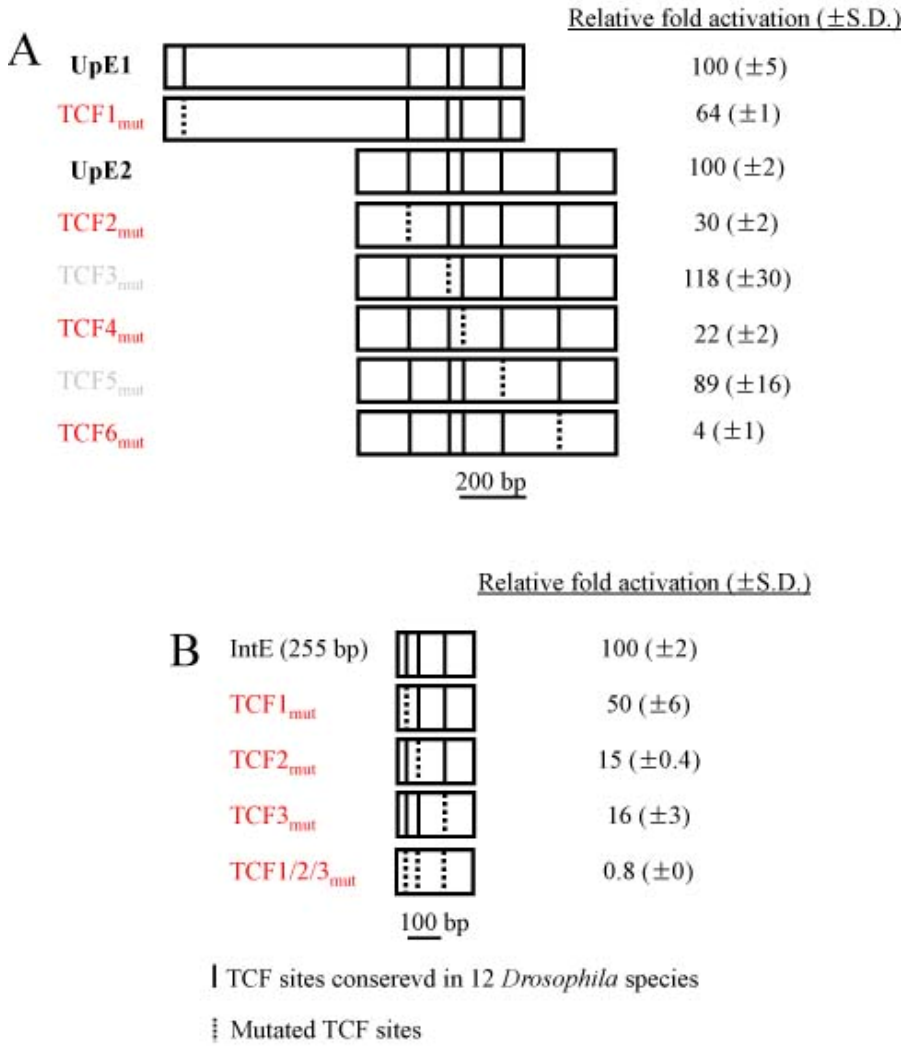

Fig. 2.5. Not every TCF binding site is functional or contributes to the WRE activity equally. The indicated reporter constructs were activated by Arm* in Kc cells. The fold activation of UpE1, UpE2 and IntE (255bp) were normalized to 100. (A) Compared to UpE1 reporter, the $TCF1_{mut}$ construct showed a 36% reduction in the Wg signaling-responsiveness. The functionality of other TCF sites was tested in UpE2 context whose activity was normalized to 100. While mutation of $TCF2_{mut}$, 4_{mut}, or 6_{mut} reduced the activity of UpE2 by different degrees, $TCF3_{mut}$ or 5_{mut} were similar to wild type UpE2. Despite these different functionalities of individual TCF sites, there is no significant difference in sequences and conservation in regions surrounding TCF sites (Fig. 2.4). (B) In the IntE (255bp) WRE, three TCF sites all contribute to Wg signaling-responsiveness. Individual mutation of TCF sites reduces the activation by Arm* IntE incompletely but 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.

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

Wg is expressed in many embryonic tissues (e.g., Fig. 2.6A, F) and larval imaginal discs (e.g., Fig. 2.6K, P, U). In all tissues examined, *nkd* transcripts are found in patterns similar to that of Wg (e.g., Fig. 2.6E, J, O, T, Y). In general, the *nkd* expression domain tends to be broader than that of Wg, consistent with non-autonomous 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*-UpE 1, UpE2 and IntE 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 patterns reminiscent of *nkd* transcript distribution. UpE1 was active in multiple imaginal discs (Fig. 2.6L, Q, V) but displayed no activity during embryogenesis (Fig. 2.6B, G). UpE2 partially recapitulates the epidermal striped *nkd* pattern during germ band extended embryos (Fig. 2.6C) but was not expressed in older embryos (Fig. 2.6H). UpE2 also has active in several imaginal discs (Fig. 2.6M, R, W). IntE did not express LacZ reporter at germ band extension (Fig. 2.6D), but was active in the mesoderm and endoderm in older embryos (Fig. 2.6I). This reporter also displayed activity in imaginal discs (Fig. 2.6N, S, X). In sum, 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 leg imaginal discs and the larval eye, the activities of all three reporter are very similar (Fig. 2.6Q, R, S, V, W, X). However, in all other tissues the WREs display a fair degree of specificity. In the wing pouch, hinge region and antennae

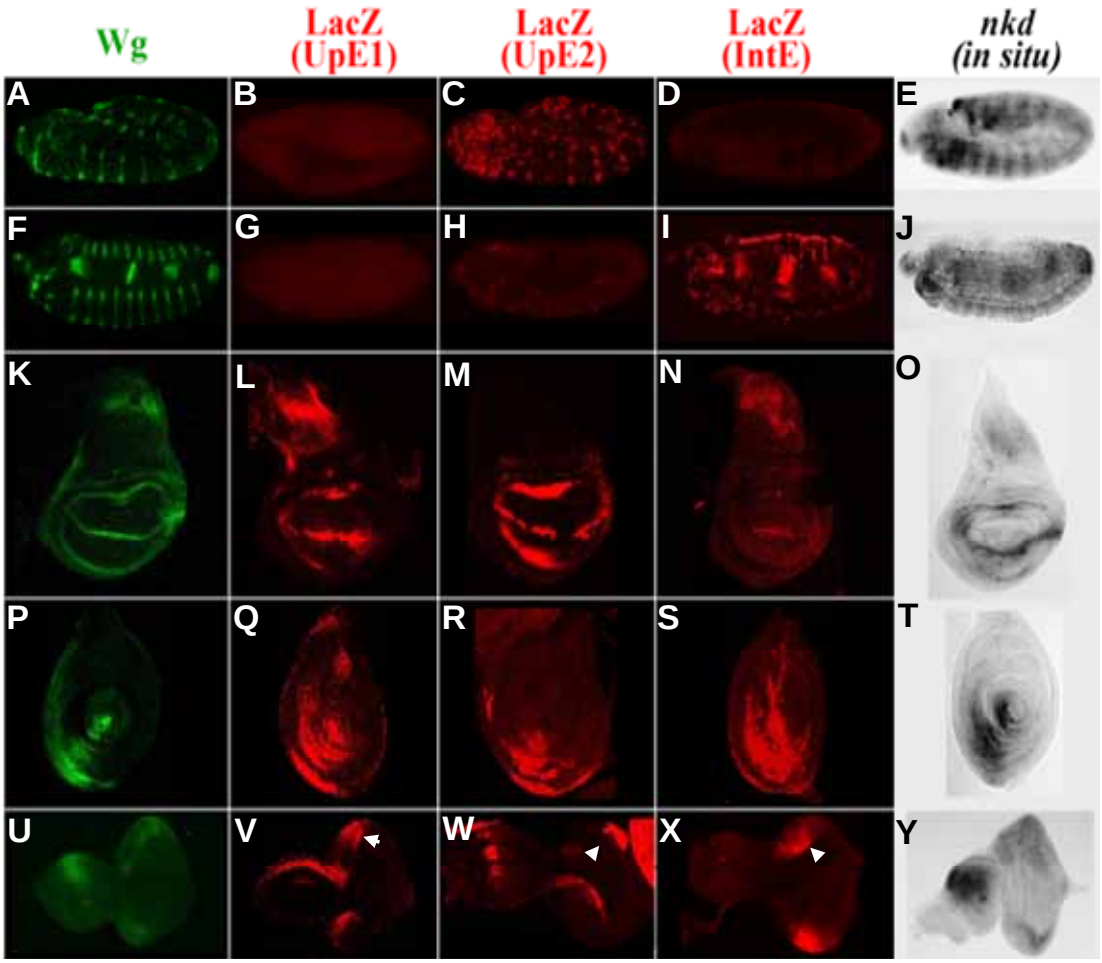

Fig. 2.6. *nkd*-WRE reporter transgenes are expressed in partially overlapping patterns similar to *nkd* transcript distribution

The patterns of Wg protein in various tissues 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*). Wing (*K*-*O*), leg (*Q*-*S*) and eye-antennal (*U*-*Y*) imaginal discs from late third larval instars are also shown. LacZ expression in eye imaginal discs is marked with arrowheads. The WRE reporters each show overlapping expression domains containing a subset of the endogenous *nkd* pattern. Three independent lines of each WRE reporter showed similar results.

Table 2.2. Summary of LacZ expression driven by various *nkd-WREs*

Tissues		*nkd-WREs*		
		UpE1	UpE2	IntE
Over-lapping	Leg discs	+	+	+
	Eye discs	+	+	+
Distinct	Early embryos (stage 10-12)	-	+	-
	Late embryos (stage 13-14)	-	-	+
	Notum	+ +	-	+
	Wing pouch	+ +	+ +	+
	Wing hinge	+	+ +	-
	Antennae	+	+ +	-

primordia, UpE1 and UpE2 are active (though UpE2 significantly stronger) while IntE is very weak or not detected. Conversely, in older embryos, IntE is very active in several mesodermal and endodermal tissues (Fig. 2.6I) similar to nkd (Fig. 2.6J) but UpE1 and UpE2 have no activity (Fig. 2.6G, H). In the notum, UpE1 is most active (Fig. 2.6L), IntE has intermediate expression (Fig. 2.6N) while UpE2 is not active (Fig. 2.6M). These data demonstrate that these WREs have partially overlapping patterns, but that they are also selectively used in many tissues as well.

All the lacZ expression patterns described for the *nkd*-WREs are consistent with being activated by Wg signaling. This was demonstrated experimentally for several incidences. When the IntE reporter (869 bp) was crossed into a wg null mutant background, the pattern expression was lost in several tissues (compare Fig. 2.7B with 2.7A), except for the activity at the leading edge of dorsal closure (arrow in Fig. 2.7B). Wg regulation of the 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 *engrailed* (*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 Fig. 2.7L; compare to Fig. 2.7K), a known Wg target (Parker et al., 2002). UpE1 and UpE2 expression was markedly reduced by TCF^{DN} (arrowheads in Fig. 2.7F and 2.7I). Conversely, expression of an active form of *Arm* (Arm^{SC10}) (Pai et al., 1997) via *patched* (*ptc*)-*Gal4* causes a dramatic increase in UpE1 expression in the wing pouch (arrows in Fig. 2.7G). Similar activation by Arm^{SC10} was observed for UpE2 and IntE (data not shown). In all cases examined, loss of Wg signaling dramatically reduces nkd-WRE activity while activation of the pathway increases reporter expression.

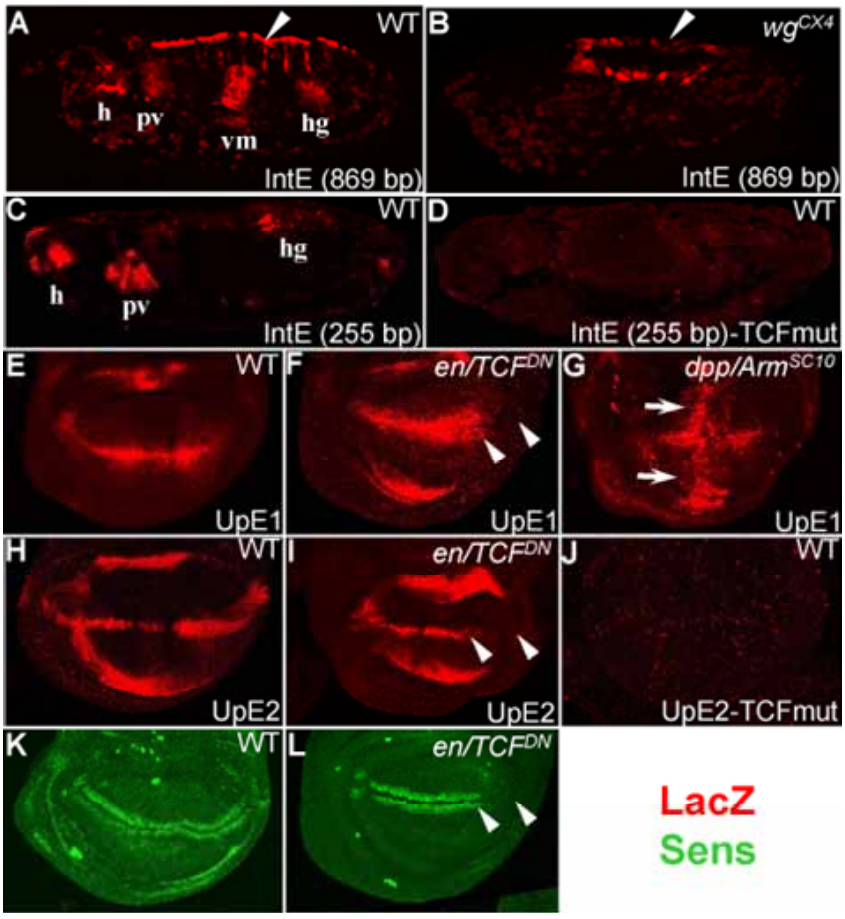

Fig. 2.7. The *nkd*-WRE reporters are positively regulated by Wg signaling . (A) Stage 14 embryo containing a 869 bp fragment containing *nkd*-IntE stained for *LacZ* (red). Several expression domains consistent with positive regulation by Wg signaling are evident, including regions of the head (h), proventriculus (pv), visceral mesoderm (vm), posterior midgut (pmg) and hindgut (hg). (B) IntE (869 bp) 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. 2.3B are mutated. (E-L) The *nkd*-UpE1 and UpE2 WREs requires Wg signaling in the wing imaginal disc. (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 the Wg readout Sens (L). (G) Conversely, expression of a stable form of Arm (Arm^{SC10}) along the anterior/posterior boundary of the wing pouch (via Dpp-Gal4; white arrows) results in marked expansion of *nkd*-UpE1 expression. (J) Mutation of the two TCF binding sites in UpE2 (the same ones indicated in Fig. 2.3A) 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.

To demonstrate the UpE2 and IntE are directly regulated by TCF and Arm in fly tissues, lacZ reporters mutating the functional TCF sites that were critical in cell culture (Fig. 2.3A, B) were created. 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 (Fig. 2.7C and data not shown) in most tissues. Mutation of the three TCF sites in IntE (255 bp) abolished the expression of LacZ in stage 13 embryos (Fig. 2.7D) and various imaginal discs (data not shown), indicating direct activation by Wg signaling. In the case of UpE2, altering two TCF sites drastically reduced lacZ expression in the wing disc (Fig. 2.7J) and all other imaginal discs examined (data not shown). Thus, IntE and UpE2 are activated by TCF-Arm in many diverse 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 (Fig. 2.6). However, the sum of these WREs accounts for only part of the endogenous embryonic pattern of *nkd* (Fig. 2.6). 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 (Fig. 2.8A) were created and monitored for lacZ expression. This combined WRE had expression in the wing disc that was the sum of the individual UpE1, UpE2 and IntE patterns (Fig. 2.8B). This was the case in embryos as well, where the combined WRE was active in weak stripes at germ band extension (Fig. 2.8C), a pattern similar to UpE2 (Fig. 2.6C). In older embryos, the pattern of the combined WRE (Fig. 2.8D) was similar to that found in IntE (Fig. 2.6I). The additive nature of the expression patterns indicates

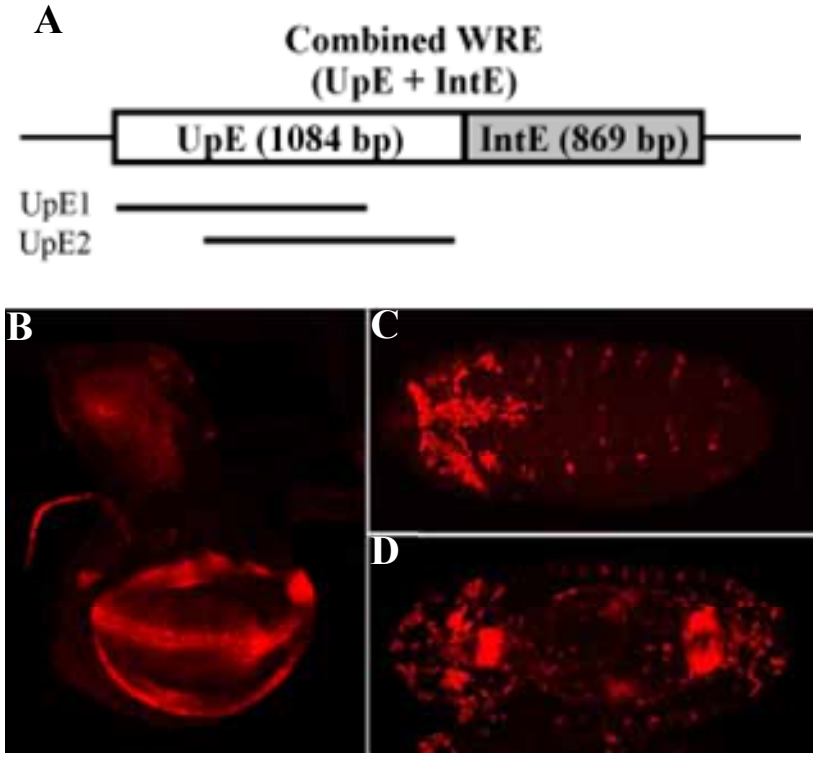

Fig. 2.8. The combination of UpE and IntE recapitulate the endogenous *nkd* pattern in the wing imaginal disc but not the embryo
(A) The combined WRE contains the overlapping regions of UpE1 and UpE2 upstream of the 869 bp IntE in the *pH-Pelican* lacZ vector. (B) The expression of the combined WRE reporter in the wing disc is very similar to the endogenous *nkd* pattern (Fig. 2.6O) and appears to be the sum of the three individual WREs (compare with Fig. 2.6L –N). (C, D) Ventral views of stage 11 (C) and 14 (D) embryos. The pattern is additive of the UpE2 and IntE (869 bp) WREs (Fig. 2.6C, I and Fig. 2.7A) and does not recapitulate the endogenous *nkd* pattern (Fig. 2.6E, J).

no detectable cooperative interactions between UpE and IntE, suggesting the existence of at least one other WRE active in the embryo.

Deletion of UpE significantly reduces endogeneous *nkd* expression in larval tissues

The data obtained from reporter assay done in cultured cells and fly tissues strongly support the model that UpE and IntE are bona fide WREs of *nkd.* However, this evidence is based entirely on the expression of luciferase and lacZ reporters in cultured cells and fly tissues. To determine whether UpE or IntE were required for expression of endogenous *nkd*, two deletions were created in the locus. Using transposons 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*; Fig. 2.10A) was engineered (see Materials and Methods and Fig. 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 sequence including IntE (Fig. 2.10B).

To test the effect of Δ*UpE* and Δ*IntE* alleles on *nkd* expression, these alleles were placed over *Df(3L)ED4782*, a large (175 kB) deletion removing *nkd* and several surrounding genes. These transheterozygotic backgrounds produced viable fertile adults at about the same frequency as +/*Df(3L)ED4782* individuals (data not shown). *nkd* mutants display an embryonic lethal mutation due to mispatterned of the epidermis due to elevated Wg signaling (Zeng et al., 2000; Waldrop et al., 2006). Therefore, the viability of the Δ*UpE*/*Df(3L)ED4782* and Δ*IntE*/*Df(3L)ED4782* transheterozygotes 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

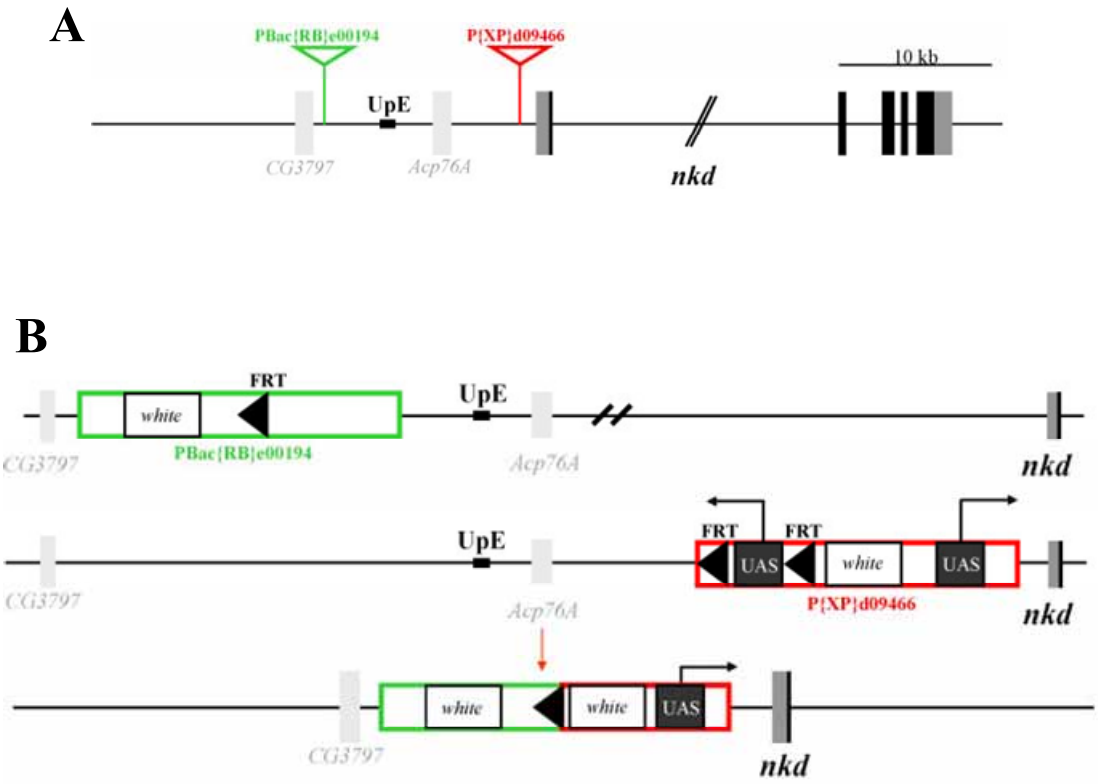

Fig. 2.9. Schematic diagram showing the generation of Δ*UpE* deletion allele
(A) Two insertions, *PBac{RB}e00194* and *P{XP}d09466*, were used to generate Δ*UpE*. (B) The FRT site present in *PBac{RB}e00194* was recombined to one of two FRTs present in *P{XP}d09466* and as a consequence, the intervening sequences having UpE and other flanking sequences were removed from one chromosome, while another pair of chromosome had duplicated sequences of UpE and the *Acp76A* gene. Flies having the Δ*UpE* chromosome were selected based on the enhanced eye color and the mutation confirmed by PCR. To test whether UAS sequences present upstream of *nkd* influence *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).

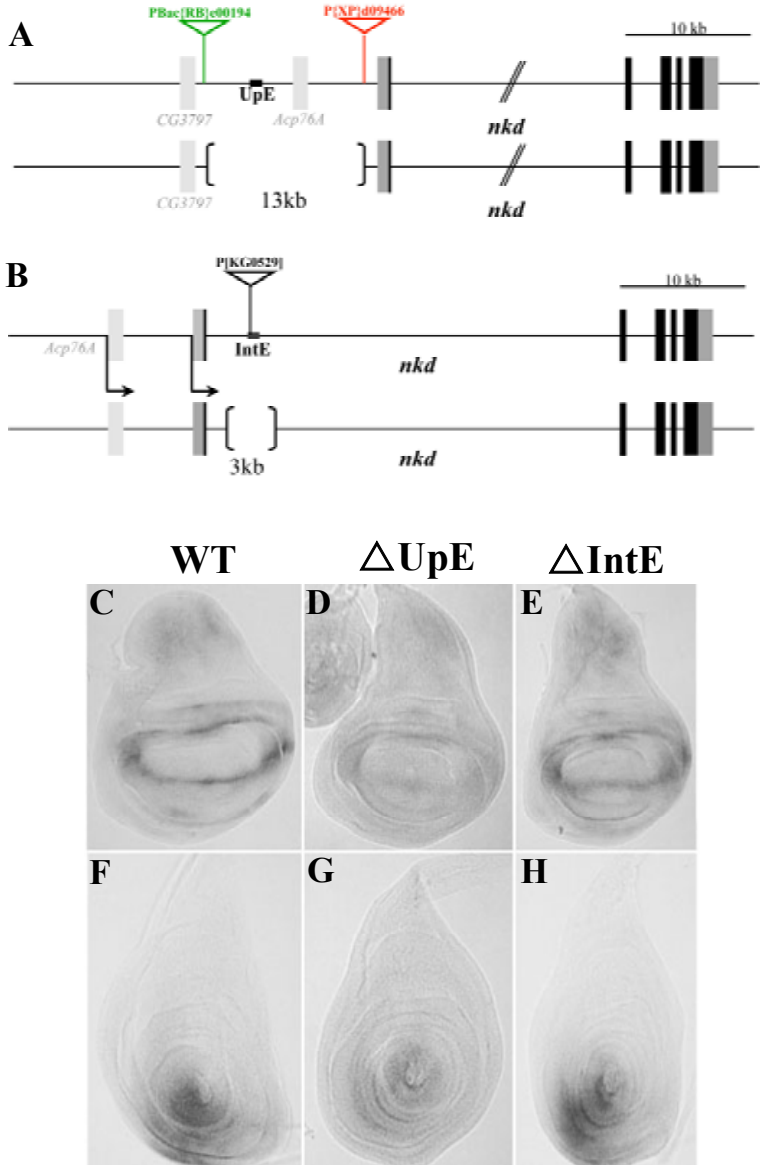

Fig. 2.10. Deletion of the IntE does not affect *nkd* expression, but a large deletion containing UpE reduces *nkd* expression in wing and leg imaginal discs
(A, B) Cartoons of the Δ*UpE* and Δ*IntE* deletions. See Materials and Methods and Fig. 2.9 for details of the deletion construction. (C-H) *nkd* transcripts in wing (C-E) and leg (F-H) imaginal discs from +/ *Df(3L)ED4782* (C, F) Δ*UpE/Df(3L)ED4782* (D, G) and Δ*IntE/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. Δ*UpE/Df(3L)ED4782* transheterozygotes had 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 reduction was consistently observed from three independent *in situ* analyses and was also confirmed by qRT-PCR (see text).

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 effect *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 (Fig. 2.10C, D, F, G). A similar reduction was observed in the eye-antennal discs (data not shown). qRT-PCR quantification revealed that Δ*UpE*/*Df(3L)ED4782* wing discs had 41% (S.D. $\pm$ 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 (Fig. 2.10E, H) and pRT-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*.

Discussion

TCFs are recruited to two distinct genomic regions near and in the *nkd* transcription unit

In this study, we used ChIP along genomic regions bound by TCF proteins in an effort to find WREs and found UpE and IntE. The recruitment of TCF to *nkd*-IntE was reported previously (Fang et al., 2006; Li et al., 2007; Chang et al., 2008; Parker et al., 2008). TCFs are present on IntE in the absence of Wg stimulation (Fig. 2.2), consistent with the model that TCF acts as a transcription repressor when signaling is off. When

cells are treated with Wg-conditioned media, TCF (Fig. 2.2) and Arm (Parker et al., 2008) recruitment to IntE increases significantly. Interestingly, from *Axin*-depleted cells, we found a novel TCF binding region upstream of *nkd* TSS (Fig. 2.2). The noticeable but modest TCF ChIP signal from UpE has been seen from Wg conditioned media-treated cells (Parker et al, 2008). It becomes more prominent when Wg signaling is activated by the depletion of *Axin* which normally activates the signaling significantly higher than the treatment of Wg conditioned media.

This UpE region, however, behaves differently from IntE. While TCF appears to be present on IntE regardless of the signaling, it seems to be recruited to UpE only when the signaling is on. In the current prevailing model, TCF acts as a transcription repressor in the absence of the signaling and acts as an activator upon the signaling. Therefore, the presence of TCF to DNA is expected regardless of the signal (reviewed Parker et al., 2007). According to this model, our data may indicate that TCF sites in UpE is dedicated to the transcriptional activation, whereas those in IntE can be used for activation and repression, at least in fly Kc cells.

Functionality tests of individual TCF sites suggest the existence of another genetic code besides TCF sites (Fig. 2.4). When individually mutated, TCF sites on UpE show different contribution to the activity of UpEs. Mutation of the TCF 6 site almost abolishes the activity of UpE2, whereas mutation of TCF 1, TCF 2, or TCF 4 site reduces Wg responsiveness of UpE1 or UpE2 by 36 to 78%. Interestingly, the TCF 3 site, which has the exact same sequences to the functional the TCF 2 site, appears to be dispensable for the activity of UpE2. We reasoned that TCF proteins may be recruited to TCF 2 site

but not to TCF site3 and such selection of functional TCF sites may require TCF sites and unknown sequences. This is under investigation.

Nkd needs multiple tissue-specific WREs

When tested in flies, UpE1, UpE2 and IntE recapitulate the pattern of endogenous *nkd* in multiple tissues (Fig. 2.6, Table 2.2). All WREs are active in the leg and antennae imaginal discs, however, each WRE has unique activity in other tissues. In embryonic epidermis, only UpE2 is active. In later stages of embryos, only IntE shows the activity in multiple primordial cells. UpE1 and UpE2 can recapitulate the *nkd* pattern in the wing and eye imaginal discs, whereas IntE shows no or minimal expression. This unique pattern of each WRE strongly suggests that WREs can respond to Wg signaling in a tissue-specific manner.

We reason that this tissue-specificity may be dependent on novel *cis*-elements that have not been discovered yet. UpE1 is active in the notum but UpE2 is not. While UpE1 shares more than 400 bp with UpE2, it has unique sequences in the 5' region. It is conceivable that there are sequences in this UpE1-unique region and they may recruit notum-specific positive factors. Conversely, notum-specific Wg inhibitors may be recruited to the unique sequences present in UpE2. As *nkd*-WREs show different activities in 6 of 8 examined tissues (Table 2.2), there can be several *cis*-elements acting as tissue-specific elements.

There are several known transcription factors that are recruited to the locus of fly and mammalian *nkd* and regulate the transcription. Some of these factors might be recruited to tissue-specific *cis*-elements and regulate *nkd* expression. Such examples

include CtBP (C-terminal Binding Protein) (Fang et al., 2006), CBP (CREB-Binding Protein) (Li et al., 2007), a homeodomain protein, Hoxc8 (Lei et al., 2007) and an ATP-dependent chromatin remodeling complex, Acf1/ISWI (Liu et al., submitted). CBP, however, is less likely to be recruited to novel sequences, as all known mechanisms of CBP recruitment are dependent on TCF and Arm (Waltzer and Bienz, 1998; Miyagishi et al., 2000; Sun et al., 2000; Li et al., 2007). DNA sequences responsible for the recruitment of CtBP and Acf1/ISWI are unknown (Fang et al., 2006; Liu et al., submitted). Several known sequences that Hoxc8 can recognize in flies and mice (Lu et al., 1995; Manak et al., 1995; Lei et al., 2007) are not found from *nkd*-WREs. However, Hox gene is known to recognize sequences that have "TAAT" core and various flanking sequences, by interacting different binding partners (Lu et al., 1995; Manak et al., 1995). Thus it would be interesting to mutate some of TAAT sequences present in *nkd*-WREs and test whether any of those sequences are required for the expression of *nkd*-WREs in certain fly tissues.

Multiple WREs may act redundantly to activate the endogenous *nkd* gene

Our data from genomic deletion assays support the existence of multiple WREs and their redundant function in *nkd* transcription. The data from reporter assays show that UpE and IntE are sufficient to respond to the Wg signal in multiple tissues (Fig. 2.6, Table 2.2), yet IntE is dispensable for endogenous *nkd* expression (Fig. 2.10E, H). Deletion of a large fragment harboring UpE reduces the *nkd* level significantly in imaginal discs (Fig. 2.10D, G), however, 41% of the *nkd* transcript is still detected from ΔUpE wing imaginal discs. This can be explained by redundancy between UpE and IntE.

In reporter assays, UpE and IntE activities are overlapping in several imaginal discs, consistent with them being redundant in such tissues (e. g. leg, eye, notum and wing pouch). Previously, we propose the existence of unknown WREs that are mainly active in embryonic cells because the combined WREs cannot recapitulate the striped *nkd* pattern in embryos. Assuming such additional WREs are present, the observation that there is no obvious effect on *nkd* embryonic expression by loss of UpE or IntE may be due to redundancy between UpE, IntE and missing WREs.

Acknowledgements

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CHAPTER III

NUCLEAR RRM PROTEINS SPLIT ENDS AND SPENITO ACT REDUNDANTLY TO PROMOTE WINGLESS SIGNALING IN A CELL-SPECIFIC MANNER IN *DROSOPHILA*

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Abstract

Wingless (Wg)/Wnt signaling directs a variety of cellular processes during animal development by promoting the association of Armadillo/β-catenin with TCFs on Wg regulated enhancers (WREs). Split ends (Spen), a nuclear protein containing RNA Recognition Motifs (RRMs) and a SPOC domain, is required for optimal Wg signaling in several fly tissues. In this report, we demonstrate that Spenito (Nito), the only other fly protein containing RRMs and a SPOC domain, acts together with Spen to positively regulate Wg signaling. The partial defect in Wg signaling observed with *spen* RNAi was enhanced by simultaneous knockdown of *nito* while it was rescued by expression of *nito* in wing imaginal discs. In cell culture, depletion of both factors causes a greater defect in the activation of several Wg targets than RNAi of either *spen* or *nito* alone. These

nuclear proteins are not required for Armadillo stabilization or the recruitment of TCF and Armadillo to a WRE. Loss of Wg target gene activation in cells depleted for *spen* and *nito* was not dependent on the transcriptional repressor Yan or Suppressor of Hairless, two previously identified targets of Spen. We propose that Spen and Nito act redundantly downstream of TCF and Armadillo to activate many Wg transcriptional targets.

Introduction

The *Drosophila* gene Wingless (Wg) encodes a member of the Wnt family of secreted and palmitoylated glycoproteins that are conserved throughout the animal kingdom (Cadigan and Nusse, 1997; Clevers, 2006; Mikels and Nusse, 2006). Wg influences cell behavior through stabilization and nuclear translocation of Armadillo (Arm), known as β-catenin in vertebrates (Kikuchi et al., 2006). This Wnt/β-catenin pathway controls numerous cell fate decisions in development and adult tissues (Logan and Nusse, 2004). Misregulation of this signaling cascade leads to a variety of diseases including several forms of human cancer (Logan and Nusse, 2004; Clevers, 2006; Johnson and Rajamannan, 2006).

In the absence of Wg/Wnt stimulation, cytosolic Arm/β-catenin is constitutively phosphorylated by the coordinated action of a group of proteins including the scaffolds Axin and Adenomatous polyposis coli protein (APC), as well as Casein kinase I and Glycogen synthase kinase-3 (GSK-3). Phosphorylated Arm/β-catenin is then ubiquitinated and degraded by the proteosome (Kikuchi et al., 2006). Wg/Wnt signaling inhibits this process (Cadigan and Liu, 2006), leading to the accumulation and nuclear

translocation of Arm/β-catenin. Once in the nucleus, Arm/β-catenin associates with members of the TCF/Lef1 (TCF) family of DNA-binding proteins. Arm/β-catenin converts TCFs from transcriptional repressors to activators in part by displacing the transcriptional co-repressor Groucho (Gro; Städeli et al., 2006; Parker et al., 2007).

Several positive regulators of TCF and Arm/β-catenin-mediated transcription have been identified (Willert and Jones, 2006; Städeli et al., 2006; Parker et al., 2007). One of the potential positive factors is Split ends (Spen), a nuclear protein that is required for Wg signaling (Lin et al., 2003). The *spen* gene encodes several large isoforms (>5500 amino acids) that contain three RNA recognition motifs (RRMs) in their N-terminal portions and a Spen Paralog and Ortholog C-terminal (SPOC) domain at their C-termini (Willette et al., 1999; Rebay et al., 2000; Kuang et al., 2000). *spen* is required for optimal Wg signaling in several imaginal discs in fly larvae, but Wg signaling occurs normally in embryos lacking maternal and zygotic *spen* activity (Lin et al., 2003). Spen appears to be a tissue-specific positive regulator of the Wg pathway.

SHARP, the human ortholog of *Spen*, was recently shown to be required for Wnt/β-catenin signaling in several human cell lines (Feng et al., 2007). SHARP could potentiate the ability of Lef1 (a vertebrate TCF) to activate reporter genes independently of β-catenin (Feng et al., 2007). This suggests that SHARP promotes Lef1 DNA binding or acts through a transcription activation mechanism that does not require β-catenin.

In addition to Wg signaling, Spen has been implicated in several other processes in flies. In the developing eye, *spen* is required to inhibit Notch signaling and promote Epidermal Growth Factor (EGFR)/Ras/MAP Kinase (MAPK) signaling (Doroquez et al.,

2007). In addition, loss of *spen* results in increased levels of the ETS-domain transcriptional repressor Yan (Doroquez et al., 2007), an antagonist of EGFR signaling (Rebay and Rubin, 1995). *spen* is also required for Hox-mediated repression of head identity in the embryonic trunk (Wiellette et al., 1999), specification, survival and axonal guidance of specific neurons (Chen and Rebay, 2000; Kuang et al., 2000), cell cycle regulation (Lane et al., 2000), and epidermal wound repair (Mace et al., 2005). The molecular mechanisms underlying the pleiotropic phenotype of *spen* mutants have not been elucidated.

In mammals, SHARP (known as Mint in the mouse) has been shown to act as a co-repressor for nuclear hormone receptors (Shi et al., 2001) and RBP-Jκ, a DNA-binding protein critical for Notch signaling (Oswald et al., 2002; Kuroda et al., 2003; Oswald et al., 2005; Tsuji et al., 2007; Yabe et al., 2007). The SPOC domain appears to be essential for the ability of Spen proteins to repress transcription and can physically interact with various proteins including the universal co-repressors SMRT and NCoR, histone deacetylases, the CtIP and CtBP corepressors and the E2 ubiquitin-conjugating enzyme UbcH8 (Shi et al., 2001; Ariyoshi and Schwabe, 2003; Oswald et al., 2005; Li et al., 2006). In contrast to these repression roles, Mint has been shown to act with Runx2 to activate *osteocalcin* (*OC*) transcription, possibly through specific DNA-binding to a G/T rich element in the *OC* control region mediated by its RRMs (Newberry et al., 1999; Sierra et al., 2004).

The Spen family is defined by the presence of N-terminal RRMs and a C-terminal SPOC domain (Wiellette et al., 1999; Kuang et al., 2000). In addition to Spen and its homologs, there are several smaller (<1000 aa) family members that include Spenito

(Nito) in flies (Rebay et al., 2000), as well as One-Twenty-Two/RNA-binding motif protein 15 (OTT1/RBM-15) in mammals. OTT1/RBM-15 (referred to as OTT1 hereafter) was originally identified as a fusion protein at a recurring chromosomal translocation in megakaryocytic acute leukemia (Ma et al., 2001; Mercher et al., 2001).

Although they share similar domains, several reports indicate that the smaller Spens are functionally distinct from their larger relatives. Like, SHARP and Mint, OTT1 can bind to RBP-Jκ and repress Notch signaling, but in some cell types it activates the pathway (Ma et al., 2007). Even more dramatic differences were noted in the fly, where *nito* was shown to act antagonistically with *spen* in the developing eye (Jemc and Rebay, 2006).

In this report, we demonstrate that Nito is a physiological regulator of Wg signaling. In contrast to other processes where they act antagonistically, Spen and Nito act redundantly to promote Wg signaling in flies and cultured cells. Simultaneous depletion of both genes results in a more severe loss of Wg activation of target genes than either gene alone and expression of *nito* can rescue the defect in cells with reduced *spen*. Consistent with their nuclear localization, Spen and Nito act downstream of Arm stabilization/nuclear translocation but WRE recruitment of TCF and Arm are unaffected in *spen*, *nito* depleted cells. Spen and Nito do not appear to act on Wg targets through regulation of the transcriptional repressor Yan or Suppressor of Hairless (Su(H)), the fly homolog of RBP-Jκ. However, loss of the Wg target *naked cuticle* (*nkd*) in *spen* and *nito* RNAi treated cells was reversed by depletion of *groucho* (*gro*). This interaction between *gro* and *spen-nito* was not seen at another Wg target, and some targets of the pathway are

independent of *spen* and *nito*. Thus, these factors act redundantly downstream of TCF-Arm complex formation to regulate Wg targets in a gene-specific manner.

Materials and Methods

Fly strains

The P[*da-Gal4*], P[*arm-Gal4*], P[*GMR-Gal4*], P[*UAS-wg*], P[*GMR-Arm**], and P[*UAS-EGFR^{DN}*] stocks are previously described (Lin et al., 2003). The P[*GMR-hid^{l0}*], P[*GMR-rpr^{2}*] stocks and P[*ptc-Gal4*], P[*dpp-lacZ*] and P[*UAS-lacZ*] strains are previously described (Guan et al., 2007; Li et al., 2007). P[*UAS-yan*] (Rebay and Rubin, 1995) was obtained from the Bloomington Stock Center.

P[*UAS-spen^{RNAi}*] and P[*UAS-nito^{RNAi}*] (*UAS-nito^{RNAi}-2*) strains were constructed as follows. *spen* was amplified by PCR with primers 5'CATGTCTAGACTTACCAGGCCATCAACT CATC3' and 5'CATGTCTAGAGAATAACCTGCGAGGCGTTTCC3' and *nito* with 5'CATGTCTAGACAATGTCCCCACCGGACTATGA3' and 5'CATGTCTAGACTCCGTATT TGCCAAAGATG CGA3' (*Xba*I sites used for cloning are underlined). The regions targeted in each gene are indicated in Fig 3.1 A. The amplified PCR products were cut with *Xba*I and ligated into the *Avr*II and *Nhe*I ends of the pWIZ vector in opposite directions (Lee and Carthew, 2003). The P[*UAS-nito*] strains was generated by amplifying the *nito* ORF from cDNA RE36227 (obtained from *Drosophila* Genomics Resource Center at Indiana University) with primers 5'CAGGAATTCATGAGTAGTCATCGAGACGGAG CCGGA3' (*Eco*RI site is

underlined) and 5'TGTGGTACCTCAGGCCGTTCC-GCCGCGCACCACC-A3' (*Kpn*I site is underlined) and was ligated into the *Eco*RI and *Kpn*I sites in *pUAST* (Brand and Perrimon, 1993). Transgenic flies were generated following the standard P element methodology using w^{1118} embryos. An additional P[*UAS-nito*^{RNAi}] line (*UAS-nito*^{RNAi}*-1*) targeting a non-overlapping region of *nito* transcripts than *UAS-nito*^{RNAi}*-2* (see Fig. 3.1 A) was obtained from Ilaria Rebay (Jemc and Rebay, 2006).

Whole-mount staining and microscopy

In situ hybridization and immunostaining were performed as previously described (Lin et al., 2004). The probe against *nito* was generated using a PCR product from genomic DNA with the following oligos: 5'GGTGGCTATTCTCCGTATCCACCTA3' and 5'GTAATACGACTCACTATAGGGCGACCACAATCACCAGGTGATCCT CCTT3'. Guinea pig anti-Sens antibody (1:1000) was described previously (Fang et al., 2006). Mouse monoclonal Wg antibody (4D4; 1:100) was from the Developmental Studies Hybridoma Bank (University of Iowa). Cy3 conjugated antibodies and Alexa Flour 488-conjugated antibodies were purchased from Jackson Immunochemicals (West Grove, Pennsylvania) and Molecular Probes (Eugene, Oregon), respectively. All confocal fluorescent pictures with Z-stack projection were obtained with a Zeiss Axiophot coupled to a Zeiss LSM510 confocal apparatus. Micrographs of *in situ* hybridizations were taken with a Nikon Eclipse 800 and adult fly heads with a Leica MZ APO light microscope. All images were processed as Adobe Photoshop files.

Cell culture and RNAi

Drosophila Kc167 (Kc) cells were cultured as previously described (Fang et al., 2006; Li et al., 2007). A S2 cell line that is expressing Wg under the control of a constitutive *tubulin* promoter was obtained from R. Nusse and control and Wg-conditioned media (WCM) were prepared as described (www.stanford.edu/~rnusse). RNA interference (RNAi) was performed as previously described (Fang et al., 2006; Li et al. 2007) with the modifications outlined below. All primers used for PCR amplification contained a T7 RNA polymerase binding site (5'GTAATACGACTCACTATAGGGCGA3') at their 5' ends. Gene specific sequences of the primers were as follows: *spen*, 5'CTCTAGATGCAAAATCCACTTGAAGC3' and 5'TGGTCTATTTCATGAGTACAAAAAGCA3' (targeting a region in the 3rd exon); *nito^{RNAi}-3*, 5'CGACGCATACAGAATTTGCAAAG3' and 5'CTTGTACACCGGCTCCACAATG3' (targeting a region in the 1st to 2nd exons); *nito^{RNAi}-4*, 5'TATGAACGTTACCACTACTCGAGGT C3' and 5'CCCTTCTGGTATTCAATCTT-CTTGA3' (targeting a region in the 3rd exon); *nito^{RNAi}-5*, 5'GCGACGCCCGCCAGGAGAATCGTA3' and 5'TTTGTCCTCCCATCCCCACCGTCGCC A3' (targeting the C-terminal region of Nito); *Axin*, 5'ATGGCCTTCCATGAACACAG3' and 5'AGTCTTCGTTTCGCCTCCTC3'; *Su(H)* 5'TTTAATTTAAACGCCGCCGC3' and 5'AGGT AACGGGTGGAGACAGTCTGGGA3'; *yan*, 5'GCA GTGGCACCTGCCTAATAGTCCAGT3' and 5'GCTGTTGCGTCCAATTCG GCTTAAT3'. The primers used for generating dsRNAs of *TCF, arm*, *gro* and *control*

were as described (Fang et al., 2006). PCR products of each gene were used as templates for the *in vitro* transcription of RNAi with Megascript kit (Ambion, Austin, TX).

When *Axin* RNAi was used to activate Wg signaling, 5 μg of dsRNAis of *spen, nito, TCF* and *arm* and 10 ug of *Axin* RNAi were applied per 1×10^6 cells and the total amount of dsRNA was normalized by *control* dsRNA to 20 μg per sample (as in Fig 3.4 A &B, Table 1, and Fig 3.7). The dsRNA was added to serum-containing media and cultured for 4 days. On day 4, cells were diluted 1:4 and then cultured 2 additional days before harvesting for further analysis. For experiments using WCM (Fig. 3.7 C), 10 ug of total dsRNA/1×10^6 cells was used and cells were cultured as described above. On day 6 the cells were treated with WCM or control media for 5 hours before processing.

Nito antibody production and Western blotting

Full length Nito was fused to Glutathione S-transferase (GST) and expressed in BL21-CodonPlus(DE3)-RP cells (Stratagene, La Jolla, CA) using the pET42a expression vector (Novagen, Darmstadt, Germany). The fusion protein was purified using Glutathione Sepharose 4 Fast Flow (GE Healthcare, Piscataway, NJ) and injected in rabbits (Cocalico, Reamstown, PA). α-Nito antibody was purified with an affinity column made by immobilized GST-Nito with an AminoLink Plus Immobilization kit (Pierce, Rockford, IL). The fractions eluted from the affinity column were combined and concentrated using a Centricon-Plus 20 centrifugal filtration device (Millipore, Billerica, MA).

Western blot analysis was performed with the ECL-plus Western Blotting Detection kit as described by the manufacturer (GE Healthcare, Piscataway, NJ). α-Nito

antibody was used at 1:2000. Polyclonal anti-TCF antibody (1:2000) was generated from rabbits as described (Fang et al., 2006). Mouse monoclonal Arm antibody (N27A1, 1:1000) was from the Developmental Studies Hybridoma Bank at the University of Iowa. The mouse monoclonal antibody against β-tubulin (1:10,000) and the rabbit antibody against acetylated Histone 4 (at K8) were purchased from Sigma (St. Louis, MO) and Millipore (Billerica, MA), respectively.

Quantitative RT-PCR (qRT-PCR)

qRT-PCR was performed as described previously (Fang et al., 2006). The primers used to amplify *nkd, CG6234*, and *β-tubulin56D* can be found in Fang et al. (2006). Other primers sequences are: *homothorax* (*hth*), 5'GCCTAACGATACTGCAAGTGA3' and 5'GGACCT GGATGCG GTGTATAG3'; *crumbs*, 5'ACCCTATTGCGGAGCCTACT3' and 5'TAATCG GTGCTGTGTG CAATTC3'; *Frizzled 3* (*Fz3*), 5'TCACACCAATCAGCTGGAGG3' and 5'GAGACGAGCAGAGCAAA AAGC3'; *arm*, 5'GATGAGTTACATGCCAGCCCA3' and 5'GATACCATCGGCGGCAGAT3'*; TCF,* 5'CGTTTCGGACTAGATCAACAGAG3' and 5'CATCTTCGTCGTCATCACTTAGC3'; *mkp3*, 5'ATGAACATGAATGCCCGTAG3' and 5'CGTAGCTGACCTGACT3'. All data were normalized to the transcript levels of *β-tubulin*56D.

Chromatin Immunoprecipitation (ChIP) analysis

ChIP was performed as described (Fang et al., 2006). 15ul of TCF or Arm antisera was used per 5×10^6 cells for one ChIP experiments. Antibodies were precipitated by Immunoglobulin(Ig)A-coupled agarose beads purchased from Upstate. Immunoprecipitates were analyzed using Q-PCR using primer sets directed to the intronic WRE or ORF of *nkd* (sites #N4 or #N0, respectively from Fang et al., 2006). The data are presented as a percentage of input DNA.

Results

Nito is an ubiquitously expressed nuclear protein

In our previous study, a dominant negative form of *spen* (*spen*^{DN}) caused a more severe defect in Wg signaling than strong (possibly null) *spen* mutants (Lin et al., 2003). One explanation for this observation is the existence of a gene(s) that acts redundantly with *spen*. The protein known as Nito (Rebay et al., 2000) or Spen-like protein (SSLP or DmSSp ; Ariyoshi and Schwabe, 2003; Kuang et al., 2000; Wiellette et al., 1999) is an obvious candidate, as it is the only other predicted protein in *Drosophila* that has both RRMs and a SPOC domain (Fig. 3.1 A).

Spen is predominantly found in the nucleus and is expressed ubiquitously in fly embryonic and larval tissues (Kuang et al., 2000; Lin et al., 2003; Wiellette et al., 1999). Similarly, Nito is highly enriched in the nucleus of Kc cells (Fig. 3.1 B) and is expressed throughout the wing imaginal disc (Fig. 3.1 C). Consistent with another report (Jemc and Rebay, 2006), we also find ubiquitous expression in the eye imaginal disc (data not

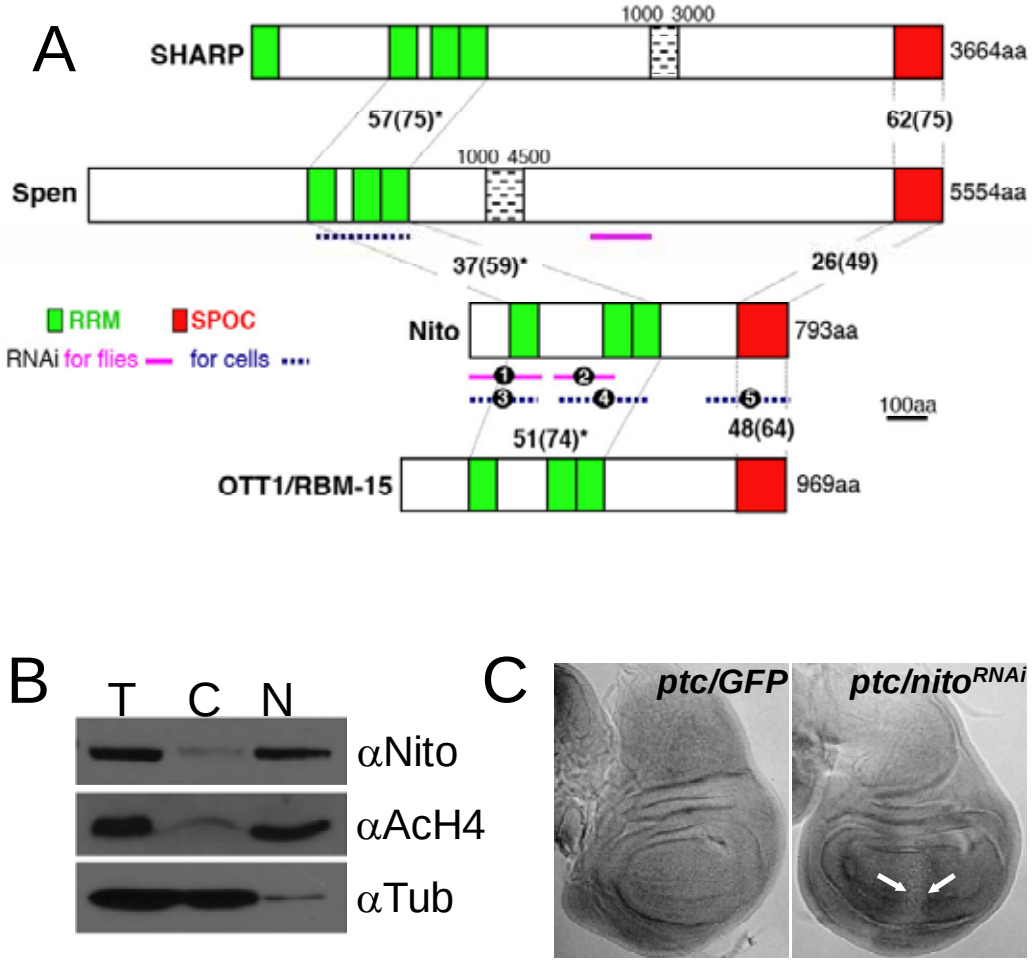


Fig. 3.1. Nito is a ubiquitously expressed nuclear protein related to Spen.
(A) Cartoon comparing Spen, Nito and their human orthologs, SHARP and OTT1/RBM-15. Number represents % of identity (similarity) between the different Spen family members. The asterisks denote that the identity/similarity of those regions is for the RRM domains only. Sequences outside the RRMs and SPOC domains showed no detectable similarities. Magenta lines indicate the regions targeted for RNAi in flies and the dashed lines the dsRNAs used in Kc cells. The multiple dsRNAs used for *nito* are numbered. (B) Western blot analysis showing a predominant nuclear localization of Nito in fly Kc cell extracts. The distribution of acetylated Histone 4 (AcH4) and Tubulin are used to mark nuclear and cytoplasmic fractions, respectively. T: total lysate, C: cytoplasmic fraction, N: Nuclear fraction. (C) Expression of *nito* was detected ubiquitously in wing imaginal discs, judged by *in situ* hybridization with *nito*-specific probes. Discs expressing a RNA hairpin targeting *nito* (*nito*^{RNAi}*-2*) showed a strong reduction of *nito* in the anterior/posterior border of wing imaginal discs where the *ptc*-Gal4 driver is active (marked by arrows).

shown). Thus Nito is broadly expressed and present in the right subcellular location to act redundantly with Spen.

nito and *spen* act redundantly in the eye

To address whether *nito* is functionally related to *spen* in flies, we generated UAS-RNAi transgenes expressing RNA hairpins targeting either *spen* or *nito* mRNAs. As will be described below (Figs. 3.2 and 3.3), *UAS-spen*^{RNAi} gives phenotypes in the eye and wing imaginal discs consistent with those obtained with *spen* mutant alleles or *spen*^{DN} (Lin et al., 2003). The ability of *UAS-nito*^{RNAi} to downregulate *nito* expression was tested by expression of the hairpin in wing imaginal discs, where it causes an inhibition of *nito* transcript levels as judged by *in situ* hybridization (Fig. 3.1 C). These reagents allow the depletion of either gene alone or in combination in several contexts where Wg signaling regulates development.

When Wg is ectopically expressed throughout the developing eye (via a *GMR* promoter), a dramatic reduction in adult eye size is observed (Parker et al., 2002; Fig. 3.2 A). A similar phenotype is seen upon expression of a constitutively active form of Arm (Arm*), which cannot be degraded (Freeman and Bienz, 2001; Fig. 3.2 F). Consistent with having a positive role in the pathway, *spen* RNAi causes a suppression of these small eye phenotypes (Fig. 3.2 B, G). Reduction of *nito* also causes an increase in GMR-*wg* or GMR-*arm** eyes (Fig. 3.2 C, H).

Simultaneous reduction of *spen* and *nito* produces a much greater suppression than either single RNAi (Fig. 3.2 D, I). These data were quantified by measuring the width at

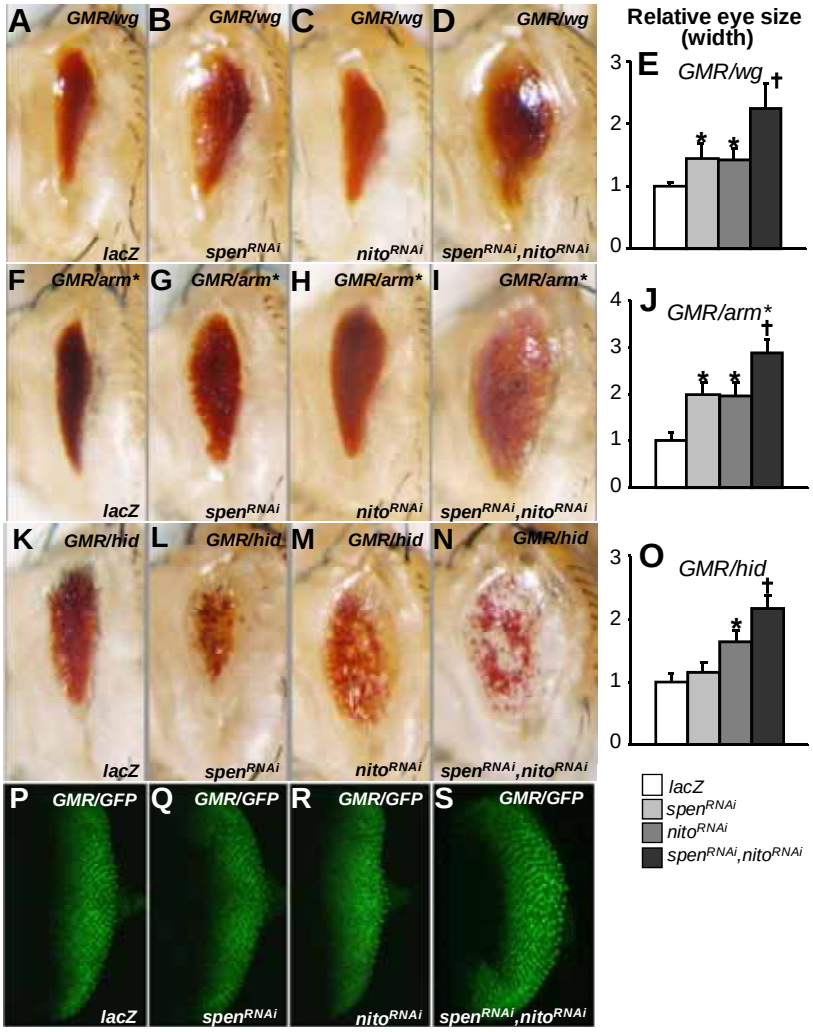


Fig. 3.2. Spen and Nito function cooperatively to promote the small eye phenotypes caused by ectopic activation of Wg/Arm signaling or the pro-apoptotic factor Hid. Micrographs of adult fly eyes all containing P[*GMR-Gal4*] and the following transgenes: P[*UAS-wg*] (A - D); P[*GMR-arm**] (F - I); P[*GMR-hid*] (K - N); P[*UAS-GFP*] (P – S). In addition, P[*UAS-lacZ*] (A, F, K, P), P[*UAS-spen*^{RNAi}] (B, G, L, Q), P[*UAS-nito*^{RNAi}*-2*] (C, H, M, R) or P[*UAS-spen*^{RNAi}]/P[*UAS- nito*^{RNAi}*-2*] (D, I, N, S) were present. *GMR*-driven overexpression of *wg*, *arm**or *hid* severely reduced eye size and these phenotypes are suppressed by RNAi depletion of *spen* or *nito*, with the greatest suppression observed when both genes were inhibited. These results were quantified by measuring the width of the eye equator (the widest point) in twenty micrographs for each condition, normalizing the lacZ control to 1. Asterisks indicate the significance ($P < 0.01$) of the difference between the lacZ control and the single knockdowns. † denotes the significance ($P < 0.01$) for the difference between *nito* RNAi alone and the *spen*, *nito* double depletion. Knockdown of *spen* and *nito* did not cause a detectable reduction in GFP expression from the *GMR-Gal4* driver (P – S).

the equator of 20 eyes from each phenotype, demonstrating that the increase in eye size is reproducible and significant (Fig. 3.2 E, J). These results are consistent with Spen and Nito acting redundantly and the suppression of GMR-*arm** suggests that they are required downstream of Arm stabilization.

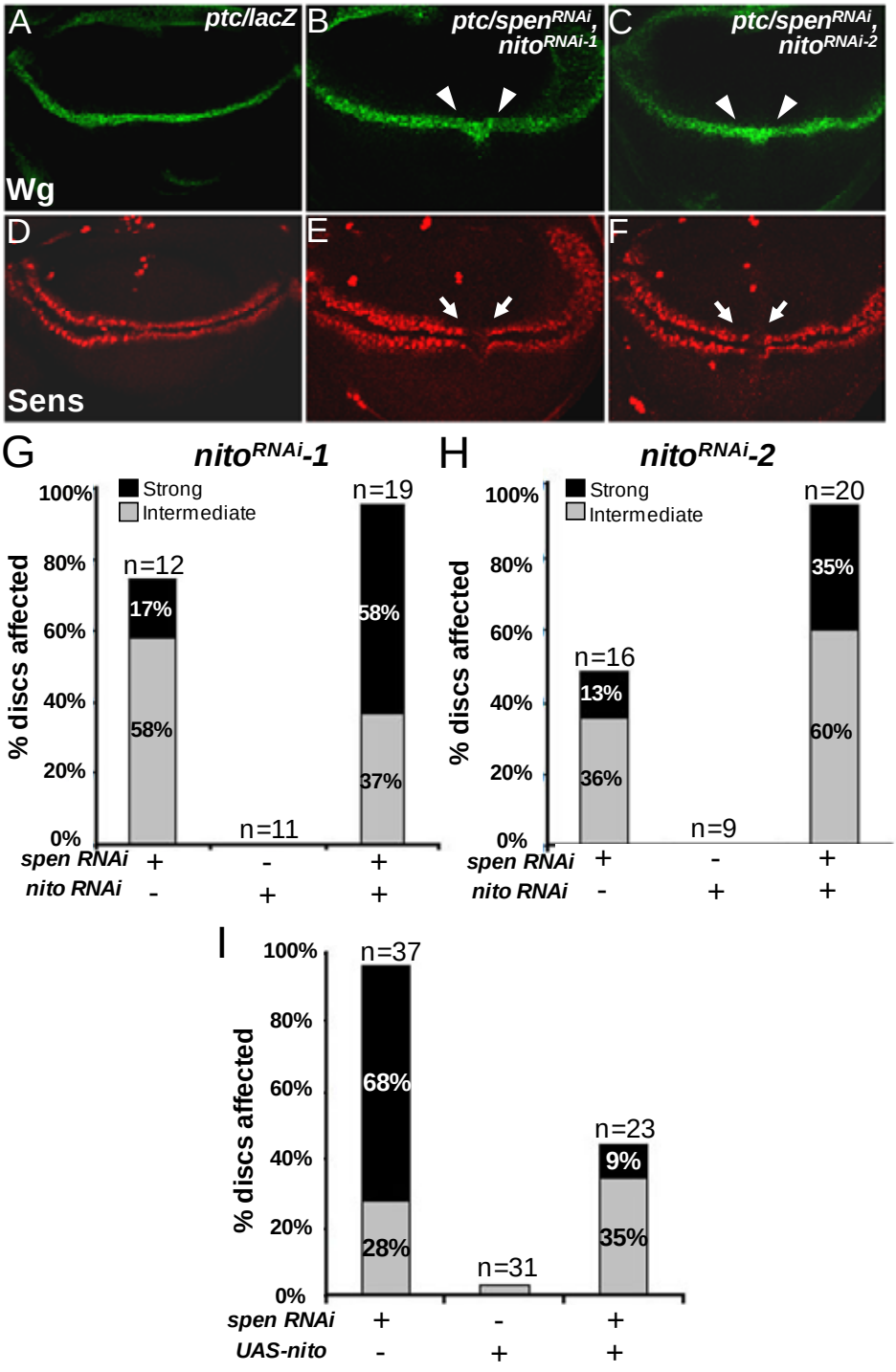
The effect of expressing *spen* and *nito* RNA hairpins through the *GMR* promoter is not specific for Wg/Arm signaling. Reduction of *spen* and *nito* suppress the phenotype caused by the pro-apoptotic factors Head involuted defective (Hid; Fig. 3.2 K-O) and Reaper (data not shown). As was the case for Wg and Arm*, knockdown of both *spen* and *nito* produces the greatest effect (Fig. 3.2 N, O; data not shown). Reducing Wg/Arm signaling does not suppress *GMR-hid* or *GMR-rpr* (Parker et al., 2002). This raises the possibility that Spen and Nito are suppressing these phenotypes because it is required for the activity of the *GMR* promoter. To address this, the ability of *spen* and *nito* RNAi to affect *GMR-Gal4*-driven GFP in the developing eye was examined. Reduction of *spen* and *nito* had no detectable effect on GFP expression (Hid; Fig. 3.2 P-S). This suggests that the suppression of Wg, Arm*, Hid and Reaper misexpression was not through the *GMR* promoter. These results also indicate that Spen and Nito are required for other processes besides Wg signaling, such as Hid or Reaper-induced apoptosis.

spen **and** ***nito*** **are redundantly required for Wg signaling in the wing imaginal discs**

To test the physiological role of *spen* and *nito* in Wg signaling in another tissue, we turned to the wing imaginal disc, where Wg is expressed at the dorsal/ventral (D/V) margin of the wing pouch (Fig. 3.3 A). Wg signaling activates expression of the zinc-finger protein Senseless (Sens) on either side of the D/V boundary

Fig. 3.3. *spen* and *nito* act redundantly in Wg signaling in the developing wing.

(A - F) Confocal images of wing discs from third instar larvae, containing P[*ptc-Gal4*] and P[*UAS-lacZ*] (A, D) P[*UAS-spen*^{RNAi}], P[*UAS-nito*^{RNAi}*-1*] (B, E) or P[*UAS-spen*^{RNAi}], P[*UAS-nito*^{RNAi}*-2*]. Wing discs were immunostained for Wg (A - C) or Sens (D - F). Sens expression was significantly reduced where *spen* and *nito* dsRNAs are expressed (white arrows), while Wg expression was often slightly expanded (white arrowheads). (G, H) Graphs summarizing the effects of *ptc-Gal4* expression of *spen*, *nito* or *spen* & *nito* dsRNA on Sens expression at 22˚C. P[*UAS-nito*^{RNAi}*-1*] (G) or P[*UAS-nito*^{RNAi}*-2*] (H) were used. The number of discs assayed is shown on top of each bar. Wild type (not shown), intermediate (gray bars) and strong loss of Wg signaling phenotypes (black bars; similar to the disc shown in panels E and F) are indicated. The strongest phenotypes were consistently observed with the double RNAi knockdown. (I) Graph summarizing the Sens expression phenotype of wing discs containing *ptc-Gal4* and *UAS-spen*^{RNAi}*, UAS-nito* or *UAS-spen*^{RNAi} & *UAS-nito*. All cultures were reared at 25˚C and the data is presented as in G and H. Almost all discs (96%) had a reduction or complete absence of Sens with *spen* RNAi, which was significantly reduced by co-expression of *nito*. On its own, *nito* overexpression had almost no effect on Sens expression.


A
ptc/lacZ
B
ptc/spen^RNAi, nito^RNAi-1
C
ptc/spen^RNAi, nito^RNAi-2
Wg
D
E
F
Sens
G
nito^RNAi-1
Strong
Intermediate
% discs affected
100%
80%
60%
40%
20%
0%
n=12
17%
58%
n=11
n=19
58%
37%
spen RNAi + - +
nito RNAi - + +
H
nito^RNAi-2
Strong
Intermediate
% discs affected
n=16
13%
36%
n=9
n=20
35%
60%
spen RNAi + - +
nito RNAi - + +
I
% discs affected
n=37
68%
28%
n=31
n=23
9%
35%
spen RNAi + - +
UAS-nito - + +

(Parker et al., 2002; Fig. 3.3 D). This activation is compromised in *spen* mutant clones or discs expressing $spen^{DN}$ (Lin et al., 2003), making Sens a good readout to study Spen and Nito function.

If *nito* has a redundant role with *spen* in Wg-induced activation of Sens, then knockdown of *nito* should enhance the effect of *spen* RNAi. To drive the expression of *UAS-spen* and/or *nito* RNA hairpins, *patched* (*ptc*)-*Gal4* was used, which is active in a stripe on the anterior side of the anterior/posterior border of the wing imaginal disc (Johnston et al. 1995). To control for off-target effects with *nito* RNAi, two different *UAS-*$nito^{RNAi}$ constructs were examined ($nito^{RNAi}$*-1* and $nito^{RNAi}$*-2*) which target distinct portions of the *nito* mRNA (Fig. 3.1 A and Materials and Methods). In addition, the cold sensitivity of Gal4 (Phelps and Brand, 1993) was used to modulate the strength of expression.

When these RNAi experiments were performed at 22°C (where Gal4 has intermediate activity), 62% of the *ptc-*$spen^{RNAi}$ wing discs had a reduction (47%) or complete loss (15%) of Sens expression in the *ptc* domain (Fig. 3.3 G, H). Knockdown of *nito* alone had no effect on Sens expression (Fig. 3.3 G, H). This was true even at 25˚C or 29˚C where Gal4 is more active (data not shown). However, simultaneous depletion of *spen* and *nito* caused a significant decrease in Sens expression in almost every (95%) disc (Fig. 3.3 G, H). In addition, the frequency of complete loss of Sens is higher (47%) than with *spen* RNAi. Similar results were obtained with both *nito* RNA hairpins. In addition, none of these manipulations had a detectable reduction on Wg expression; rather a slight increase in Wg protein was often observed (arrowheads in Fig.

3.3 B, C). These data indicate that *spen* and *nito* are required for Wg activation of Sens expression in the developing wing.

To test whether the increased severity of the *spen*, *nito* double knockdowns was due to functional redundancy, the ability of exogenously expressed *nito* to rescue the *spen* RNAi phenotype was tested. At 25˚C, *ptc-spen*^{RNAi} wing discs had a stronger defect in Sens expression than observed at 22˚C (compare Fig. 3.3 I with Fig. 3.3 G, H). Expression of *nito* had no significant effect on Sens expression on its own but was able to dramatically reduce the effect of *spen* knockdown (Fig. 3.3 I). These results support the view that Nito performs the same biochemical function as Spen in promoting Wg signaling in the wing imaginal disc.

Spen and Nito act redundantly in Wg signaling in Kc cells

To extend our studies of the role of Spen and Nito in the Wg pathway, their relationship in Kc cells was explored. These *Drosophila* cells respond to Wg conditioned media (WCM) by stabilizing Arm and inducing transcript levels of target genes (Fang et al., 2006). Two of these transcriptional targets, *nkd* and *CG6234*, have been shown to be directly activated by TCF/Arm (Fang et al., 2006; Li et al., 2007). In this report, the Wg pathway is activated either by *Axin* RNAi (e.g., Fig. 3.4 A, B), a component of the Arm destruction complex (Kikuchi et al., 2006) or stimulation with WCM (Fig. 3.4 C, D).

Activation of *nkd* and *CG6234* expression by *Axin* knockdown requires *spen* and *nito*. The greatest block in activation occurred when both *spen* and *nito* were inhibited by RNAi (Fig. 3.4 A, B). *spen and nito* were also required for the activation of *nkd* and

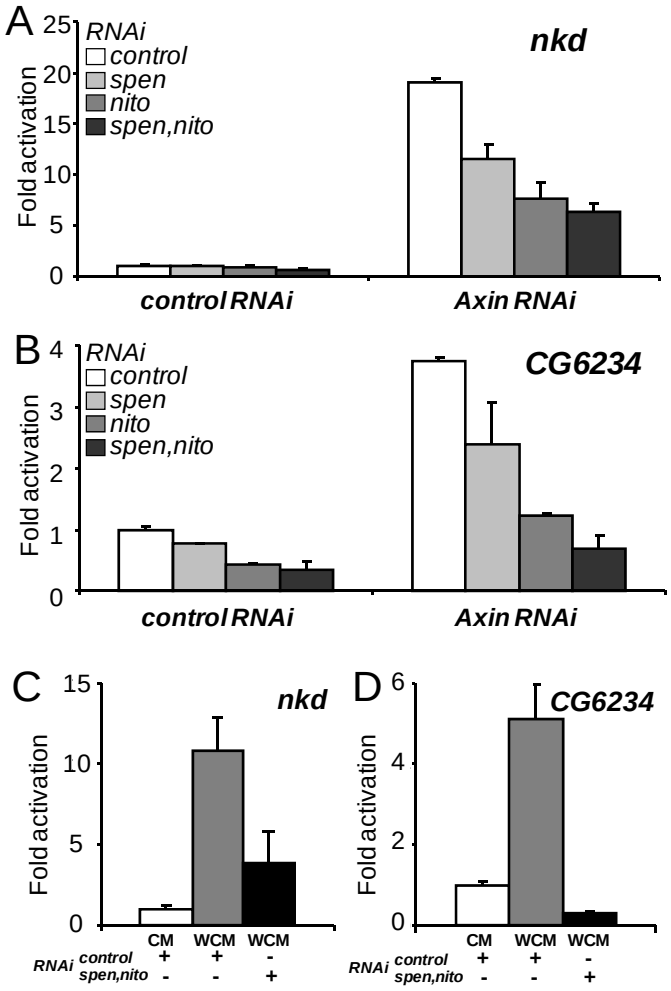


Fig. 3.4. *spen* and *nito* act redundantly to activate the expression of Wg target genes in Kc cells. (A, B) Kc cells were treated with *control* dsRNA or duplexes corresponding to *Axin, spen* and/or *nito* for 6 days. The total amount of total RNAi treated was normalized by *control* RNAi. Transcript levels of *nkd* and *CG6234* were measured by qRT-PCR, which was normalized to the expression of *β-tubulin56D*. As shown in (A), *nkd* induction by depletion of *Axin* was severely impaired by *spen*, *nito* RNAi, while individual RNAi treatments showed a smaller loss of activation. Similar results were obtained with expression of *CG6234* (B). (C) Wg signaling was activated by the addition of conditioned media containing active Wg protein (WCM). After 6 days of treatment with *control* or *spen*, *nito* dsRNAs, WCM or control media (CM) was added to the cells for 5 hours before processing for qRT-PCR. The inductions of *nkd* and *CG6234* by WCM treatment were severely reduced by *spen*, *nito* RNAi. All data in the graphs are the means of triplicate samples with standard deviations indicated by the lines. Similar results were observed in several separate experiments.

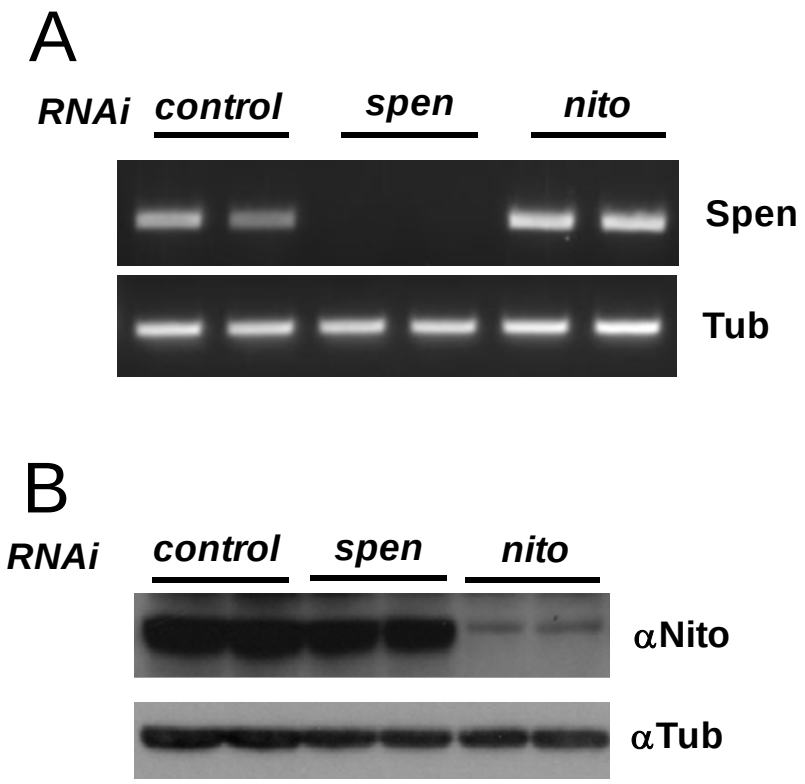


Fig. 3.5. dsRNAs for *spen* and *nito* are efficient for depletion and do not show cross reactivity. (A) RT-PCR measurement of *spen* and a *β-Tub56D* expression (the loading control) from Kc cells treated for 6 days with dsRNA corresponding to *spen* or *nito* (*nito*^{RNAi}*-4*). *spen* dsRNA dramatically reduces *spen* transcripts but *nito* dsRNA does not. (B) Western blots probed for Nito or Tubulin from Kc cells treated with dsRNAs as described for A. Nito expression is efficiently reduced by *nito* dsRNA but not by *spen* dsRNA.

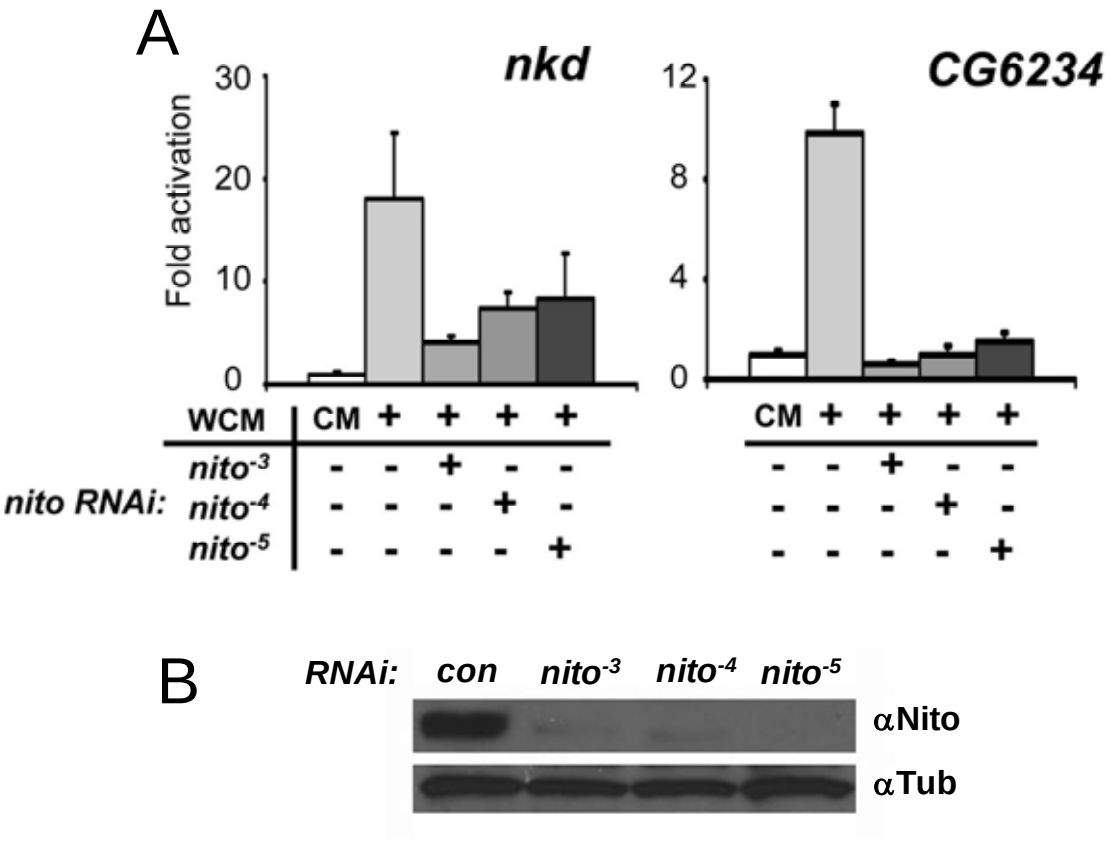


Fig. 3.6. Several dsRNAs corresponding to different parts of the *nito* mRNA reduce Wg activation of transcriptional targets.
(A) Kc cells were treated with the distinct *nito* dsRNAs (see Fig. 1A for the appropriate region of each duplex) for 6 days prior to a 5 hr treatment with CM or WCM. Cells were then harvested and *nkd* and *CG6234* transcript levels were determined by qRT-PCR. All three dsRNAs had a strong effect on Wg signaling. (B) Western blot showing that all three dsRNAs efficiently depleted Nito protein.

CG6234 by WCM (Fig. 3.4 C, D). The dsRNAs for *spen* and *nito* efficiently knocked down the corresponding gene, but displayed no cross-reactivity for the other, e.g., *nito* RNAi had no effect on *spen* expression (Fig. 3.5). dsRNAs corresponding to three distinct regions of *nito* were able to inhibit WCM activation of *nkd* and *CG6234* (Fig. 3.6; data not shown). *Axin* transcripts were equally depleted with *Axin* RNAi alone and *Axin*, *spen*, and *nito* RNAi (data not shown), and Arm levels were equally high in both treatments (Fig. 3.7 A). This indicates that the use of multiple dsRNAs did not dilute the efficiency of *Axin* knockdown. Activation of *CG6234* by *Axin* RNAi or WCM was more sensitive to *spen*, *nito* RNAi than activation of *nkd* (Fig. 3.4 and Table I).

To extend these studies to other Wg targets, three other Wg responsive genes identified in a microarray screen (T. Blauwkamp and K. Cadigan, unpublished) were also tested for a *spen*, *nito* requirement. Two of the three, (*fz3* and *crumbs*) have a greatly reduced activation by *Axin* RNAi in *spen*, *nito* depleted cells, while a third, (*hth*), is largely independent of these factors (Table I). Three genes not regulated by Wg signaling, *mkp3*, *TCF* and *arm* are not significantly affected by *spen*, *nito* knockdown (Table I). These results suggest that Spen and Nito are needed for many, but not all, Wg targets to respond to pathway activation.

Spen and Nito act downstream of the TCF and Arm complex on target gene chromatin

Where in the Wg pathway do Spen and Nito act? One possibility is at the level of Arm stabilization or nuclear translocation. *Axin* RNAi causes a dramatic increase in both cytoplasmic and nuclear Arm (Fig. 3.7 A). However, these increases also occur when

Table 3.1. *spen* and *nito* are required for the maximal activation of Wg targets in Kc cells

Gene group	Gene name	Fold activation (± S.D.)[1]	
		RNAi	
		Axin	*Axin,spen,nito*
Wg-targets	*nkd*	53.9 (± 11.9)	23.2 (± 4.4)
	CG6234	4.7 (±0.05)	0.3 (± 0.04)
	Fz3	21.8 (± 3.1)	6.2 (± 0.4)
	crumbs	26.4 (± 3.0)	8.7 (± 1.7)
	hth	22 (± 1.1)	18.1 (± 1.1)
Non Wg-targets	*mkp3*	1.1 (± 0.07)	1.6 (± 0.3)
	TCF	1.1 (± 0.2)	1.8 (± 0.4)
	Arm	1.0 (± 0.06)	1.1 (± 0.2)

Kc cells were treated with dsRNA and transcript levels measured as described in Fig. 5 are expressed as fold activation over control RNAi. Four targets (*nkd, CG6234, fz3,* and *crumbs*) showed severe reduction in their activation by Wg signaling when *spen* and *nito* (*nito*^{RNAi}*-4*) are depleted (ranging from 57% to 94% reduced activities compared to *Axin* RNAi only condition). In contrast, *hth* activation appears to be largely independent of *spen*/*nito*. Three genes that are not regulated by Wg signaling show no change in expression upon *spen*, *nito* RNAi treatment.

Axin, *spen* and *nito* are depleted (Fig. 3.7 A). The levels and subcellular location of TCF, which is predominately nuclear in Kc cells, are not affected by depletion of *Axin*, *spen* and *nito* or all three genes simultaneously (Fig. 3.7 A). These data argue that Spen and Nito are not required for Wg pathway-dependent nuclear accumulation of Arm or the nuclear localization of TCF. In addition, Nito is found almost exclusively in the nucleus in control or *Axin* RNAi treated cells (Fig. 3.7 A), consistent with a nuclear function for the protein.

TCF is thought to bind to specific DNA sequences in the WREs of Wg targets, where it recruits Arm in response to pathway activation (Städeli et al., 2006; Parker et al., 2007). We have previously identified a WRE in the first intron of the *nkd* gene that contains functionally important TCF sites (Li et al., 2007). In addition, Chromatin Immunoprecipitation (ChIP) demonstrated that this region of the *nkd* gene is bound by TCF in the absence or presence of WCM treatment (Fang et al., 2006; Li et al., 2007). Arm is also bound to the same region upon WCM stimulation (Li et al., 2007). As previously found, TCF is preferentially bound to the area containing the intronic WRE compared with the *nkd* ORF (Fig. 3.7 B). TCF binding is slightly increased in *Axin* depleted cells, and additional knockdown of *spen* and *nito* had no effect on TCF binding (Fig. 3.7 B). In contrast to TCF, Arm is only preferentially bound to the *nkd* WRE upon *Axin* RNAi (Fig. 3.7 C). This 10-20-fold increase in Arm binding to the WRE was still observed in *spen*, *nito* depleted cells (Fig. 3.7 C).

The data described above suggest that Spen and Nito are required downstream of TCF-Arm complex formation on WREs. To test whether Nito was directly associated with the *nkd* WRE, ChIP was performed with α-Nito antisera. No preferential binding of

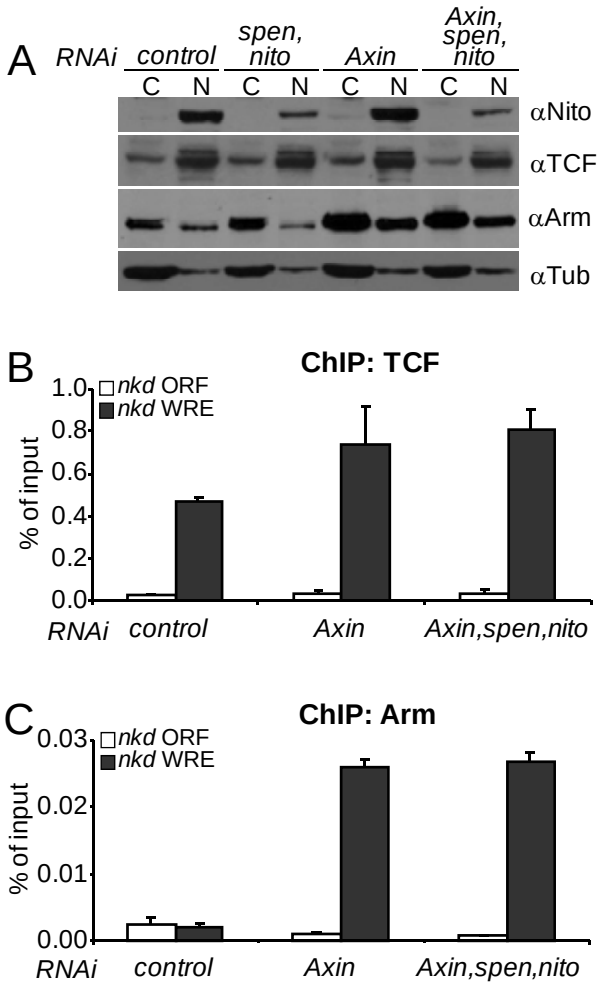


Fig. 3.7. Arm and TCF are still recruited to a Wg target gene in *spen*, *nito* depleted cells. (A) Western analysis of the cytoplasmic (marked by C) and nuclear (N) fractions for Nito, TCF and Arm protein from cells where *Axin*, *spen-nito* (*nito*^{RNAi}*-4*) or all three genes were knocked down. *Axin* RNAi caused an increase of Arm protein in both fractions, but had no effect on TCF or Nito levels or nuclear localization. Depletion of *spen* and *nito* (with or without *Axin* RNAi) had no obvious change in the Arm or TCF profile. (B, C) TCF and Arm binding to the ORF and intronic WRE in the *nkd* locus were monitored. The amount of DNA pulled down by α-TCF or α-Arm was monitored by qPCR and the data are expressed as the percent of input DNA. (B) Enriched binding of TCF to the WRE was observed in the controls or cells where Wg signaling was activated by *Axin* RNAi. (C) A dramatic increase in Arm binding to the WRE was observed upon *Axin* RNAi. Binding of TCF or Arm to the WRE was not affected by *spen*, *nito* RNAi. The bars represent the mean of triplicate ChIP samples, with standard deviations indicated by the lines. Similar results were observed in several separate experiments.

Nito to the *nkd* intronic WRE was observed (data not shown). However, the lack of binding may be due to the quality of the antibody as we do not have a positive control. We were also unsuccessful in demonstrating an association between Nito and Arm or TCF using co-immunoprecipitation (data not shown). While there are technical caveats with these negative results, they raise the possibility that Nito does not act directly in the transcriptional activation of Wg targets.

The Spen-Nito requirement for Wg signaling does not require Yan or Su(H), but may involve Gro

While Spen has been implicated in many processes in flies (Chen and Rebay, 2000; Kuang et al., 2000; Lane et al., 2000; Mace et al., 2005; Wiellette et al., 1999), the most mechanistic description to date is in the developing eye, where Spen antagonizes Notch signaling and promotes EGFR signaling (Doroquez et al., 2007). This report also found that loss of *spen* resulted in increased levels of the Ets-domain transcriptional repressor Yan. This raises the possibility that an increase in Yan expression accounts for the loss of Wg signaling in cells depleted of *spen* and *nito*. However, reduction of *yan* by RNAi did not reverse the loss in activation of the Wg targets *nkd* and *CG6234* in *Axin*, *spen*, *nito*-depleted Kc cells (Fig. 3.8 A, B). *yan* transcript levels were reduced more than 10-fold by RNAi in these experiments (data not shown). In addition, expression of Yan had no effect on Sens expression in wing discs (Fig. 3.8 D; data not shown). Therefore, it does not appear that the levels of Yan influence Wg signaling, indicating that Spen repression of Yan cannot account for the positive role of Spen and Nito in the pathway.

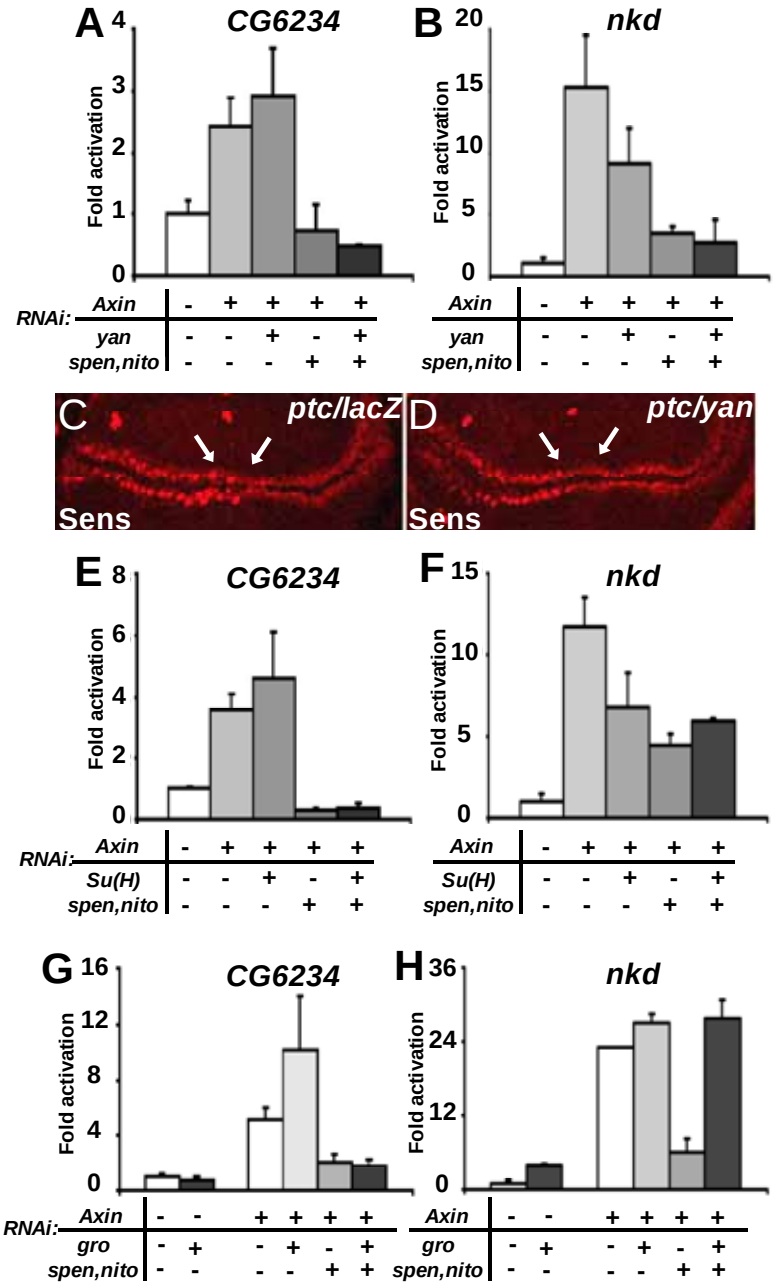


Fig. 3.8. Spen and Nito promote Wg signaling independently of Yan and Su(H), but may act in part through Gro.
(A, B) Kc cells were depleted of *Axin*, *spen*, *nito* (*nito*^{RNAi}*-4*) and *yan* as indicated for 6 days before *CG6234* (A) and *nkd* (B) transcripts were determined as described in Fig. 4. Inhibition of *yan* did not rescue the lack of activation of the Wg targets in *spen*, *nito*-depleted cells. (C, D) Confocal images of wing imaginal discs from animals containing P[*ptc-Gal4*] and P[*UAS-lacZ*] (C) or P[*UAS-yan*] (D) stained for Sens. Discs expressing

Yan showed no noticeable reduction of Sens compared to wild type cells (n = 21). The arrows indicate the area where *ptc-Gal4* is active. (E – H) Kc cells depleted of *Axin*, *spen*, *nito* and either *Su(H)* (E, F) or *gro* (G, H) and processed as described for panels A and B. Inhibition of *Su(H)* did not rescue the Wg signaling defect observed in *spen*, *nito* double knockdowns. The same is true for *CG6234* expression when *gro* is depleted (G). However, *gro* RNAi did rescue the block in *nkd* activation by Wg signaling in *spen*, *nito* depleted cells (H). All data in the graphs are the means of triplicate samples with standard deviations indicated by the lines. Similar results were observed in several separate experiments.

In mammalian cell culture, the Spen homolog MINT binds to RBP-Jκ and suppresses Notch signaling (Oswald et al., 2002; Kuroda et al., 2003; Oswald et al., 2005; Tsuji et al., 2007; Yabe et al., 2007). This fits with the data in the fly eye (Doroquez et al., 2007) and raises the possibility that loss of *spen* and *nito* results in hyperactivation of Su(H), the fly ortholog of RBP-Jκ. This was tested in Kc cells as described above for Yan. When *Su(H)* transcripts were reduced more than 10-fold by RNAi, no rescue of Wg signaling was observed in *spen*, *nito*-depleted cells (Fig. 3.8 E, F). This suggests that Spen and Nito do not act on the Wg pathway through Su(H).

One aspect of activation of Wg targets by Arm is displacement of the co-repressor Gro from TCF. To test whether Gro plays a role in Spen-Nito function in Wg signaling, the effect of *gro* depletion was examined in Kc cells. *gro* RNAi caused a 3-4 fold increase in *nkd* expression in the absence of signaling (Fig. 3.8 H; see also Fang et al., 2006) and a mild increase in *CG6234* expression in *Axin*-depleted cells (Fig. 3.8 G). Inhibition of *gro* did not rescue the loss of Wg activation of *CG6234* in *spen*, *nito*-depleted cells (Fig. 3.8 G) but did reverse the defect for *nkd* activation by the pathway (Fig. 3.8 H). This suggests that Spen and Nito may influence Wg signaling in part by antagonizing Gro-mediated repression of some Wg targets (see below for further discussion).

Discussion

Nito functions redundantly with Spen in Wg signaling and apoptosis.

In *Drosophila*, Spen and Nito are the only two proteins containing both RRMs and a SPOC domain. However, the sequence similarity of these domains is low (Fig. 3.1 A) and they are unrelated outside of these motifs. Spen and Nito show higher conservation to their respective orthologs in humans than to each other (Fig. 3.1 A), suggesting that they evolved functional specificity after a duplication event (Jemc and Rebay, 2006). Consistent with this, Spen and Nito have been shown to act antagonistically in the developing fly eye. Overexpression of *nito* disrupted eye development, and this phenotype was enhanced by a reduction in *spen* gene activity. Conversely, a small eye phenotype caused by overexpression of $spen^{DN}$ was enhanced with *nito* overexpression and suppressed with *nito* RNAi (Jemc and Rebay, 2006). As Spen has been shown to regulate Notch and EGFR signaling pathways in the eye (Doroquez et al., 2007), it may be that Spen and Nito have opposing functions in these pathways.

In our study we provide strong evidence for functional redundancy between *spen* and *nito* in the context of Wg signaling. Our single and double RNAi analysis indicated that both genes positively regulate the pathway in the fly eye (Fig. 3.2 A-J), the wing imaginal disc (Fig. 3.3) and in Kc cells (Fig. 3.4 and Table I). Expression of *nito* can rescue the *spen* RNAi phenotype in the wing disc (Fig. 3.3 I), strongly suggesting that Nito and Spen perform a similar biochemical function in the Wg pathway. There is a report concluding that loss of *spen* has no role in Wg signaling in the developing wing (Mace and Tugores, 2004). While the discrepancy may be due to the use of different Wg

signaling readouts, it is likely that redundancy with *nito* can explain the negative results obtained with *spen* mutant clones in that study.

In humans, there is evidence that the *spen* and *nito* homologs possess both similar and distinct functional activities in the Notch pathway. SHARP can repress Notch signaling through interaction with RBP-Jκ, acting as a transcriptional corepressor (Oswald et al., 2002; Oswald et al., 2005). OTT1 can also bind to RBP-Jκ, but this interaction can lead to either repression or activation of a Notch/RBP-Jκ reporter gene, depending on the cell line used (Ma et al., 2007). Taken together with our data and those of Jemc and Rebay (2006), these data suggest that Spen and Nito share some biochemical activities but also have distinct, antagonistic properties, depending on the molecular context.

In addition to acting redundantly in Wg signaling, we found that *spen* and *nito* are required for the activity of pro-apoptotic factors Hid and Rpr in the fly eye (Fig. 3.2 K-O; data not shown). The fact that the suppression was greatest when both *spen* and *nito* were inhibited suggests that they act redundantly, though this requires further study and the molecular relationship between Spen, Nito and apoptosis is not clear.

The mechanism of Spen-Nito action in Wg-mediated transcriptional activation

Previous work from our lab (Lin et al., 2003) and this study (Fig. 3.2 F-J) demonstrate that *spen* and *nito* are required for Arm* to activate Wg signaling, suggesting that they act downstream of Arm stabilization. In Kc cells, *spen*, *nito* depletion did not effect the formation of a TCF-Arm complex on the *nkd* intronic WRE (Fig. 3.7), even though they are required for activation of *nkd* expression by Wg

signaling (Fig. 3.4; Table I; Fig. 3.6). Taken together, these data indicate that Spen and Nito act downstream or in parallel of TCF and Arm to promote transcriptional activation of Wg targets.

Our results are consistent with those recently reported for SHARP in human cells (Feng et al., 2007). SHARP was required for maximal activation of Wnt transcriptional targets and reporter constructs, acting downstream of β-catenin stabilization. SHARP could potentiate the ability of Lef1 to activate transcription, independently of Lef1's ability to bind to β-catenin (Feng et al., 2007). Whether there is a direct biochemical interaction between SHARP and Lef1 is not known.

Interestingly, *SHARP* expression was elevated in several types of carcinomas with constitutively active Wnt/β-catenin signaling, suggesting that it is part of a positive feedback loop regulating the pathway (Feng et al., 2007). This circuit does not appear to be present in flies, where *spen* and *nito* are ubiquitously expressed (Fig. 3.1 C; Wiellette et al., 1999; Lin et al., 2003).

Consistent with a role in transcription, Spen and Nito contain domains that could be involved in DNA-binding. The murine Spen counterpart Mint has been shown to bind to a G/T-rich element in the FGF-responsive minimal enhancer of the *OC* promoter via its RRMs (Newberry et al., 1999; Sierra et al., 2004). While the recognition site is not well defined, it is interesting to note that there are several G/T rich regions near some of the functional TCF sites in *nkd* intronic WRE. We are exploring whether the RRMs of Nito or Spen can recognize these sequences.

While Spen and Nito may play a direct positive role in the activation of transcriptional targets by Wg signaling, it is also possible that the functional requirement

is indirect. The fact that loss of *spen* elevates Notch signaling in the fly eye (Doroquez et al., 2007) fits with the vertebrate data showing that SHARP, Mint and OTT1 can associate with RBP-Jκ and inhibit its ability to activate Notch target genes (Oswald et al., 2002; Kuroda et al., 2003; Oswald et al., 2005; Ma et al., 2007). However, RNAi inhibition of the fly RBP-Jk homolog Su(H) did not restore activation of Wg targets to *spen*, *nito* depleted cells (Fig. 3.8 E, F). This suggests that activation of Notch signaling is not the mechanism by which Spen and Nito promote Wg signaling.

Upstream of Su(H), Notch signaling has been reported to repress Wg signaling in flies and fly cell culture (Lawrence et al., 2001; Hayward et al., 2005; Hayward et al., 2006). However, this cross-talk has been shown to occur at the level of Arm stabilization (Hayward et al., 2005; Hayward et al., 2006). Since Spen and Nito do not effect Arm protein levels (Fig. 3.7 A), this mechanism appears not be involved in the promotion of Wg signaling by Spen and Nito.

Spen is also required to downregulate protein levels of the Ets-domain transcriptional repressor Yan in the eye and wing imaginal discs (Doroquez et al., 2007). Could an increase in Yan levels explain the block in Wg signaling observed when *spen* and *nito* are depleted? This does not appear to be the case, since inhibition of *yan* does not reverse the *spen*, *nito* requirement for Wg signaling in Kc cells (Fig. 3.8 A, B). In addition, overexpression of Yan in the wing imaginal disc did not affect the expression of the Wg target Sens (Fig. 3.8 D), which is highly dependent on *spen* and *nito* (Fig. 3.3).

In contrast to *Su(H)* and *yan*, depletion of *gro* did reverse the *spen*, *nito* defect in Wg activation of *nkd* (Fig. 3.8 H). Gro is a transcriptional co-repressor that is thought to bind to TCF in the absence of Wg signaling (Cavallo et al., 1998) and is known to be

important in silencing *nkd* expression (Fang et al., 2006). *In vitro*, binding of β-catenin and TLE1 or TLE2 (vertebrate homologs of Gro) to TCF are mutually exclusive (Daniels and Weis, 2005). β-catenin and TLE1 binding to WREs in cells is also exclusive (Wang and Jones, 2006). This suggests a model where Gro displacement by Arm is defective in *spen*, *nito* depleted cells, leading to reduced activation of *nkd*. However, we have found that Arm recruitment to the *nkd* WRE is not affected by *spen*, *nito* knockdown (Fig. 3.7 C). In addition to this discrepancy, Gro has no apparent role in Spen, Nito regulation of *CG6234* by the Wg pathway (Fig. 3.8 G). This suggests that Gro displacement is not the major mechanism by which Spen and Nito function in Wg signaling.

Spen and Nito are target-specific regulators of Wg signaling

One interesting aspect of Spen-Nito regulation of Wg signaling is that all targets of the pathway do not appear to require these proteins for activation. In our original report, Spen was required for Wg function in several imaginal discs, but not in embryos (Lin et al., 2003). This could be explained by redundancy with Nito, but ubiquitous knockdown of both genes throughout the embryonic epidermis caused no detectable defect in Wg signaling (data not shown). In the wing imaginal discs, *spen* and *nito* clearly are required for Sens expression (Fig. 3.3, 3.4), but two other Wg targets, *Distal-less* and *fz3* are expressed normally under the RNAi conditions used (data not shown). In Kc cells, several Wg targets required *spen* and *nito* for activation, but *hth* did not (Table I). These results have to be interpreted cautiously, because residual *spen* and/or *nito* activity may

be sufficient for regulation of some Wg targets. However, the data do support a model where Spen and Nito regulate Wg-mediated transcriptional activation in a gene-specific manner.

In mice, disruption of *Mint* or *OTT1* results in early embryonic lethality (Kuroda et al., 2003; Raffel et al., 2007). It is interesting to note that a conditional deletion of *OTT1* in the hematopoietic compartment caused a defect in pro/pre-B cell differentiation (Raffel et al., 2007). Loss of *Lef1* results in poor survival/growth of pro-B cells (Reya et al., 2000). While the phenotypes are not identical, this could be due to redundancy between *Lef1* and other *TCF*s or between *Mint* and *OTT1*. The existence of fluxed alleles of *Mint* and *OTT1* (Yabe et al., 2007; Raffel et al., 2007) should allow the relationship between these factors and the Wnt/β-catenin pathway to be more fully explored in the mouse.

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CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

PART I – IDENTIFICATION AND CHARACTERIZATION OF WINGLESS RESPONSE ELEMENTS FROM A DIRECT TARGET GENE, *NAKED CUTICLE (NKD)* IN *DROSOPHILA*

The Wnt signal travels from the cell surface to the nucleus and ultimately control downstream target genes by a direct or indirect manner. To have better understanding of how Wnts regulate transcription, the direct Wnt target *nkd* was studied using *Drosophila* as a model organism. *nkd* was chosen not only because it is an essential component of Wnt signal transduction, but also because it has uniquely high sensitivity to the pathway. The majority of direct Wnt targets are activated by the pathway only in certain cells. This cell-specific response to Wnts is possible because most Wnt targets utilize multiple regulatory sequences, each of which works only in certain cells. In contrast, *nkd,* which functions as a negative feedback Wnt inhibitor, is activated by Wnt signaling regardless of the cell type. Therefore, I hypothesized that *nkd* may have a single WRE (Wingless Response Element) which is active in all *nkd*-expressing cells. The alternative hypothesis was that *nkd* also utilizes multiple WREs, each of which is active in a tissue-specific manner.

Data from biochemical and functional assays demonstrated that *nkd* needs multiple and tissue-specific WREs to fully respond to Wnts. Recruitment of TCF proteins, an

essential transcription factor of Wnt signaling, via the ChIP assay in cultured fly cells revealed two distinct genomic regions in the *nkd* locus. When these two TCF-bound sequences, named UpE and IntE, were tested as reporter genes in cells, they exhibited high sensitivity to Wnt stimulation. Consistent with the ChIP data, several functionally important TCF sites in UpE and IntE were identified by reporter gene analysis. To test the *in vivo* function of these sequences, IntE and two derivatives of UpE (UpE1 and UpE2) were integrated into fly genome as reporter genes. UpE1 and UpE2 share 402 bp out of 868 bp in common but contain unique sequences as well. The reporter expression controlled by UpE1, UpE2 and IntE revealed three things (1) the three WREs in combination largely, but not completely, recapitulate the expression pattern of *nkd* in multiple cell types (2) the activity of the three WREs are overlapping in some cells, suggesting the redundancy between WREs, and (3) each WRE responds to the signaling only in certain cell types but not others, suggesting they are cell/tissue-specific WREs.

In the following section, some experiments will be suggested to address a few more questions of *nkd* transcription regulation.

Are TCF sites all that nkd needs? (Identification of novel *cis*-elements)

My data strongly suggest the presence of two kinds of unknown *cis*-elements. One should be the sequences that are important for determining functional TCF sites. The other are sequences that determine the cell-specific activity of WREs.

When individually mutated, 6 conserved putative TCF sites in UpE revealed different degree of contributions to the activity of WREs (Fig 2.5). Most strikingly, the mutation of TCF site 2 and 3 (which have identical sequences) showed clearly different

results (Fig. 2.4, 2.5). Disruption of TCF site 2 reduces the activity of UpE2 70% whereas disruption of TCF site 3 does not inhibit the activity of UpE2. This indicates that there is more information needed to determine which site is functional. Such information is probably present in DNA sequences as *cis*-elements. Our lab has already been pursuing this idea and found a novel 7 bp sequence that is necessary for the activity of multiple WREs. While the function of this 7 bp sequence, named the Helper site, is not entirely clear yet, it may be an extended TCF recognition sequences which increases the binding specificity and affinity of TCF. Similar sequences were also found from the study of certain isoforms of mammalian TCF1 and TCF4. The C-terminal E tail domain that is present in alternative spliced isoforms of TCF-1 and 4 can recognize RCCG (R= preferentially G/C, but A/T can be possible) which should be 0-3 bp distant from TCF sites (Atcha et al., 2003 and 2007). While our data do not entirely agree with the data from mammalian TCFs, both notions from the fly and the mammalian system consistently suggest the requirement of additional sequences in Wnt-mediated transcription.

The functional importance of sequences similar to the Helper site has been shown in WREs of known direct Wnt targets such as *notum* and *slp*. These data suggest that there may be a general requirement for Helper sequences in Wnt target activation. However, there is a WRE that does not contain sequences close to helper sequences. The CG6234 gene, whose molecular function is unknown, was identified as a direct Wnt target in fly cultured cells (Fang et al., 2006). A 539 bp CG6234 WRE contains functional TCF sites, but no sequences similar to helper sites. This suggests that different classes of helper sites may exist in *CG6234*, which remain to be discovered.

Discovery of novel *cis*-elements essential for Wnt-mediated transcription may help us to explore Wnt-mediated transcription in many ways. For instance, novel *cis*-elements may be binding sites of unknown trans-acting factors that perhaps can lead us to find new Wnt signaling components. Identifying more *cis*-elements can also greatly improve the quality of the search for Wnt targets using bioinformatics. Searches for the 7 to 11 b bp TCF consensus is not very useful for identifying new Wnt targets from the entire genome as they yield too many hits.

Assuming novel and functionally important *cis*-elements are found from WREs of *CG6234* or other direct Wnt targets through a series of mutagenesis approaches, one can try to find *trans*-acting factors that can bind these new *cis*-elements. Several screening methods can be applied. For instance, using tagged *cis*-element sequences as a bait, one can try to pull down interacting proteins from cell lysates, which can be identified by mass spectroscopy. Another method is a yeast one-hybrid screen in which proteins encoded from a fly cDNA library will be screened for their binding to cis-elements used a bait. Cell lysates for affinity purification or cDNA library for a yeast one-hybrid assay can be prepared with cells where target *cis*-elements are functionally required for the activity of WREs.

Tandem repeats of *cis*-elements alone can often activate transcription with or without Wnt stimulation, like the case for TCF sites. If novel *cis*-elements can induce transcription when multimerized, stable cell lines containing a reporter genes of *cis*-elements can be generated. Then, a screen for any factors that are required for reporter activation can be performed using a fly RNAi library. This RNAi screen can identify any transcription factors important for reporter activation. As a control, a reporter that is

activated by unrelated sequences will be used to screen out any factors regulating promoters and other parts of the plasmid backbone. Once any interesting candidates such as DNA binding proteins are found, their requirement in Wnt signaling will be tested in cells using RNAi-mediated depletion.

A second kind of novel yet undiscovered *cis*-elements could be sequences important for tissue-specific activity of WREs. In the developing notum, UpE1 is active but UpE2 is not. Two explanations for this different activity of UpEs are that (1) the 5' unique region of UpE may have sequences where notum-specific activators bind or (2) the 3' unique sequences of UpE2 may contain notum-specific inhibitor elements. If the second explanation is true, the adding UpE2 sequences to UpE1 may inhibit UpE1 activity in the notum, which is not the case (the entire UpE that has all sequences of UpE1 and 2 is still active in the notum). These data suggest that the unique sequences of UpE1 may have *cis*-elements required for Wnt responsiveness in notum cells.

In order to test this, one can start to generate a series of deletions from the 5' of UpE1, and test their activities using reporter genes in flies. Once the notum-specific region is narrowed down to a small piece, serial mutagenesis can be done to find minimal *cis*-elements. If *cis*-elements that are required for the activity of UpE2 in the notum are found, one can fuse these sequences to UpE2 and test whether these *cis*-elements are also sufficient to respond to Wnt signaling in the notum. Similar approaches can be adopted to locate embryonic epidermis-specific sequences from UpE and embryonic midgut-specific *cis*-elements from IntE.

Do *nkd* WREs act with the endogenous *nkd* promoter?

The sum of UpE and IntE patterns covers most, if not all, larval tissues that express *nkd*. However, none of the *nkd* WREs or the combined WRE showed the complete striped pattern in embryonic epidermis that is seen in endogenous *nkd*. This suggests that there is at least one more undiscovered *nkd* WRE, which is active in the embryonic epidermis. Alternatively, this would indicate that *nkd* WREs have a promoter preference, in that the heterologous *hsp70* minimal promoter in the reporter plasmid may not be fully comparable with the endogenous *nkd* promoter. One possible mechanism that supports this idea is the model of DNA looping between the regulatory sequences and the promoter. DNA looping has been proposed to explain long-distance transcription control by some enhancers like the Locus Control Region (LCR) of the β-globin gene (Bulger and Groudine, 1999). It is possible that looping between *nkd* WREs and the endogenous promoter is required for expressing *nkd* in embryos. A piece of evidence supporting this looping idea is that often TCF appears to be present on the promoter region of *nkd* judged by ChIP (Ni and Cadigan, unpublished). This TCF ChIP signal on the promoter may be seen because TCFs on WREs are recruited to the promoter via an interaction with the basal transcription machinery on the promoter. To test this idea, one can substitute the *hsp70* minimal promoter with the *nkd* endogenous promoter from the *nkd* WRE-reporter and test the activity in flies. If the *nkd* endogenous promoter does not enhance the activity of WREs in embryonic tissues, it further supports the existence of other unidentified WREs. Possible ways to locate unknown WREs are explained in the next section.

How to find other *nkd* WREs?

Our lab has found several genomic regions that contain TCF binding sites that are indistinguishable from known functional sites by sequences, but showed no response to Wnt signaling when tested in reporter assays in tissue culture. While these data may indicate that those non Wnt-responsive regions are not *nkd*-WREs, it is also possible that they may be active in certain cells, but not in a cell line used for the reporter assays. One could test at least a few of these non-responsive reporters in flies to see if they have any Wnt responsive activity in the developing fly tissues.

Alternatively, comparative genomic approaches can be employed to locate as yet unidentified *nkd*-WREs. Comparative analyses of conserved elements between species, for instance fish to mammal alignments, have been successfully used to search for regulatory sequences on a genome-wide scale (Elgar, 2001 and reference therein; Woolfe and Elgar, 2007). By adopting a similar strategy, one could compare the *nkd* locus of *Drosophila* with that of other species. Alignment of *Drosophila* sequences with those of vertebrates may be technically challenging due to a great degree of variation. Conversely, alignment of 12 *Drosophila* species that across about 50 million years of evolution (Russo et al., 1995; reviewed in Powell, 1997) was not informative, however, as it identified too many conserved sequences.

Among other invertebrate genomic sequences that are available, the mosquito genome may be useful. While there has been no obvious *nkd* homolog found in the *C. elegans* genome, the mosquito *Anopheles gambiae* genome has a putative *nkd* that has 29% identity at the amino acid sequence level with the fly *nkd* (Walrop et al., 2006). Preliminary attempts using a public online tool (via UCSC genome bioinformatics site)

to find conserved sequences between the fly and mosquito were not successful. However, more efforts are needed, perhaps by expanding searching parameters. The alignment between fish and human sequences showed that the length of conserved patches of DNA can be as small as 24 bp (Woolfe and Elgar, 2007). Other potentially informative organisms are the sea urchin and the hydra. While whether they have the *nkd* ortholog is not known, these simpler animals do have functional Wnt signaling and contain many Wnt component genes (Croce et al., 2006; Guder et al., 2006). As this comparative genomic approach can perform searches over the large genomic regions, it can be highly useful if unidentified WREs are distant from the *nkd* transcription unit.

Part II – NUCLEAR RRM PROTEINS SPLIT ENDS AND SPENITO ACT REDUNDANTLY TO PROMOTE WINGLESS SIGNALING IN A CELL-SPECIFIC MANNER IN *DROSOPHILA*

Wnt/TCF/β-catenin signaling displays manifold roles in a huge variety of physiological and disease conditions. Such pleiotropy of Wnt activity is mainly achieved by regulating specific target genes in a context-dependent manner. Previously, a fly nuclear protein, Split ends (Spen), has been shown to promote Wnt signaling in a cell- and gene-specific manner. In this thesis, another fly gene encoding Spenito (Nito), a predicted redundant partner of Spen, was studied. Spen and Nito are the only two fly genes containing three common domains found in all other Spen family proteins; 3 repeats of RRM (RNA recognition motif), a NLS (Nuclear localization signal), and a conserved C-terminal SPOC domain (Fig. 3.1). Consistent with this sequence similarity, Spen and Nito act redundantly to activate Wnt signaling in cultured fly cells and fly tissues. Depletion of Nito by RNA interference enhanced Wnt defects caused by depletion of Spen in fly larval tissues and cultured cells (Fig. 3.2, 3.3, 3.4). Conversely misexpression of Nito can rescue the phenotype caused by the reduced Spen expression, strongly suggesting their redundancy in Wnt signal transduction (Fig. 3.4). Interestingly, the activity of Spen and Nito is required for Wnt signaling from fly cells and larval tissues, but not from embryos (Fig. 3.3, 3.4, data not shown). This suggests that Spen and Nito have tissue specific roles in Wnt signaling. Moreover, in fly cells and larval tissues, only a subset of Wnt targets requires Spen and Nito to be activated by the Wnt signal (Table, 3.1, data not shown). Collectively, these data strongly suggest that Spen and Nito act redundantly to promote Wnt signaling in a cell- and gene-specific manner.

While several attempts to show the direct action of Spen and Nito in the pathway have not been successful, I found that Spen and Nito activity on *nkd* is dependent on Gro (Fig. 3.8), which is a known transcriptional repressor of some Wnt targets (Fang et al., 2007). Thus a current model is that Spen and Nito may play roles in inhibition of Gro activity. In the following section, Gro and the known mechanisms of Gro-mediated repression are briefly reviewed. Then, several experiments to test the possible roles of Spen and Nito in the regulation of Gro and/or HDAC, which can associate with Gro and Spen proteins, will be discussed.

Gro inhibits transcription via at least two mechanisms and its activity can be regulated by phosphorylation

Gro proteins that lack intrinsic DNA-binding activity can be recruited to the chromatin via interaction with a variety of DNA-binding proteins. Gro proteins are well conserved from yeast to humans and can act as important regulators of several signaling mechanisms, including the Wnt pathway. Gro represses transcription via at least two possibly coordinated processes involving inhibition of the RNA polymerase II complex and chromatin remodeling (Fig. 4.1; reviewed in Buscarlet and Stifani, 2007).

The first so-called "Gro repressosome" model suggests that Gro present on the regulatory sequences can interacts with the basal transcriptional machinery and the mediator complex (reviewed in Chen and Courey, 2000; Courey and Jia, 2001). These associations may inhibit transcription by interfering with interactions between transcriptional activators and the transcriptional machinery/mediator complex. This

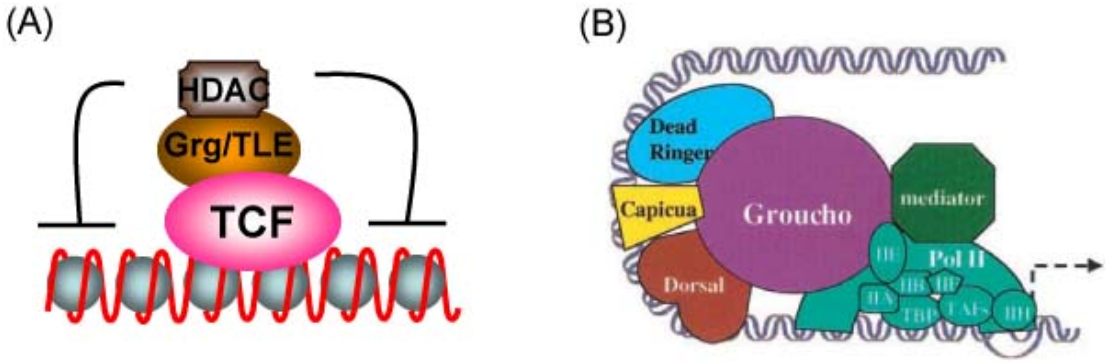

Fig. 4.1. Schematic diagrams of two possible mechanisms of transcriptional repression by Gro proteins. (A) Gro with HDAC and other components of chromatin remodeling complexes are believed to repress transcription through modification of the histone acetylation and/or through other chromatin-remodeling mechanisms. The docking of Gro to the chromatin should be done via DNA-binding proteins like TCF in this case. (B) The Groucho repressosome model. For the repression of *zen* in the ventral side of *Drosophila* embryos, DNA-binding proteins including Dorsal and Dead cooperatively recruit Gro, which is believed to block the formation or function of the basal transcription complex by interacting with components of the mediator (reproduced from Courey and Jia, 2001).

model is largely based on the data from Tup1, a yeast homolog of Gro (Gromoller and Lehming 2000). Srb7 protein, a subunit of the basal transcription machinery, can interact with Tup1 *in vivo* and a mutation of Srb7 that disrupts its interaction with Tup1 resulted in derepression of targets repressed by Tup1. More interestingly, Tup1 can compete with Med6, a component of the mediator complex that is required for the transcription, in binding Srb7. It has therefore been proposed that Gro can block transcription via preventing the association between components in various necessary transcription complexes. In *Drosophila,* a similar model has been proposed for the repression of genes like *zen* and *dpp* during early embryogenesis as seen in Fig. 4.1. In this presumptive repressosome, Gro-dependent transcriptional repressors such as Dorsal and Dead ringer may recruit Gro to repress target genes (Rey et al., 1991; Valentine et al., 1998).

The second model suggests that Gro can repress transcription either through modification of the acetylation of histone tails or through other chromatin-remodeling mechanisms (reviewed in Chen and Courey, 2000; Courey and Jia, 2001). In *Drosophila*, Gro has been shown to be in a complex with histone deacetylase Rpd3 and histones (Chen et al., 1999). Consistent with Rpd3 being a functional binding partner, overexpression of Rpd3 can promote Gro-mediated transcription repression (Chen et al., 1999). Moreover, HDAC-defective Rpd3 can no longer enhance the ability of Gro to repress transcription, suggesting that the activity of HDAC is important for Gro-mediated transcriptional repression (Chen et al., 1999).

There are three classes of phosphorylation of Gro that are known to regulate the Gro activity. The first class of phosphorylation by CK2 (casein kinase II) can activate

Gro. Nuthall and other (2002a) showed that CK2 can promote Gro-mediated transcription repression in mammalian cells, and inhibition of CK2 activity relieved this transcription repression. CK2 was shown to phosphorylate Gro at Ser239 (Nuthall et al., 2004). Mutation of this Ser to Ala made Gro less repressive and decreased Gro localization to chromatin. Taken together, these data suggest that CK2-mediated phosphorylation is required for the Gro activity, at least in part, by promoting Gro binding to chromatin where it can repress the transcription.

The second class of phosphorylation of Gro is by Cdc2, a Cell-cycle dependent kinase at the G2/M transition stage. This phosphorylation, however, inhibits Gro-mediated repression. Inhibition of Cdc2 activity or deletion of the Cdc2-phosphorylation domain from Gro enhanced the Gro-mediated reporter repression and increased the chromatin-bound pool of Gro (Nuthall et al., 2002b). These data suggest that phosphorylation by Cdc2 inhibits Gro recruitment to chromatin, thus alleviating the transcription repression.

The third class is also an inhibitory phosphorylation of Gro. A *Drosophila* homolog of homeodomain-interacting protein kinase 2(HIPK2) can phosphorylate Gro at separate sites from the CK2- or Cdc2-modification sites. As in the case of Cdc2, HIPK2 overexpression relieves Gro-mediated reporter repression and decreases the interaction between Gro and its DNA docking protein (e. g. Eyeless, Choi et al., 2005). In addition, phosphorylation of Gro by HIPK2 decreased the association between Gro and other corepressors, which may promote the removal of Gro from target sites (Choi et al., 2005).

In summary, Gro can be phosphorylated by three different kinases. Furthermore, each mechanism of Gro regulation correlates with Gro sub-nuclear localization. When

its repression ability is promoted by CK2-mediated phosphorylation, Gro binding to chromatin increases. Cdc2 and HIPK2-mediated phosphorylation make Gro less potent in transcriptional repression and less "sticky" to the chromatin. This may indicate that phosphorylation is an efficient way to regulate Gro activity.

Given the genetic interaction between Spen family protein and Gro during the regulation of *nkd*, the following specific aims may be worth testing.

(1) Can Spen and Nito regulate the expression, subcellular localization or recruitment of Gro to WREs?

(2) Is the role of Spen family proteins the inhibition of HDAC activity?

(3) Can Spen and Nito influence the activity of the Gro repressosome?

Is Gro regulated by Spen and Nito?

My data may indicate that the role of Spen and Nito in *nkd* activation is inhibition of Gro activity. The simplest way to regulate the activity of Gro may be regulating the level of the protein. To test whether Spen family proteins can reduce the level of Gro protein, one could monitor Gro level via western blot assay in the absence or the presence of Spen and Nito. If the Gro protein level is affected by Spen family depletion, Gro mRNA level and Gro protein stability may be further tested to find at which steps Spen and Nito regulate the Gro protein level.

It is also conceivable that depletion of Spen and Nito may reinforce Gro binding to chromatin, possibly via regulating Gro's phosphorylation. To test this, the sub-nuclear localization of Gro will be tested as done previously (Nuthall et al., 2002a, 2002b, 2004). In this assay, the free nuclear pool of Gro versus Gro bound on chromatin can be

separated via a commonly used nuclear fractionation method. Alternatively, Gro recruitment to *nkd* WREs may be tested from fly cells via ChIP using Gro antibody. If Gro ChIP is technically challenging in fly cells, Gro/TLE ChIP can be done in mammalian cells on *c-MYC* WRE as done previously (Sierra et al., 2006). In this case, depletion of mammalian Spen family protein should be done.

If there is any hint that Gro attachment to chromatin can be regulated by Spen and Nito, Gro phosphorylation can be further monitored. It appears that only a pan-TLE antibody available that recognizes multiple phospho-Gro without distinction (Nuthall et al., 2002, 2004). Thus one may need to use Mass-spectrometry or related methods to characterize the Gro structure.

In addition, one can test whether Gro and Spen protein can physically interact, which is consistent with their genetic interaction. Common biochemical binding assays such as Co-IP with or without overexpression of Gro and Nito (as Spen is very large, Nito is practically favorable for cloning and overexpression) can be tried.

Is HDAC activity the target of Spen and Nito action?

Alternatively, Spen and Nito may regulate the activity of HDAC with or without affecting Gro on WREs. This is particularly interesting because a mammalian Spen has also been shown to interact with HDAC (Shi et al., 2001). First, one can test whether Spen and Nito activity is dependent on HDAC activity. If this is the case, Spen and Nito depletion may not reduce *nkd* activation by Wnts from cells that are treated with HDAC inhibitors such as benzamide or hydroxamate (Beckers et al., 2007). As ChIP assays with a HDAC-specific antibody and acetylated histone 3 and 4 antibodies can be done

(Sierra et al., 2006, Parker et al., 2008), one can monitor the HDAC level and the histone acetylation on WREs from fly or mammalian cells with or without Spen proteins. If HDAC appears to be a target of Spen and Nito action, one can test whether the ability of Spen proteins to interact with HDAC is essential for their role. For that, Spen and Nito will be depleted via RNAi from fly cells, and wild type or mutant Nito lacking a HDAC-binding, SPOC domain (Shi et al., 2001) will be transfected. If wild type can rescue *nkd* expression upon Wnt signaling but mutant cannot, this may indicate that Spen proteins can regulate the activity of HDAC via its 3' conserved SPOC domain.

Can Spen and Nito influence the activity of the Gro repressosome?

Gro can repress transcription by being a member of repressosome which inhibits the assembly of transcription machineries. While known members of Gro repressosome are limited and whether they are required for Wnt-mediated transcription is not known, one can perform some preliminary experiments. For instance, whether the Gro-dependent repressors Dorsal and Death ringer are required for *nkd* regulation can be tested via RNAi-mediated loss of function assays in cells. Alternatively, similar experiment can be done in flies depending on the availability of reagents. If Dorsal or Death ringer proteins plays role in *nkd* expression, presumably in repression, one can monitor whether their recruitment to *nkd* WREs are regulated by Spen proteins. Also the specific binding sequences of Dorsal which is $GGG(W)_nCCM$ (where W = A or T, M = C or A, and *n* corresponds to either four or five W residues, Markstein et al, 2002) and/or of Death ringer which is TATTGAT (Valentine et al., 1998) can be searched in the *nkd* locus and their functional importance may be tested in reporters.

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