The Wntome: A genome-wide analysis of kinases and phosphatases that regulate Wnt signaling

by

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Abstract

Evolutionarily conserved cell signaling pathways regulate diverse and indispensable processes during metazoan development. A precise control of both the state and threshold of signaling is required for normal development to occur. Cells predominantly utilize the kinase- and phosphatase-mediated reversible phosphorylation of proteins to control signaling.

The canonical Wnt signaling pathway has homologous roles during the processes of axis polarization and stem cell maintenance in diverse metazoans. Wnt signaling is regulated through reversible phosphorylation both in its silent and active state. We have identified and characterized the function of *Drosophila* Hipk, a novel kinase component of the pathway. Genetic and biochemical analyses suggest that Hipk stabilizes the pathway effector Armadillo in a process that is dependent on its catalytic activity. Additionally, Hipk acts to promote pathway activity independent of its effect on Armadillo stability. We find that Hipk2 has a functionally conserved role to regulate Wnt signaling in mammalian cells.

Hipk and a limited number of other kinases (and phosphatases) have thus far been implicated in the regulation of Wnt signaling. We have performed an *in vivo* loss-of-function RNAi screen to identify additional enzymes that regulate this pathway through reversible phosphorylation in *Drosophila*. Our analyses have identified novel pathway components at all levels of the Wnt signaling relay.

Keywords: Wnt; Wingless; Hipk; β-catenin; Armadillo; β-TrCP

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Table of Contents

List of Figures

List of Acronyms

TGF-β	Transforming Growth Factor-β
SPRE	Signaling Pathway Response Element
Wg	Wingless
En	Engrailed
Hh	Hedgehog
Ser	Serrate
EGF	Epidermal Growth Factor
Arm	Armadillo
Sgg	Shaggy
GSK3	Glycogen Synthase Kinase 3
CK	Casein Kinase
Hipk	Homeodomain-interacting protein kinase
Gish	Gilgamesh
Dpp	Decapentaplegic
Smo	Smoothened
Sens	Senseless
Dll	Distalless
Ap	Apterous
Ci	Cubitus interruptus
DYRK	Dual-specificity tyrosine phosphorylation-regulated kinase
Sev	Sevenless
Arr	Arrow
LRP	Low-density lipoprotein receptor-related protein
Ptc	Patched
TCF	T-cell factor
β-TrCP	β-transducin repeat-containing protein
SCF	Skp1, Cullin1, F-box
Slimb	Supernumerary limbs
PP	Protein Phosphatase
GLI	Glioma-associated oncogene
Cul1	Cullin1
Porc	Porcupine
TLE	Transducin-like Enhancer of Split
LEF1	Lymphoid enhancer factor 1
GFP	Green Fluorescent Protein
OTE	Off-target effect

1. Wnt signaling

1.1. Signal transduction pathways

Evolutionarily conserved signal transduction pathways are reiteratively implemented spatiotemporally to regulate the physiology and differentiation of cells during the embryonic, juvenile, and adult stages of metazoan development (Gerhart, 1999). These signaling pathways are used as a means of communication between cells, and together with the evolution of cell adhesion molecules facilitated the transition from a unicellular protozoan ancestor to multicellular animals (King et al., 2003; Nichols et al., 2006). Multiple essential cellular behaviours as diverse as metabolism, growth, proliferation, apoptosis, survival, motility, polarity, and differentiation are regulated by signaling pathways. As a result, the deregulation of signaling results in a multitude of human developmental disorders and cancers (Hanahan and Weinberg, 2011; Tan et al., 2009). Signal transduction is initiated when a secreted or surface bound ligand (first messenger) from a signal-producing cell binds a specific transmembrane or intracellular receptor of a signal-receiving cell. The ligand may assume the form of a protein, peptide, lipid, small molecule derivative, or gas (Freeman and Gurdon, 2002). Based on the proximity of the responding cell to the source of the ligand, signaling is categorized as autocrine (cell autonomous), juxtacrine (cell-to-cell contact), paracrine (short-range), or endocrine (long-range) (Freeman and Gurdon, 2002). Binding of the ligand to its cognate receptor induces one or more intracellular signaling relays through the combinatorial use of second messenger, intermediary, and effector molecules (Figure 1.1.1). The primary consequence of most signaling events is the regulation of a molecule defined as the effector which directs the cellular response through the transcription factor-mediated expression of target genes of the pathway (Downward, 2001) (Figure 1.1.1). The signal-receiving cell may activate signaling in a graded induction (when the response is proportional to the concentration of the ligand) or in an all-or-none induction (when the response occurs only on reaching a certain threshold

concentration of the ligand) (Freeman and Gurdon, 2002). This conversion of an extracellular signal from a signal-producing cell into an intracellular response in a signal-receiving cell is termed intercellular signal transduction. The amplitude and duration of signaling may be regulated through a combination of feedback mechanisms that are initiated in the signal-receiving cell. Positive feedback occurs when signaling induces the production of one or more molecules that serve to stabilize, prolong, or amplify the initial signaling event. The signal-receiving cell may additionally undergo a reversible process of desensitization through one or more negative feedback mechanisms that dampen signaling. Desensitization of the signal-receiving cell can occur through several ways such as through the production of a ligand antagonist, inactivation of the ligand-receptor complex, degradation of the second messenger, or the nuclear export of the effector (Freeman, 2000; Perrimon and Mcmahon, 1999) (Figure 1.1.1).

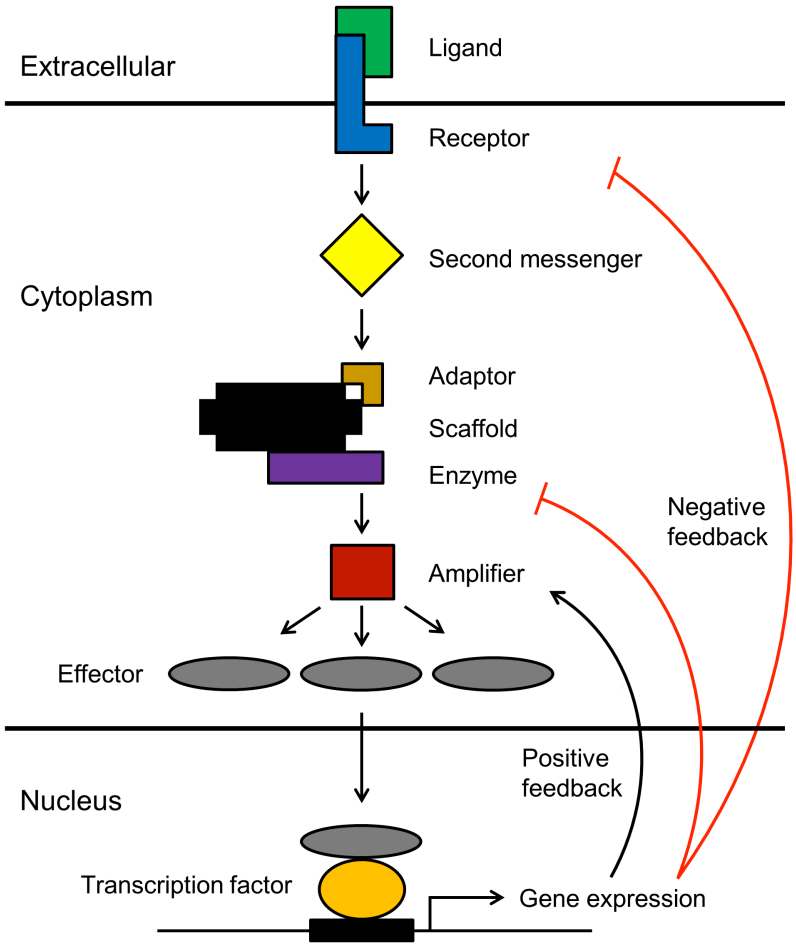


Figure 1.1.1 Signal transduction pathway

The binding of a secreted ligand to a transmembrane receptor relays information intracellularly to the nucleus through the use of second messenger, intermediary, and effector components. The intermediary components of a pathway may include a combination of an adaptor, scaffold, enzyme, or amplifier. The pathway induces feedback mechanisms that regulate the amplitude and duration of signaling. (Figure based on Downward, 2001)

1.2. Principles of developmental signaling

Cellular differentiation in metazoans is predominantly regulated by the following developmental signaling pathways: Wnt, Transforming Growth Factor (TGF)-β, Hedgehog (Hh), Notch, Receptor Tyrosine Kinase (RTK), Janus Kinase (JAK)/Signal Transducer and Activator of Transcription (STAT) and Nuclear Hormone Receptor. All other signaling pathways for the most part regulate the physiological functioning of cells (Gerhart, 1999). Fundamental elements from each of these signaling pathways are present in all multicellular animals ranging from diploblastic poriferans (Srivastava et al., 2010) to triploblastic chordates (Lander et al., 2001). Although divergent in their molecular mechanisms of signal transduction, each pathway functions to facilitate the association of an effector with a signaling pathway response element (SPRE) to regulate the expression of specific target genes. Moreover, there are at least three inherent properties shared between developmental signaling pathways that determine the spatial and temporal specificity of expression of target genes: effector insufficiency, cooperative activation, and default repression. The combination of these properties permits a signaling pathway to control the specification of a variety of different cell fates (Barolo and Posakony, 2002) (Figure 1.2.1).

The activation of a signaling pathway in any one instance is insufficient to cause the expression of all of its target genes. Rather, a signaling pathway controls the expression of distinct (but some common) target genes depending on the developmental context. This is due to the existence of *cis*-regulatory elements, besides the SPRE, that are bound by context-specific coactivators and confer a target gene competent to be regulated by the effector of the pathway (Affolter et al., 2001; Flores et al., 2000; Halfon et al., 2000; Hepker et al., 1999; Prieve and Waterman, 1999; Singson et al., 1994; Xu et al., 2000). As a result of this requirement for additional regulatory elements, an artificial pathway reporter composed of only a minimal SPRE does not always recapitulate the expression pattern of a native target gene *in vivo* (Barolo et al., 2000; Furriols and Bray, 2001; Guss et al., 2001; Hepker et al., 1999; Riese et al., 1997; Xu et al., 1998). This inability of a SPRE and thereby a signal-regulated effector alone to activate target gene expression *in vivo* is referred to as effector insufficiency (Figure 1.2.1). Signaling does however robustly activate SPRE-dependent target gene expression in appropriate

developmental contexts through the use of cooperative activation (or combinatorial transcription). Functional coactivators that bind *cis*-regulatory elements of a target gene are spatially and/or temporally restricted. The synergistic effect between a signal-regulated, context-independent effector and signal-independent, context-specific coactivators enables a pathway to regulate the expression of different target genes in different contexts (Affolter et al., 2001; Flores et al., 2000; Giese et al., 1995; Halfon et al., 2000; Hepker et al., 1999; Pearce et al., 1998; Schule et al., 1988) (Figure 1.2.1). A drawback of cooperative activation is that signal-independent, context-specific coactivators are often sufficient to weakly direct the expression of a target gene even in the absence of signaling input via the SPRE (Barolo et al., 2000; Müller and Basler, 2000). Signaling pathways utilize the property of default repression to prevent this problem of inadvertent expression of target genes. In this scenario, transcription and other auxiliary factors direct the SPRE-mediated repression of a target gene in the absence of signaling (Affolter et al., 2001; Cavallo et al., 1998; Méthot and Basler, 1999; Morel and Schweisguth, 2000; Wotton and Massague, 2000; Xu et al., 2000). This strategy ensures that a SPRE-containing target gene is silenced until signaling is activated, despite the presence of context-specific coactivators (Figure 1.2.1). The properties of transcriptional control discussed here contribute to the context-specific responses to developmental signaling pathways (Barolo and Posakony, 2002).

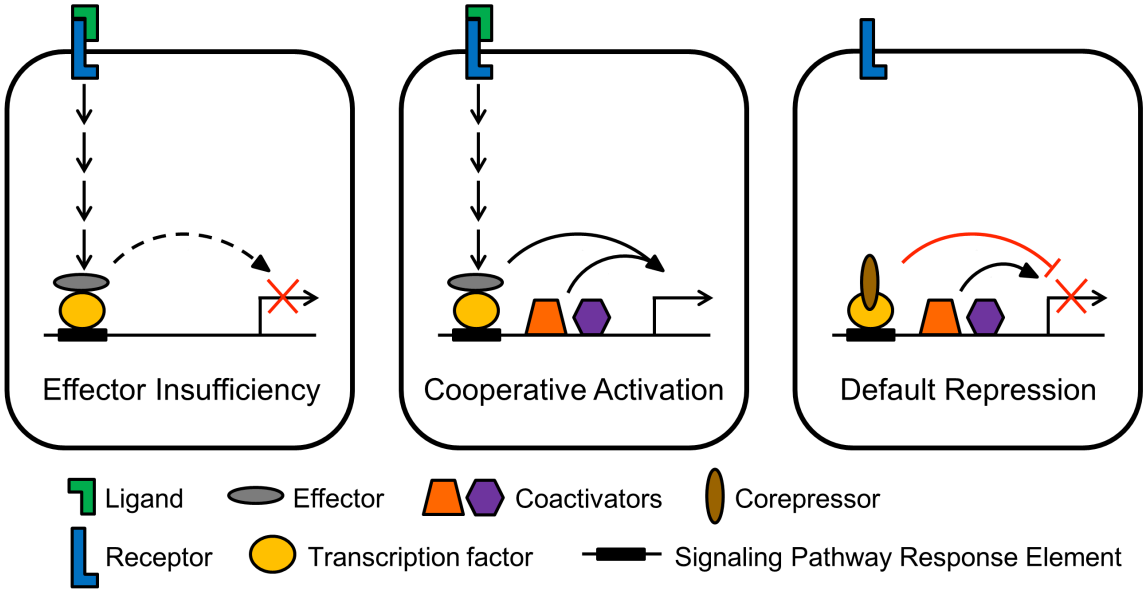


Figure 1.2.1 Principles of developmental signaling

A signaling pathway can specify a variety of different cell fates through the expression of distinct subsets of target genes. This process is mediated through the combinatorial use of a signal-dependent, context-independent effector and signal-independent but context-dependent coactivators. (Figure adapted from Barolo and Posakony, 2002)

1.3. Evolution of Wnts

The comparison of representative genomes across divergent metazoan phyla suggests that the aforementioned developmental signaling pathways did not arise completely *de novo* in the proto-metazoan lineage (Adams et al., 2000; Chapman et al., 2010; Lander et al., 2001; Srivastava et al., 2008, 2010; Venter et al., 2001). Rather, there were pre-existing functional elements of these pathways that preceded multicellularity and provided a foundation for their emergence in metazoans (King et al., 2003, 2008). Through co-option and domain shuffling of pre-existing components combined with the addition of novel domains and molecules, developmental signaling pathways evolved in metazoans (Pires-daSilva and Sommer, 2003; Richards and Degnan, 2009). On the emergence of the core components early in the metazoan lineage, any significant further modification to these pathways has occurred through the expansion of existing gene subfamilies and not through the addition of novel components (Richards and Degnan, 2009). For example, there exists an overall trend of continual expansion of ligand, receptor, and transcription factor gene subfamilies across metazoan clades (Ben-Shlomo et al., 2003; Herpin et al., 2004; Holstein, 2012; Itoh and Ornitz, 2004; Larroux et al., 2008; Miyata and Suga, 2001).

An important morphological feature that has contributed to the phylogenetic relationships between metazoans is symmetry about a primary body axis. Every animal species as it develops during the embryo or juvenile stage is organized about a primary axis that is alternatively referred to as anterior/posterior in bilaterians or apical/basal (or oral/aboral) in pre-bilaterians. The primary axis of the animal provides a positional system for regional patterning along the body, and is polarized as a result of Wnt signaling (Niehrs, 2010). A localized expression of one or more Wnt ligands, after which the pathway in named, is present in the posterior of bilaterians and in a similar polarized manner along the primary axis in pre-bilaterians during development. Conversely, Wnt antagonists are expressed in the anterior of bilaterians and at the opposite pole to that of Wnt expression in pre-bilaterians. This pattern of posterior Wnt signaling and anterior Wnt inhibition is a core determinate of primary axis polarity in metazoans (Petersen and Reddien, 2009).

The Wnt family of ligands is specific to metazoans. No homologues of Wnts have been identified in unicellular fungi, protists, or choanoflagellates, the latter of which are presumed to share a common ancestor with animals (King et al., 2003, 2008; Sebé-Pedrós et al., 2011). Wnts have been grouped into 13 subfamilies across metazoan phyla based on sequence homology (Holstein, 2012; Prud et al., 2002; Sidow, 1992) (Figure 1.3.1). Parazoans possess only a few identifiable Wnts (Lapébie et al., 2009; Pang et al., 2010; Srivastava et al., 2008, 2010). However, a significant expansion in ligand diversity appears to have occurred soon after on the evolutionary scale in cnidarians within the genome of *Nematostella* that encodes Wnts from 12 of the 13 subfamilies (Kusserow et al., 2005; Lengfeld et al., 2009) (Figure 1.3.1). Protostomes do not have a representative of the Wnt3 subfamily and there is an apparent loss of Wnt subfamilies in ecdysozoans as observed in the genomes of *Caenorhabditis* and *Drosophila.* Deuterostomes have Wnts from all 13 subfamilies, while humans and other chordates are characterized by the loss of WntA but also by the gain of a number of Wnt paralogues (Cho et al., 2010; Janssen et al., 2010) (Figure 1.3.1). The diversity of Wnts in early eumetazoans is indicative of a robust expansion of the genetic repertoire in accordance with the evolution of multicellularity and animal body plans (Davidson and Erwin, 2006).

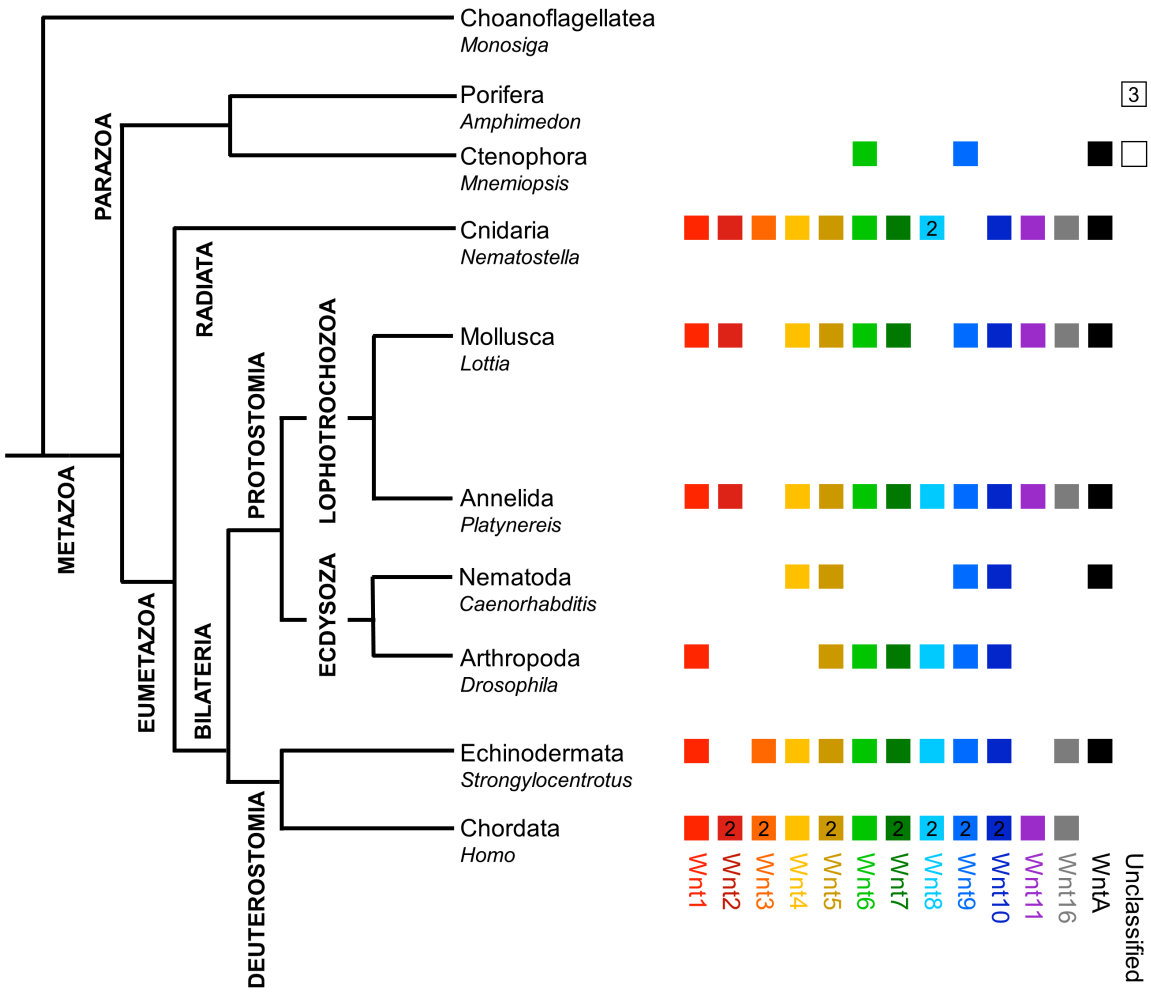


Figure 1.3.1 Evolution of Wnts in metazoans

The Wnt family of ligands has been grouped into 13 subfamilies across metazoans. The number of Wnt paralogues of each subfamily is indicated. The number of Wnt subfamilies diversified early in the metazoan lineage and were subsequently lost in ecdysozoans. (Figure based on Janssen et al., 2010)

The evolutionary relationships among the different Wnt subfamilies are unclear thus far. Nonetheless, a clustering of the Wnt1, Wnt6, and Wnt10 subfamilies has been identified from phylogenetic analyses and is also observed in the syntenic organization of these genes in *Drosophila* and humans (Nusse, 2001; Prud et al., 2002). In the *Drosophila* genome, *wnt1*, *wnt6*, and *wnt10* are physically adjacent to one another on the chromosome and transcribed in the same orientation. This organization of genes is conserved in humans where the cluster has been duplicated and modified (Nusse, 2001) (Figure 1.3.2). This cluster has subsequently been shown to be present in the genomes of cnidarians (Kusserow et al., 2005; Lengfeld et al., 2009) and *Lottia* (Cho et al., 2010; Janssen et al., 2010). A second conserved cluster comprising the *wnt5* and *wnt7* genes has recently been discovered in different species (Cho et al., 2010; Janssen et al., 2010; Sullivan et al., 2007) (Figure 1.3.2).

In addition to their syntenic organization, genes of the Wnt family are expressed in serial and overlapping domains along the primary body axis of diverse species (Guder et al., 2006; Kusserow et al., 2005; Lapébie et al., 2009). These features are reminiscent of the Hox family of transcription factors. The Hox system of collinearity in gene organization and expression is used to specify regional identity along the primary axis in bilaterians (Garcia-Fernàndez, 2005), and there is a lack of bona fide Hox genes in pre-bilaterians (Chourrout et al., 2006; Finnerty et al., 1996; Kamm et al., 2006; Larroux et al., 2007). As patterning along the primary body axis occurs in pre-bilaterians as well, a more primitive system must exist that fulfills this function in these animals. The Wnt signaling pathway has been proposed to be the ancestral metazoan axial patterning system (Guder et al., 2006; Lee et al., 2006; Ryan and Baxevanis, 2007). If this is indeed the case, it is possible that Hox genes on their emergence were co-opted into the Wnt pathway. Recent evidence from diverse clades suggests that Wnt signaling can function in tandem with Hox genes to specify regional identity (Arata et al., 2006; Bilder et al., 1998; Merabet et al., 2005). Thus, it is possible that the coordination of these two systems present in extant bilaterians is indicative of a co-option event that occurred in the past (Figure 1.3.3).

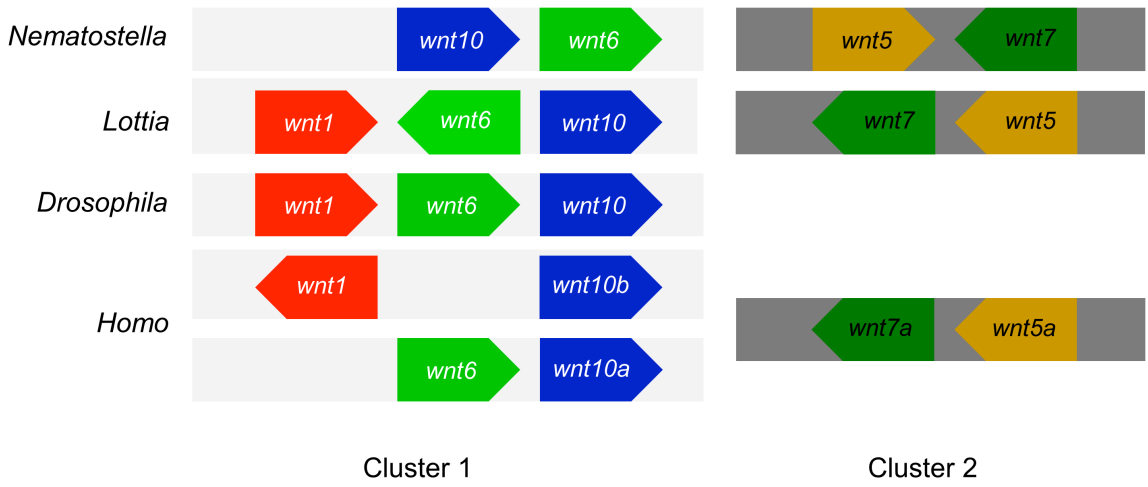


Figure 1.3.2 Synteny of genes from Wnt subfamilies

The relative position and orientation of genes from different Wnt subfamilies is displayed in two conserved clusters. The syntenic organization of *wnt1*, *wnt6*, *wnt10* and *wnt5*, *wnt7* across species is in agreement with the clustering of theses gene from phylogenetic analyses. (Figure adapted from Holstein, 2012)

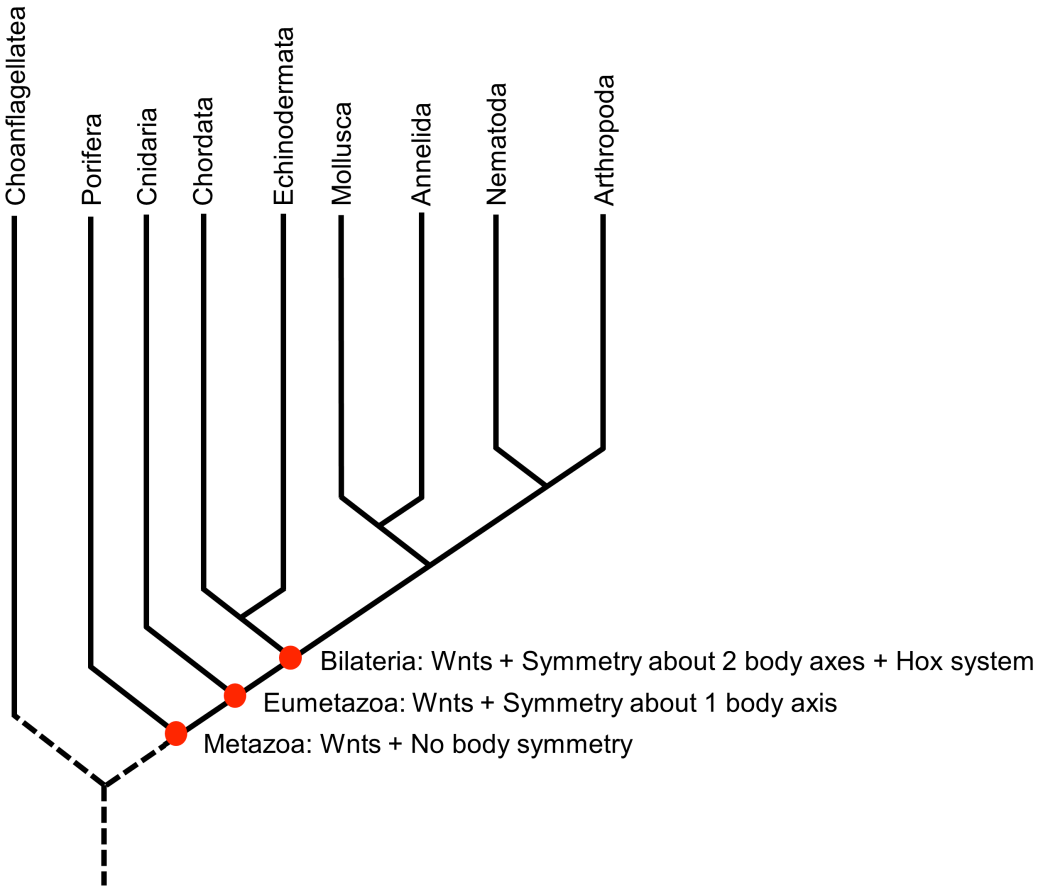


Figure 1.3.3 Wnt and Hox system of axial patterning

Wnt signaling may represent the ancestral axial patterning system that operates in pre-bilaterians. The Hox system evolved in bilaterians and may function in tandem with Wnt signaling to pattern the primary axis in these animals. (Figure adapted from Guder et al., 2006)

1.4. The history of Wnts

The Wnt signaling pathway has essential biological roles in the regulation of cell proliferation, cell migration, cell polarity, and cell fate specification during divergent stages of metazoan development, from embryo to adult (Cadigan and Nusse, 1997; MacDonald et al., 2009; Veeman et al., 2003). Wnts are secreted glycoprotein ligands that act both at short-range (Bafico et al., 2004; Hooper, 1994) and as long-range morphogens (Charron and Tessier-Lavigne, 2005; Kiecker and Niehrs, 2001; Neumann and Cohen, 1997a; Zecca et al., 1996). Members of this family are defined by sequence homology to the founding *wnt1* gene (Nusse and Varmus, 1982; Nusse et al., 1984). The characteristic feature of all Wnts is the nearly invariant positioning of 23-25 cysteine residues within the protein sequence (Miller, 2001). Wnts have traditionally been classified as canonical (β-catenin-dependent) or non-canonical (β-catenin-independent) based on the ability of the former, but not the latter, to induce a secondary axis in *Xenopus* embryos (Du et al., 1995; McMahon and Moon, 1989) or transform the mouse mammary epithelial cell line C57MG (Nusse and Varmus, 1982; Wong et al., 1994). A canonical Wnt-mediated pathway regulates the stability of the effector β-catenin, while non-canonical Wnt-mediated pathways signal independently of β-catenin (Niehrs, 2012) (Figure 1.4.1). However, this categorization as one of two types is incongruous as a Wnt ligand may have the capacity to initiate both canonical and non-canonical signaling based on the developmental context. For example, the prototypical non-canonical Wnt11 ligand is required for convergent extension movements during gastrulation in vertebrates (Heisenberg et al., 2000; Marlow et al., 2002; Tada and Smith, 2000), but has subsequently been shown to regulate *Xenopus* axis formation through β-catenin-dependent signaling (Tao et al., 2005). Similarly, the non-canonical Wnt5A ligand (Moon et al., 1993) has also been demonstrated to independently inhibit canonical signaling (He et al., 1997; Mikels and Nusse, 2006; Topol et al., 2003; Westfall et al., 2003). The ability of a Wnt to signal through canonical or non-canonical pathways is therefore not an intrinsic property of the ligand itself but dependent on the types of available receptors (van Amerongen and Nusse, 2009). The various non-canonical Wnt signaling pathways will not be discussed in this thesis and any reference to Wnt signaling relates only to the canonical pathway.

Wnt signaling has evolutionarily conserved roles in the regulation of axial polarity, posterior growth, and stem cell maintenance during metazoan development (Martin and Kimelman, 2009; Petersen and Reddien, 2009; Reya and Clevers, 2005). Much of our understanding of the molecular mechanism and biology of this pathway has come as a result of genetic and biochemical analyses performed predominantly using *Drosophila* and other model systems including *Xenopus*, *Caenorhabditis*, and *Mus*. The *Drosophila wnt1* or *wingless* (*wg*) gene is required to pattern the wings and other adult body structures. It was originally identified through a hypomorphic wg^{1} allele which causes the variable transformation of the adult wings to nota (Babu, 1977; Morata and Lawrence, 1977; Sharma and Chopra, 1976). Coincidentally, although unknown at the time, the first *wg* mutant dates even further back to the early part of the twentieth century. The *Glazed* (*Gla*) mutation that disrupts photoreceptor formation and creates a reduced eye surface in *Drosophila* has recently been identified to be allelic with *wg* (Brunner et al., 1999; Morgan et al., 1936). Analogous to the Mouse Mammary Tumor Virus (MMTV) insertion adjacent to the *wnt1* locus first identified (Nusse and Varmus, 1982), the *Gla* mutant has a retro-transposon insertion in a regulatory element of the *wg* gene that causes a gain-of-function phenotype (Brunner et al., 1999). Subsequent to characterization of the viable wg^{1} allele, saturation mutagenesis screens performed in *Drosophila* yielded embryonic lethal loss-of-function alleles of *wg* (Nusslein-Volhard and Wieschaus, 1980; Nusslein-Volhard et al., 1984). The *wg* gene was identified to be homologous to *wnt1* (Baker, 1987; Cabrera et al., 1987; Rijsewijk et al., 1987), and through the use of conditional mutants, mosaic analyses, and ectopic expression it was demonstrated to have important roles in multiple tissues in *Drosophila* including the embryonic ectoderm and wing imaginal disc (Klingensmith and Nusse, 1994; Siegfried and Perrimon, 1994). The identification and characterization of components of the Wg signaling pathway in *Drosophila* have since been performed in either one or both of these tissues.

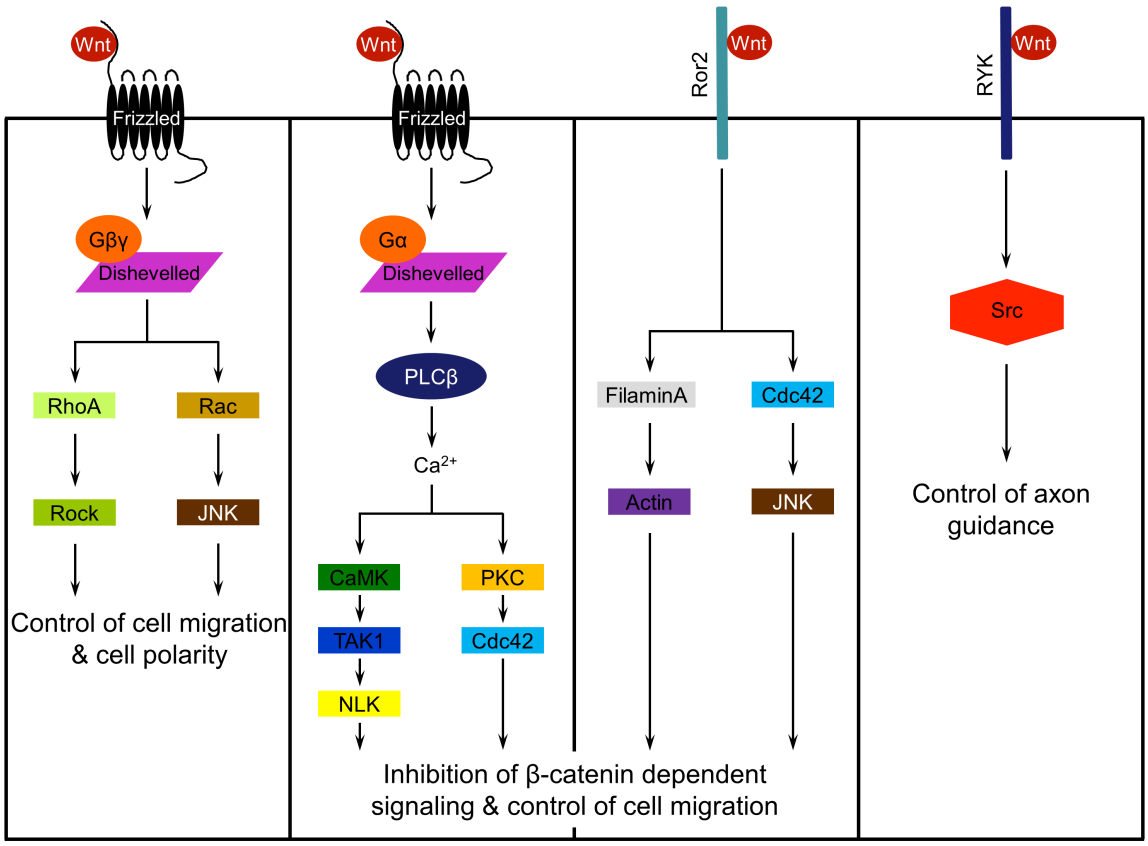


Figure 1.4.1 Non-canonical Wnt signaling pathways

Non-canonical or β-catenin-independent Wnt signaling pathways regulate diverse processes such as cell migration, cell polarity, and axon guidance through the use of different combinations of ligand, receptor, and downstream effector molecules. (Figure based on Niehrs, 2012)

1.5. Wg signaling in the *Drosophila* embryonic ectoderm

During *Drosophila* embryogenesis, a hierarchy of maternal and zygotic (gap, pair-rule, and segment polarity) genes progressively subdivide the embryonic syncytium into transverse regions that determine the anterior/posterior axis (Ingham and Martinez Arias, 1992; St. Johnston and Nuesslein-Volhard, 1992). The cellular blastoderm is formed towards the end of embryogenesis and coincides with the division of the anterior/posterior axis into segmental units as directed by the segment polarity genes *wg* and *hh*. These segment polarity genes interact with one another to define the segment boundaries and intra-segmental pattern of the embryo (Perrimon, 1994). Wg-mediated specification events generate a stereotypical pattern of naked cuticle that is separated by hair-like protrusions called denticles on the ventral epidermis of the embryo (Figure 1.5.1). This process of tissue patterning can be divided into four successive events: establishment of the organizer, asymmetric signaling from the organizer, subdivision of each segment into signaling domains, and cell fate specification by the signaling domains (Figure 1.5.1).

The expression of *wg* and *hh* is initiated by the pair-rule genes in adjacent, non-overlapping domains during mid-embryogenesis and they subsequently reciprocally regulate each other to stabilize their expression (DiNardo et al., 1994). Wg secreted from an anterior row of cells maintains the expression of a transcription factor, *engrailed* (*en*), in adjoining posterior rows of cells. The *en*-expressing cells in turn produce and secrete the Hh ligand which maintains *wg* expression in the neighbouring anterior cells (Bejsovec and Martinez-Arias, 1991; DiNardo et al., 1988; Hidalgo and Ingham, 1990; Martinez-Arias et al., 1988). The interface between these two adjacent signaling domains defines the parasegment boundary or organizer, with *en*/*hh* transcribed at the anterior and *wg* at the posterior end of each parasegment (Baker, 1987; Lee et al., 1992; Mohler and Vani, 1992) (Figure 1.5.1).

After the parasegment boundary is first established, the distribution of Wg is bidirectional and initiates signaling at equivalent levels in both the anterior and posterior direction (Peifer et al., 1994; Riggleman et al., 1990). As embryogenesis progresses, the distribution of the ligand becomes graded only anterior to the *wg*-expression domain and is almost completely lost in the posterior direction (González et al., 1991; van den

Heuvel et al., 1989). This asymmetry in distribution is a consequence of Hh signaling-mediated endocytosis and degradation of Wg in cells posterior from which it is secreted (Dubois et al., 2001; Sanson et al., 1999). Wg signaling in turn attenuates Hh signaling anterior to the *hh*-expression domain, thereby allowing its activity in only the posterior direction (Gritzan et al., 1999). Thus, a pattern of polarized signaling of Wg and Hh at the parasegment boundary is formed that can direct cells on either side to follow distinct developmental programs. The expression of *wg* and *en*/*hh* become independent of one another during this stage of embryogenesis (Bejsovec and Martinez-Arias, 1991; Heemskerk et al., 1991) (Figure 1.5.1).

The *wg*- and *en*/*hh*-expression domains successively specify two additional domains within each parasegment that are defined by the expression of the Notch pathway ligand *Serrate* (*Ser*) (Wiellette and McGinnis, 1999) and *rhomboid* (*rho*) which promotes Epidermal Growth Factor (EGF) signaling (Golembo et al., 1996). Wg and Hh signaling antagonize the posterior and anterior boundaries respectively of *Ser* expression, thereby restricting its domain to the centre of each parasegment (Alexandre et al., 1999; Gritzan et al., 1999; Sanson et al., 1999). *rho* is repressed by Wg signaling but also reinforced through a combination of Hh and Ser-mediated Notch signaling, thereby establishing its expression immediately posterior to the *en*/*hh* domain (Alexandre et al., 1999; Gritzan et al., 1999; Sanson et al., 1999). Each parasegment thus comes to be divided into four domains at the end of embryogenesis that express specific genes responsible for patterning the embryo. This period coincides with the formation of a segmental groove at the posterior edge of the *en*/*hh* domain that defines the segment boundary (Figure 1.5.1).

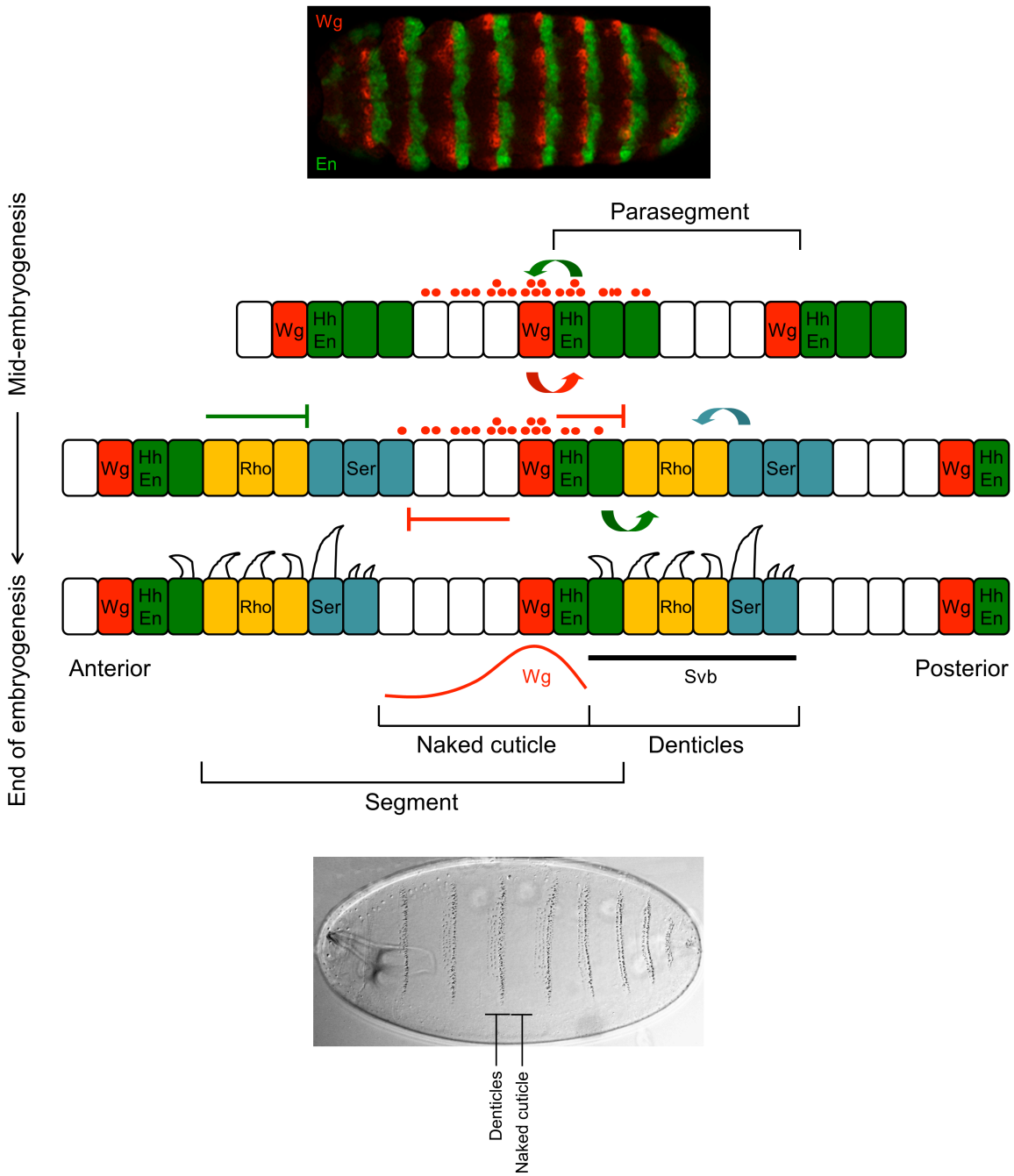


Figure 1.5.1 Wg signaling patterns the *Drosophila* embryonic ectoderm

The interaction between Wg and Hh signaling establishes the parasegment boundaries and directs the intrasegmental patterning of the embryo. On the ventral embryonic ectoderm, cells that transduce the Wg signal do not express *svb* and produce naked cuticle while cells not exposed to Wg express *svb* and produce denticles. (Figure adapted from Swarup and Verheyen, 2012)

The four signaling domains established within each segment control the binary decision between specification of naked cuticle or denticles. The outcome between these two choices is dependent on the expression of the *shaven baby* (*svb*) gene which is necessary and sufficient to direct denticle formation cell autonomously (Payre et al., 1999). Cells that specify naked cuticle inhibit the expression of *svb* while cells that produce denticles express *svb*. Wg signaling specifies naked cuticle by repressing the expression of *svb* (Bejsovec and Martinez-Arias, 1991; Lawrence et al., 1996; Noordermeer et al., 1992; Payre et al., 1999). Due to the asymmetric distribution of Wg across the posterior half of each segment, one row of cells posterior to and four rows of cells anterior to its expression domain produce naked cuticle (O'Keefe et al., 1997; Szuts et al., 1997), in addition to the *wg*-expressing cell itself (Hooper, 1994). The six rows of cells towards the anterior half of each segment do not receive Wg but instead transduce EGF signaling that promotes *svb* expression and the formation of denticles (Payre et al., 1999). The outcome of these regulated cell signaling events is a repeated mosaic of naked cuticle and denticles on the ventral epidermis of the embryo (Figure 1.5.1). *svb* expression is not repressed and naked cuticle is not specified in a *wg* loss-of-function mutant, resulting in the expansion of the number of rows of denticles relative to a *wild type* embryo. Conversely, ectopic expression of *wg* produces excess naked cuticle with a reduction or loss of denticles (Payre et al., 1999) (Figure 1.5.2). These traits that result from the aberrant specification of naked cuticle or denticles are referred to as segment polarity phenotypes.

Components of the Wg pathway were initially identified in loss-of-function genetic screens to isolate zygotic mutations that disrupt the *Drosophila* embryonic ectoderm. These screens yielded numerous genes that displayed segment polarity phenotypes when mutated, including both positive and negative regulators of the pathway (Jurgens et al., 1984; Nusslein-Volhard and Wieschaus, 1980; Nusslein-Volhard et al., 1984; Wieschaus et al., 1984). Mutants of positive regulators of signaling such as *armadillo (arm)* and *arrow (arr)* resembled the *wg* loss-of-function phenotype to specify extra rows of denticles (Nusslein-Volhard et al., 1984; Wieschaus et al., 1984), while a mutant of the negative regulator *naked* displayed a *wg* gain-of-function phenotype with an excess specification of naked cuticle (Jurgens et al., 1984). Although these genetic screens led to the identification of several components, they did not yield the entire complement of

genes involved in the Wg pathway. This is because there is sufficient maternal contribution of some gene products for a zygotic mutant to pattern a relatively normal embryo. This limitation was circumvented through the use of a technique that induces mitotic recombination in the germ line to eliminate maternally contributed gene product (Perrimon and Gans, 1983). On re-screening part of the genome for maternal effect mutations that disrupt patterning of the embryonic ectoderm, the novel positive regulators *dishevelled* and *porcupine* (*porc*), and the negative regulator *shaggy (sgg)* of the Wg pathway were isolated (Perrimon et al., 1989).

A traditional loss-of-function epistasis analysis relies on evaluating phenotypes in double mutant combinations, thus allowing one to determine which of two distinct phenotypes overrides or is epistatic to the other. This information allows the ordering of genes within a pathway since the gene that acts most downstream will be epistatic to those acting upstream. However, the similar phenotypes of many of the first identified segment polarity genes precluded such analyses. To overcome this problem, analyses were performed using combinations of ectopic expression (gain-of-function) and loss-of-function that on their own generated distinct phenotypes. These genetic interaction studies in the *Drosophila* embryonic ectoderm allowed the early components to be defined into a rudimentary pathway leading from *porc* to *wg* to *dishevelled*, *sgg*, and *arm* (Noordermeer et al., 1994; Peifer et al., 1994; Siegfried et al., 1992, 1994), and has continued to serve as a template for positioning and characterizing novel members of Wg signaling.

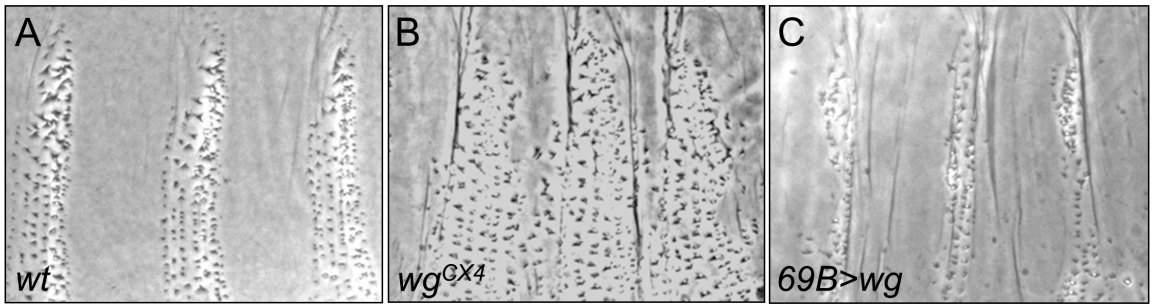


Figure 1.5.2 Wg specifies naked cuticle on the *Drosophila* embryonic ectoderm

(A) The ventral epidermis of a wild type embryo has an alternating pattern of naked cuticle and denticles. (B) A wg^{CX4} mutant embryo does not specify naked cuticle and has excess denticles while (C) ectopic expression of a *UAS-wg* transgene using the *69B-Gal4* driver specifies excess naked cuticle at the expense of denticles. (Figure adapted from Swarup and Verheyen, 2012)

1.6. Wg signaling in the *Drosophila* wing

Drosophila imaginal discs are epithelial structures formed from small groups of undifferentiated cells that are set aside towards the end of embryogenesis. These cells proliferate during the subsequent larval stages of development to produce large populations that are eventually specified to give rise to the ectodermal tissues of adult structures such as the wings, eyes, and legs (Maves and Schubiger, 1999). The wing disc is made up of 20 cells when it is first formed during embryonic development. It then proliferates during the three larval stages to generate a tissue of approximately 75000 cells before undergoing metamorphosis during pupation to give rise to the adult wing (Bate and Martinez-Arias, 1991; Cohen et al., 1991; Garcia-Bellido and Merriam, 1971). During the late third larval stage the major elements of the wing primordium that give rise to the adult structure can be identified (Figure 1.6.1). *wg* is expressed along the dorsal/ventral compartment boundary, in two ring-like domains at the periphery of the wing field, and in a wedge in the dorsal part of the disc (Baker, 1988; Phillips and Whittle, 1993). The most dorsal part of the wing disc gives rise to the notum while the ring-like domains frame the wing blade and give rise to the hinge. The dorsal/ventral compartment boundary forms the wing margin, and it is this region of the disc and adult wing that has widely been used to assay for Wg signaling (Couso et al., 1994; Neumann and Cohen, 1997a; Rulifson et al., 1996) (Figure 1.6.1).

As previously mentioned, the wg^{1} mutation displays a wing-to-notum transformation (Morata and Lawrence, 1977; Sharma and Chopra, 1976). Conversely, over-expression of *wg* leads to the ectopic induction of wing structures in the notum (Klein and Martinez-Arias, 1998; Ng et al., 1996). These phenotypic analyses suggest that *wg* has a crucial function during larval development to specify the wing field region of the disc that gives rise to the adult wing. During the second larval stage, *wg* is expressed in the ventral region of the wing disc and specifies the wing field while *vein* (*vn*), a ligand for EGF signaling, is expressed in the dorsal region of the wing disc to specify the notum. The loss of *vn* during the second larval stage results in the loss of the notum, while over-expression of *vn* in the wing field prevents wing formation and produces an ectopic notum (Baonza et al., 2000; Simcox et al., 1996; Wang et al., 2000). The complementarity of the *wg* and *vn* phenotypes is due to the mutual

antagonism between the Wg and EGF pathways that segregate the early wing disc into the wing field and notum respectively. The expression of *vn* is restricted to the dorsal region of the wing disc by the suppressive influence of Wg signaling in the ventral region. Conversely, *wg* expression is antagonized in the dorsal region of the wing disc by EGF signaling to limit its expression to only the ventral region (Baonza et al., 2000; Wang et al., 2000).

The process of tissue patterning necessitates the implementation of three sequential events. First, positional information conveyed by homeotic selector genes allocates cells into non-overlapping sets that form separate compartments. Second, cells of one compartment interact with cell in the adjacent compartment through short-range signaling to establish a compartment boundary. Lastly, cells at the boundary secrete a long-range morphogen to pattern cells of both compartments (Brook et al., 1996; Lawrence and Struhl, 1996). The formation of the wing primordium is regulated by signaling centres that are established at the boundaries of the anterior/posterior and dorsal/ventral compartments during the third larval stage (Dahmann and Basler, 1999). Prior to the establishment of these signaling centres, the selector genes *en* and *apterous* (*ap*) specify the compartments of the wing disc (Figure 1.6.2). The expression of *en* specifies the posterior compartment of the developing wing field and distinguishes it from cells in the adjacent anterior compartment (Guillen et al., 1995; Hidalgo, 1994; Simmonds et al., 1995; Tabata et al., 1995; Zecca et al., 1995). Once specified, cells of the posterior compartment produce and secrete the Hh ligand (Lee et al., 1992; Mohler and Vani, 1992; Tabata et al., 1992), that directs short-range signaling at the anterior/posterior interface to establish the compartment boundary (Mullor et al., 1997; Strigini and Cohen, 1997; Tabata and Kornberg, 1994). Cells at the compartment boundary subsequently express and secrete the vertebrate TGF-β morphogen homologue *decapentaplegic* (*dpp*) (Basler and Struhl, 1994; Capdevila and Guerrero, 1994; Posakony et al., 1990), that patterns the wing field in the anterior and posterior directions through long-range signaling (Lecuit et al., 1996; Nellen et al., 1996; Zecca et al., 1995) (Figure 1.6.2). The same paradigm of selector gene, short-range inducer, and long-range morphogen is applied to pattern the dorsal and ventral compartments of the wing disc. The expression of *ap* specifies the genetic address of cells in the dorsal compartment and distinguishes it from the adjacent ventral compartment of the wing field

(Blair et al., 1994; Diaz-Benjumea and Cohen, 1993; Williams et al., 1993). Ap directs the expression of *Ser* which initiates cell-to-cell signaling via the Notch pathway at the dorsal/ventral interface to establish the compartment boundary (Diaz-Benjumea and Cohen, 1995; Irvine and Wieschaus, 1994; Kim et al., 1995). Notch signaling at the dorsal/ventral compartment boundary induces the expression of *wg* (de Celis et al., 1996; Diaz-Benjumea and Cohen, 1995; Doherty et al., 1996; Rulifson and Blair, 1995). The Wg morphogen secreted from cells at the boundary patterns the wing field in the dorsal and ventral directions (Couso et al., 1994; Neumann and Cohen, 1997a; Zecca et al., 1996) (Figure 1.6.2).

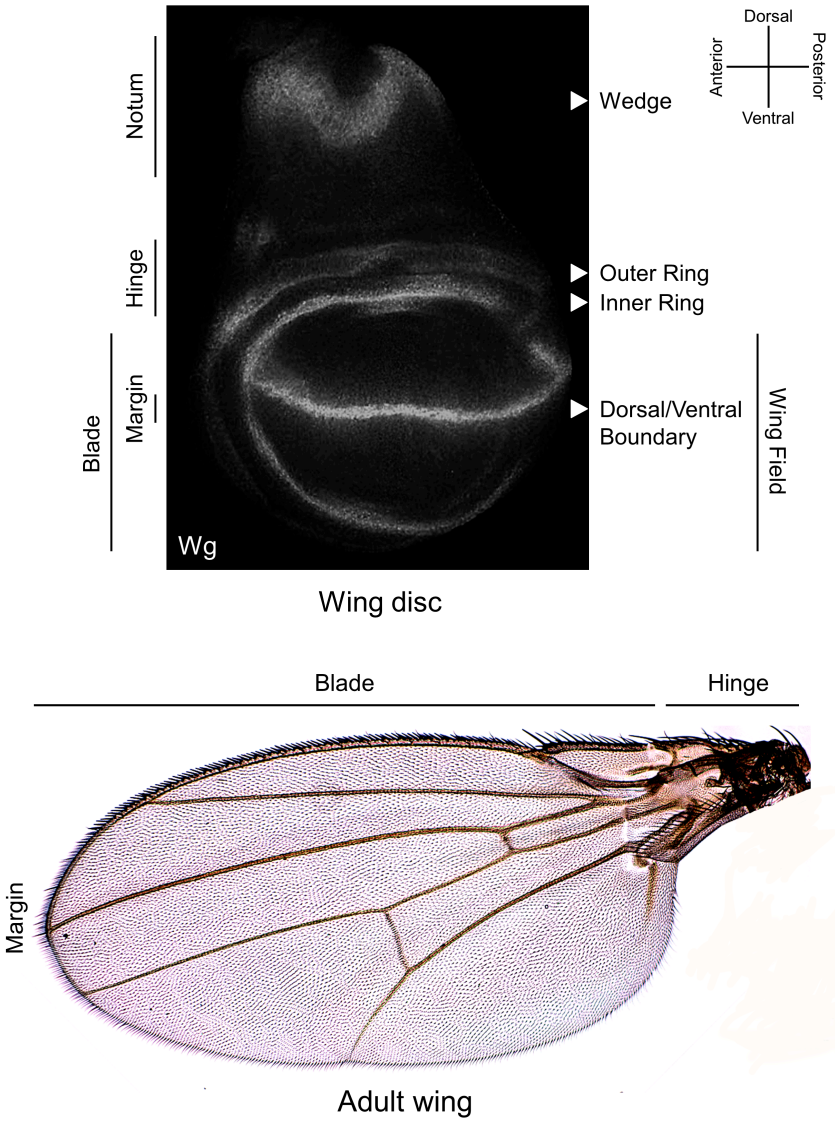


Figure 1.6.1 Wg signaling patterns the *Drosophila* wing

wg is expressed at the dorsal/ventral compartment boundary, in two concentric rings, and in a wedge in the third larval stage wing disc that give rise to the margin, hinge, and notum respectively of the adult structure.

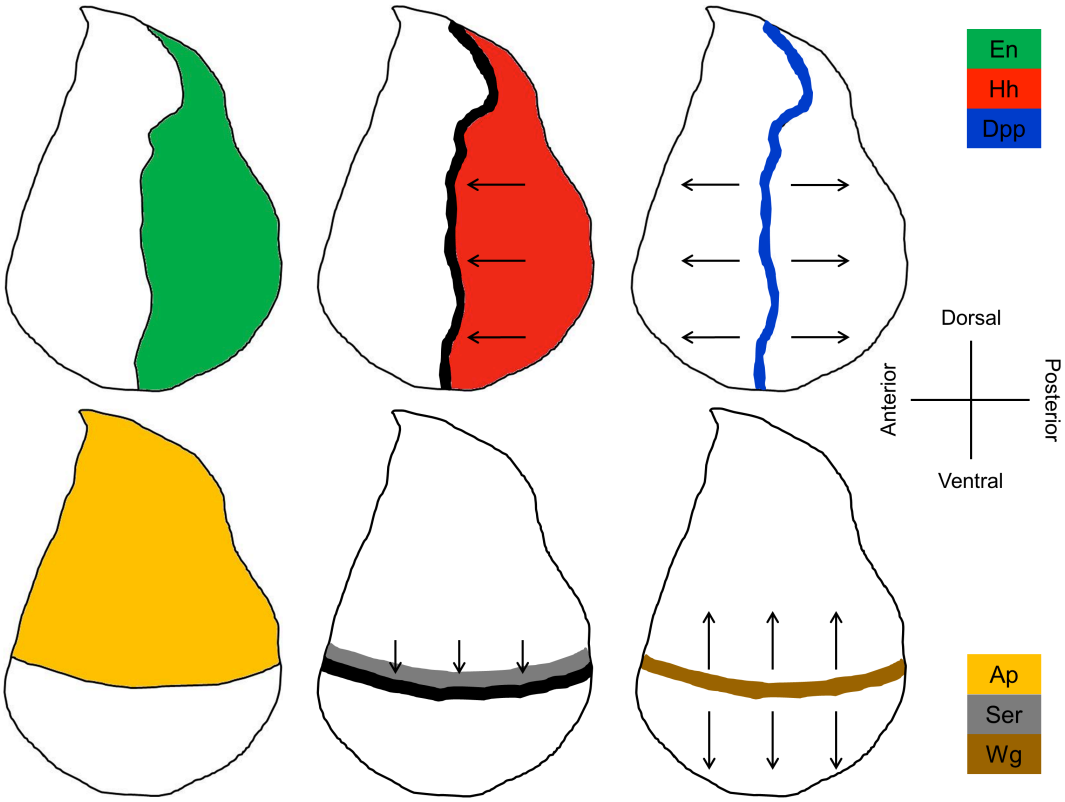


Figure 1.6.2 Compartments, boundaries and signaling in the *Drosophila* wing disc

The anterior/posterior and dorsal/ventral compartments of the wing disc are patterned through the sequential actions of a selector gene, short-range inducer, and long-range morphogen. En specifies the posterior compartment, the cells of which produces and secretes Hh to direct short-range signaling at the anterior/posterior interface to establish the compartment boundary. The Dpp morphogen is subsequently secreted from cells of the compartment boundary to pattern the wing field in the anterior and posterior directions. The dorsal/ventral compartments of the wing disc are patterned in a similar manner through the sequential actions of Ap, Ser-mediated short-range Notch signaling, and long-range signaling in response to the Wg morphogen. The anterior/posterior and dorsal/ventral compartment boundaries are indicated in black. (Figure based on Dahmann and Basler, 1999)

1.7. The Wg morphogen

A morphogen is a signaling molecule that diffuses away from a localized source to directly instruct differential cell identities in a concentration-dependent process (Gurdon and Bourillot, 2001; Neumann and Cohen, 1997b; Wolpert, 1989). For the secreted Wg ligand to qualify as a morphogen, it must form a graded distribution away from its source, directly act on cells at a distance rather than indirectly through a relay mechanism, and induce the commitment of cells as a function of their distance from the source to distinct developmental fates through the expression of different sets of genes.

Experiments performed in the *Drosophila* wing disc suggest that Wg does indeed behave as a morphogen in this tissue. During the third larval stage, Wg secreted from cells at the dorsal/ventral boundary diffuses away from its source on either side to induce the nested expression of the pathway target genes *achaete* (*ac*) (or *senseless* (*sens*)), *distal-less* (*dll*), and *vestigial* (*vg*) (Swarup and Verheyen, 2012) (Figure 1.7.1). The concentration gradient of Wg can be detected up to 10 cell diameters away from the dorsal/ventral boundary, suggesting that it forms a graded distribution away from its source and has the potential to act directly at long-range (Couso et al., 1994; Strigini and Cohen, 2000). The modulation of Wg signaling through loss-of-function clonal analyses or ectopic expression of *arm* and *dishevelled* cell-autonomously affects the expression of target genes regardless of the position of the cell from the source of the ligand (Neumann and Cohen, 1997a; Zecca et al., 1996). Additionally, unlike normal Wg, a membrane-tethered form of the ligand that is unable to diffuse away from its source on secretion activates expression of target genes only in its immediate neighbouring cells. These results confirm that Wg acts directly on cells over long-range and the expression of target genes is not regulated via a secondary or relay signal (Zecca et al., 1996). Through the use of a temperature-sensitive wg^{ts} allele it has been demonstrated that the minimum level of Wg activity required to direct expression of the high threshold target gene *ac*, the medium-low threshold target *dll*, and the low threshold target gene *vg* is progressively lower. As the activity of Wg^{ts} is decreased by raising the temperature, the expression of *ac* is lost at a temperature at which *dll* expression is retained. As the temperature is further increased, the expression of *dll* is lost with no effect on *vg* expression. In both cases there is a concomitant reduction of the expression domains of

target genes toward the dorsal/ventral boundary (Neumann and Cohen, 1997a). Consistent with these results, low levels of ectopically expressed Wg are able to activate *dll* but not *ac.* These data suggest that Wg activates different target genes in a concentration-dependent process and defines their expression domains through different activation thresholds (Zecca et al., 1996). Therefore, experimental evidence suggests the Wg morphogen has a graded distribution over a long-range to directly specify cells to distinct developmental fates in the wing disc. This does not exclude the possibility of other permissive signals that are required for the activation of the aforementioned target genes but it does argue that the threshold of the Wg ligand is the instructive signal.

Wg has also been proposed to behave as a morphogen in the *Drosophila* embryonic ectoderm but whether it actually does so in this developmental context is unclear. Wg is secreted from the parasegment boundary in the ectoderm and adopts a graded distribution in the anterior direction up to four cells away to specify naked cuticle (Bejsovec and Martinez-Arias, 1991). Although in this tissue Wg fulfills the criteria of being secreted from a localized source and forming a gradient, there is no evidence for a direct concentration-dependent induction of target genes. In fact, Wg seems to act in a gradient-independent manner in the embryonic ectoderm as the segment polarity phenotype of a *wg* mutant embryo can be rescued through the ubiquitous expression of a *wg* transgene (Sampedro et al., 1993). Moreover, the expression domain of the target gene *en* is not extended in the presence of ubiquitous Wg signaling (DiNardo et al., 1988). Lastly, in a *wg* mutant background the over-expression of a membrane-tethered form of Wg can recapitulate the normal range of signaling and suggests that the Wg ligand does not function directly over a long-range to pattern the embryonic cuticle (Pfeiffer et al., 2002). Therefore, it is possible that Wg signaling does not define the pattern of the response but rather stabilizes patterns of gene expression that have been specified through other mechanisms in the embryonic ectoderm.

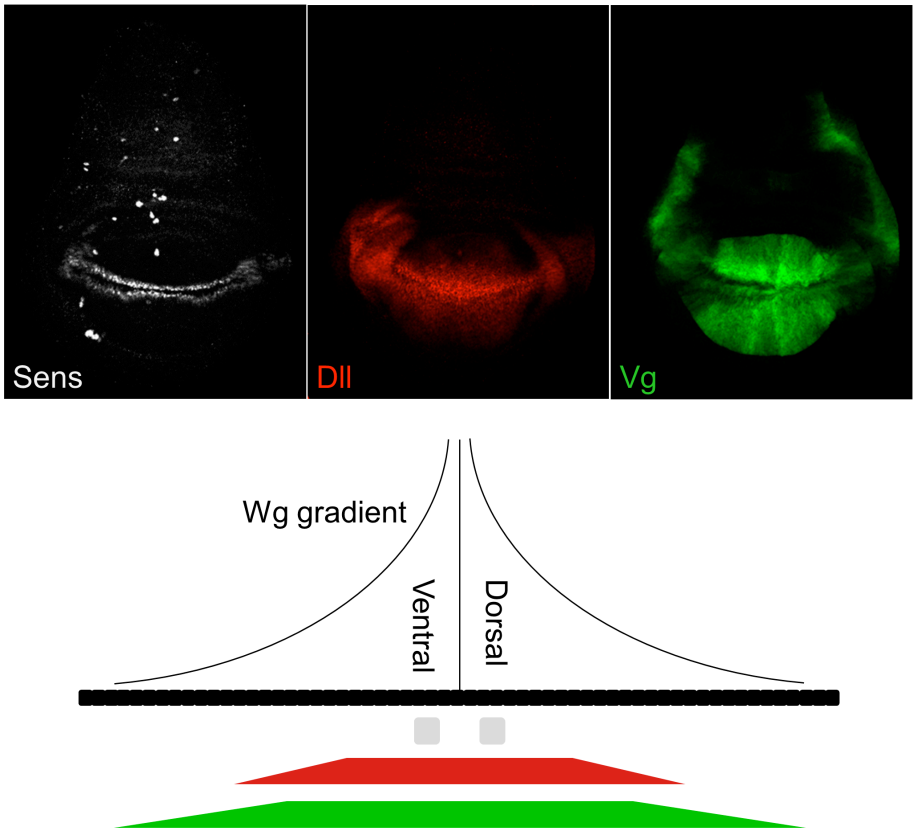


Figure 1.7.1 Wg functions as a morphogen in the *Drosophila* wing disc

Wg directly regulates the nested expression of *sens* (high threshold), *dll* (medium-low threshold), and *vg* (low threshold) target genes as a function of its concentration on either side of the dorsal/ventral compartment boundary in the wing disc. (Figure adapted from Swarup and Verheyen, 2012)

1.8. Mechanism of Wnt signaling

The regulation of the stability of the pathway effector β-catenin is the central feature of Wnt signaling (Goentoro and Kirschner, 2009; Pai et al., 1997; Papkoff et al., 1996; Peifer et al., 1994; Riggleman et al., 1990). In the absence of signaling, cytosolic β-catenin constitutively undergoes sequential phosphorylation, poly-ubiquitination, and proteolysis (Figure 1.8.1). The destruction complex composed of Axin, Adenomatous Polyposis Coli (APC), Casein Kinase 1α (CK1α), Glycogen Synthase Kinase 3 (GSK3), and Protein Phosphatase 2A (PP2A) mediates the phosphorylation of β-catenin. Although it is well established that there is a continuous flux of β-catenin through the destruction complex in the absence of signaling, the exact molecular sequence of events of an individual cycle of β-catenin phosphorylation is currently unknown. I here discuss a mechanistic model of β-catenin passage through the destruction complex based on the transient phosphorylation of its components (Kimelman and Xu, 2006). The phosphorylation state of any substrate described here is with reference to a particular kinase, phosphatase, or event and is not indicative of its absolute state of phosphorylation. The phosphorylation of the scaffolding protein Axin by GSK3 enhances complex assembly and recruits the substrate β-catenin (Ikeda et al., 1998; Jho et al., 1999; Rubinfeld et al., 1996; Yamamoto et al., 1999) (Figure 1.8.2). On binding Axin and APC within the complex (Behrens et al., 1998; Fagotto et al., 1999; Hamada et al., 1999; Hart et al., 1998; Kremer et al., 2010; Nakamura et al., 1998; Spink et al., 2001; Xing et al., 2003), β-catenin is phosphorylated by CK1α at serine 45 that is both necessary and sufficient for its subsequent phosphorylation at threonine 41, serine 37, serine 33 by GSK3 (Amit et al., 2002; Liu et al., 2002; Matsubayashi et al., 2004; Yanagawa et al., 2002) (Figure 1.8.2). Concurrently, APC is phosphorylated by the complex kinases as well (Ferrarese et al., 2007; Ha et al., 2004; Ikeda et al., 2000; Liu et al., 2006; Rubinfeld et al., 2001). The phosphorylation of Axin, β-catenin, and APC change the compositional interactions of the destruction complex over a cycle of its assembly and (partial) disassembly. In contrast to the simultaneous binding of non-phosphorylated APC and Axin to β-catenin, the phosphorylation of APC significantly enhances its affinity for the substrate to displace it from the Axin interface. This results in the APC-mediated extraction of phosphorylated β-catenin from the destruction complex and its transfer to an E3 Ubiquitin ligase complex (Ferrarese et al., 2007; Ha et

al., 2004; Liu et al., 2006; Su et al., 2008; Tickenbrock et al., 2003; Xing et al., 2004) (Figure 1.8.2).

Targeted degradation via the 26S proteasome, a multi-subunit ATP-dependent protease complex, can be achieved by the covalent addition of multiple Ubiquitin molecules (poly-ubiquitination) to one or more lysine residues on a substrate protein. Ubiquitination results in the formation of an amide bond between the C-terminus of Ubiquitin and the amino group of a substrate lysine residue. This process requires the cyclical actions of an E1 Ubiquitin activating enzyme, an E2 Ubiquitin conjugating enzyme, and an E3 Ubiquitin ligation enzyme (or ligase). The E1 activating enzyme creates a thioester bond between a conserved cysteine residue and the C-terminus carboxyl group on Ubiquitin, thereby activating it for nucleophilic attack. The activated Ubiquitin is then passed by a transthiolation reaction to a reactive cysteine residue of a specific E2 conjugating enzyme. Finally, in collaboration with an E3 ligase the Ubiquitin moiety is transferred to a lysine residue on the targeted protein (or another Ubiquitin in the case of poly-ubiquitination) (Pickart, 2001). The E3 ligase complex that poly-ubiquitinates β-catenin is $SCF^{\beta\text{-}TrCP}$ (Hart et al., 1999; Jiang and Struhl, 1998; Latres et al., 1999; Liu et al., 1999). $SCF^{\beta\text{-}TrCP}$ is composed of Roc1, Skp1, the scaffold Cullin1 (Cul1), and the F-box component β-Transducin repeat containing protein (β-TrCP) that provides substrate specificity. Phosphorylated β-catenin that is displaced from the destruction complex by APC becomes accessible to $SCF^{\beta\text{-}TrCP}$ (Figure 1.8.2). β-catenin phosphorylated at serine 33 and serine 37 provides a consensus phosphodegron recognition motif for β-TrCP that mediates its poly-ubiquitination at lysine 19 and lysine 49 (Winston et al., 1999; Wu et al., 2003). The poly-ubiquitination of β-catenin leads to its rapid proteolysis via the 26S proteasome (Aberle et al., 1997; Kitagawa et al., 1999). APC is subsequently dephosphorylated by PP2A so that the destruction complex may be reconstituted to mediate another round of β-catenin phosphorylation (Ikeda et al., 2000; Li et al., 2001; Seeling et al., 1999) (Figure 1.8.2). The sequential transfer of β-catenin from the destruction complex to the E3 ligase complex suggests that its stability can be regulated at the level of phosphorylation and/or poly-ubiquitination.

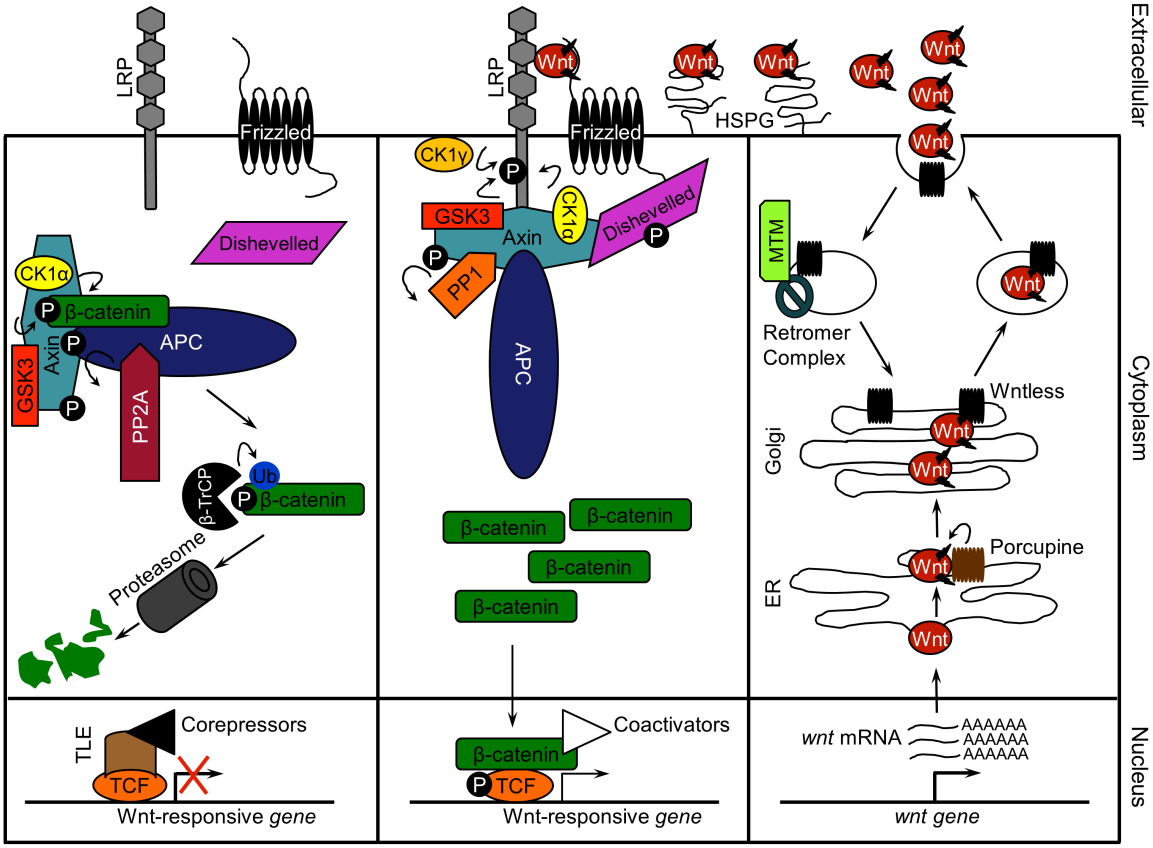


Figure 1.8.1 The Wnt signaling pathway

In the absence of pathway activity, the cytosolic effector β-catenin undergoes sequential phosphorylation, poly-ubiquitination, and proteolysis. As a result, pathway target genes are silenced by TCF with the aid of corepressors. In the presence of Wnt, the inhibitory phosphorylation of β-catenin is prevented to stabilize its levels in the cell. β-catenin subsequently forms an active transcriptional complex with TCF and coactivators to direct the expression of pathway target genes. The processing, secretion, and diffusion of the Wnt ligand is regulated prior to it initiating signaling. The pathway comprises numerous phospho-proteins including Axin, APC, β-catenin, Dishevelled, LRP and TCF. (P = Phosphate, Ub = Ubiquitin) (Figure based on MacDonald et al., 2009)

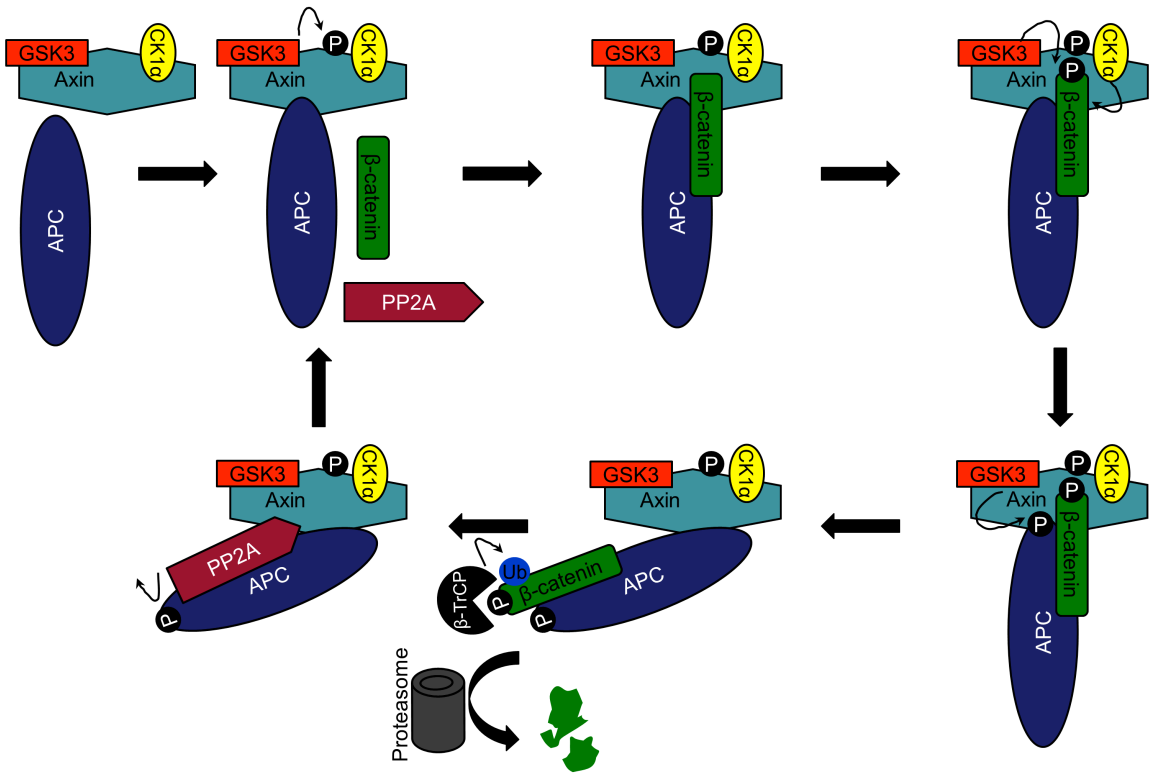


Figure 1.8.2 Model of β-catenin passage through the destruction complex

The transient phosphorylation of members of the destruction complex mediates the entry and exit of the substrate β-catenin. The phosphorylation of Axin enhances complex assembly and recruits β-catenin. Within the complex β-catenin is phosphorylated at its N-terminus and subsequently displaced from the complex by phosphorylated APC. Phosphorylated β-catenin is poly-ubiquitinated by β-TrCP and degraded via the proteasome. The PP2A-mediated dephosphorylation of APC resets the destruction complex to its basal state. (P = Phosphate, Ub = Ubiquitin) (Figure based on Kimelman and Xu, 2006)

The DNA-binding protein T-cell factor (TCF) occupies the Wnt Response Element (WRE) of pathway target genes (Atcha et al., 2007; Chang et al., 2008; Giese et al., 1991; van de Wetering et al., 1991). In the absence of signaling and stabilized levels of cytosolic β-catenin, TCF forms a repressor complex in combination with Transducin-like Enhancer of Split (TLE) and other auxiliary factors to silence the expression of pathway target genes (Brannon et al., 1997; Cadigan and Waterman, 2012; Calvo et al., 2001; Cavallo et al., 1998; Kim et al., 2000; Merrill et al., 2004; Roose et al., 1998; Thorpe et al., 1997; Yang et al., 2000b) (Figure 1.8.1).

Similar to the destabilization of β-catenin, the presence of Wnt induces the stabilization of β-catenin via phosphorylation-dependent interactions and conformational changes between pathway components (Figure 1.8.1). The simultaneous binding of Wnt to the G protein-coupled receptor (GPCR) family member Frizzled (Fz) and the single-pass transmembrane Low-density lipoprotein receptor-related protein (LRP) co-receptor initiates pathway activity (Bhanot et al., 1996; Pinson et al., 2000; Sawa et al., 1996; Tamai et al., 2000; Wehrli et al., 2000; Yang-Snyder et al., 1996). The formation of a trimeric receptor-ligand-co-receptor complex induces the phosphorylation (Lee et al., 1999; Rothbächer et al., 2000; Yanagawa et al., 1995; Yanfeng et al., 2011) and re-localization of the cytosolic protein Disheveled to the cell surface where it interacts with Fz (Tauriello et al., 2012; Umbhauer et al., 2000; Wong et al., 2003) (Figure 1.8.3). Phosphorylated Dishevelled at the plasma membrane forms oligomers that serve as a signaling platform to mediate interactions between downstream components of the pathway (Bilic et al., 2007; Rothbächer et al., 2000; Schwarz-Romond et al., 2005, 2007a). Axin (and the destruction complex) is recruited into this Dishevelled platform (Cliffe et al., 2003; Fiedler et al., 2011; Schwarz-Romond et al., 2007b) where it interacts with LRP (Mao et al., 2001; Tamai et al., 2004; Zeng et al., 2005). This dynamic assembly of protein-protein interactions at the cell surface induces the phosphorylation and activation of the cytoplasmic tail of LRP (MacDonald et al., 2008; Tamai et al., 2004; Zeng et al., 2005) (Figure 1.8.3). Remarkably, the multisite phosphorylation of LRP in response to signaling is mediated by the same GSK3 and CK1α kinases used to destabilize β-catenin in the absence of signaling. LRP is phosphorylated by these destruction complex kinases and the plasma membrane-tethered kinase CK1γ (Davidson et al., 2005; Zeng et al., 2005) (Figure 1.8.3). In contrast to the

phosphorylation of β-catenin, the phosphorylation of LRP occurs in an opposite sequence with GSK3 acting as the priming kinase for further phosphorylation by CK1 family kinases (Zeng et al., 2005). The initial phosphorylation of LRP results in the recruitment of additional Axin (and destruction complexes) to the cell surface, which in turn further enhances the phosphorylation of the co-receptor in a feed-forward loop to amplify signaling (Baig-Lewis et al., 2007; MacDonald et al., 2008; Zeng et al., 2008). Phosphorylated LRP directly binds and inhibits the activity of GSK3 towards β-catenin and Axin, presumably by accessing its catalytic pocket to act as a pseudo-substrate (Cselenyi et al., 2008; Mi et al., 2006; Piao et al., 2008; Wu et al., 2009; Zeng et al., 2005) (Figure 1.8.3). Concomitant with the inhibition of GSK3-mediated phosphorylation is the Protein Phosphatase 1 (PP1)-mediated dephosphorylation of Axin. The dephosphorylation of Axin induces a change in its conformation that causes its detachment from activated LRP and complex disassembly. The phosphorylated co-receptor can now be accessed by additional intact destruction complexes for disassembly (Kim et al., 2013; Luo et al., 2007; Willert et al., 1999a) (Figure 1.8.3). In accordance with this sequence of events, a reduced association of Axin with β-catenin and GSK3 is observed over the duration of signaling (Itoh et al., 2000; Kishida et al., 1999; Liu et al., 2005b). A subpopulation of Axin, the limiting component of the destruction complex, may also be subsequently degraded (Lee et al., 2003; Mao et al., 2001; Tolwinski et al., 2003). The inactivation of the destruction complex in response to signaling causes the accumulation of β-catenin in the cytosol which relocates to the nucleus (Figure 1.8.1).

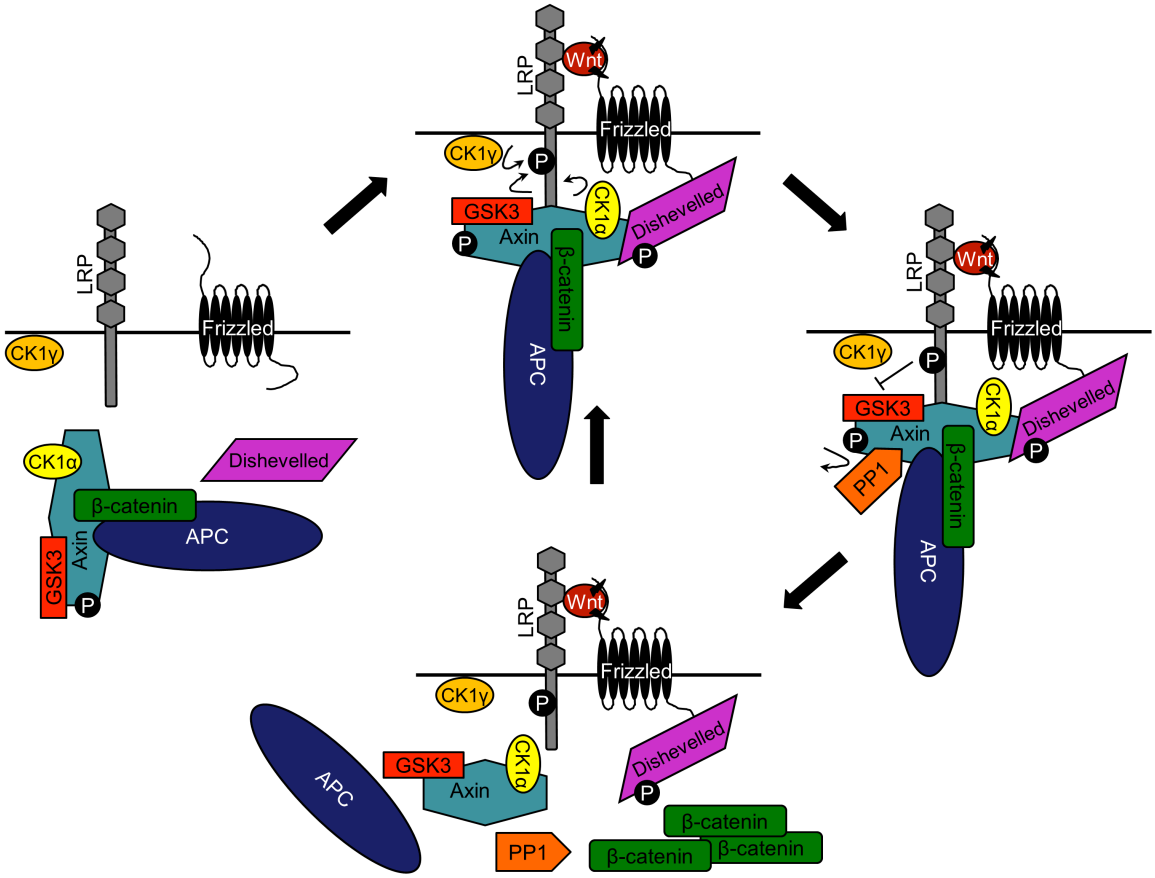


Figure 1.8.3 Model of β-catenin stabilization

The phosphorylation-dependent interactions of components of the signaling platform at the cell surface result in the stabilization of β-catenin. In response to the Wnt ligand, Dishevelled is phosphorylated and relocates to the cell surface to interact with Fz. Axin is recruited by Dishevelled which induces the phosphorylation of LRP by GSK3 and CK1 family kinases. Phosphorylated LRP in turn inhibits the ability of GSK3 to phosphorylate Axin and PP1 simultaneously dephosphorylates Axin. This causes the disassembly of the destruction complex and the stabilization of β-catenin. (P = Phosphate) (Figure based on Kim et al., 2013)

Once in the nucleus, β-catenin displaces TLE to form an active transcriptional complex with TCF to direct the expression of pathway target genes (Behrens et al., 1996; Brunner et al., 1997; Daniels and Weis, 2005; Fan et al., 1998; Graham et al., 2000; Liu et al., 2005a; Molenaar et al., 1996; Schweizer et al., 2003; van de Wetering et al., 1997) (Figure 1.8.1). The formation of an active transcriptional complex results in the recruitment of other coactivators to the WRE of target genes (Figure 1.8.1). Some of these coactivators are pathway specific, while many are non-specific and include general regulators of transcription and chromatin structure (Mosimann et al., 2009). Apart from TCF, most coactivators of the transcriptional complex bind the N- and C-termini of β-catenin (Mosimann et al., 2009). Although the molecular mechanisms by which the N- and C-termini function is incompletely understood, studies suggest that these transactivation domains alone are sufficient for directing the expression of target genes when fused to a DNA-binding protein and physically interact with one another during this process (Cox et al., 1999; Hsu et al., 1998; Orsulic and Peifer, 1996; Valenta et al., 2011; van de Wetering et al., 1997). The transcriptional complex can be regulated through phosphorylation which either promotes or inhibits signaling depending on the kinase and developmental context (Hikasa and Sokol, 2011; Hikasa et al., 2010; Ishitani et al., 1999, 2003; Lee et al., 2001a; Lo et al., 2004; Ota et al., 2012; Wang and Jones, 2006).

A historically neglected but presently active field of research addresses the post-translational modification, secretion, and diffusion of the Wnt ligand itself. Prior to its secretion, Wnt associates with the O-acyltransferase (MBOAT) family member Porcupine (Figure 1.8.1). Porcupine catalyzes the lipidation of Wnt in the endoplasmic reticulum (ER) which is essential for its secretion from the signal-producing cell and reception at the surface of the signal-receiving cell (Franch-Marro et al., 2008a; Galli et al., 2007; van den Heuvel et al., 1993; Kadowaki et al., 1996; Tang et al., 2012; Zhai et al., 2004). Lipid-modified Wnt travels along the cell's secretory pathway, and is escorted by the sorting receptor Wntless from the Golgi to the plasma membrane for secretion (Bänziger et al., 2006; Bartscherer et al., 2006; Goodman et al., 2006). Wntless is recycled back to the Golgi in a process mediated by the retromer complex (Belenkaya et al., 2008; Franch-Marro et al., 2008b; Pan et al., 2008a; Port et al., 2008; Yang et al., 2008) and the myotubularin class of lipid phosphatases (Silhankova et al., 2010) to

mediate another cycle of Wnt secretion (Figure 1.8.1). The movement of Wnt extracellularly has been proposed to be regulated by lipoprotein particles (Morrell et al., 2008; Neumann et al., 2009; Panáková et al., 2005), exosomes (Beckett et al., 2013; Greco et al., 2001; Gross et al., 2012), or through the formation of oligomers (Katanaev et al., 2008). Wnt also interacts with heparan sulfate proteoglycans (HSPGs) in the extracellular environment which regulate its diffusion (Baeg et al., 2001; Han et al., 2005; Lin and Perrimon, 1999; Tsuda et al., 1999) (Figure 1.8.1).

A distinct pool of β-catenin is associated with the plasma membrane where it is a component of cadherin-based cell structures referred to as adherens junctions. Adherens junctions mediate cell-to-cell adhesion and play crucial roles in the regulation of tissue integrity and plasticity during the development of metazoans. Cadherin, p120-catenin, β-catenin, and α-catenin comprise the adherens junctions (Heuberger and Birchmeier, 2010). The common reservoir of nascent β-catenin protein produced in the cell competitively associates with either the destruction complex that targets it for proteolysis or cadherin that sequesters it at the plasma membrane. As part of the adherens junction, β-catenin directly interacts with both cadherin (Huber and Weis, 2001; Nagafuchi and Takeichi, 1989; Ozawa et al., 1989) and α-catenin (Aberle et al., 1994; Drees et al., 2005; Pokutta and Weis, 2000; Yamada et al., 2005). Several studies have demonstrated that modulating the levels of cadherin (and the adherens junctions) influences the levels of β-catenin in the cytosol and downstream expression of Wnt pathway target genes in *Drosophila* (Cox et al., 1996; Sanson et al., 1996), *Xenopus* (Fagotto et al., 1996; Heasman et al., 1994; Torres et al., 1996), and cell culture (Marambaud et al., 2002; Maretzky et al., 2005; Orsulic et al., 1999; Reiss et al., 2005; Sadot et al., 1998; Stockinger et al., 2001). Moreover, the adherens junctions are dynamic molecular complexes and modulation of the structural integrity of these structures through phosphorylation also affects the amount of β-catenin present in the cytosol (Bek and Kemler, 2002; Dupre-Crochet et al., 2007; Lickert et al., 2000; Piedra et al., 2001, 2003; Qi et al., 2006; Roura et al., 1999). But Wnt signaling in either its silent or active state has thus far not been demonstrated to have a direct influence on the phosphorylation or poly-ubiquitination of β-catenin associated with the plasma membrane.

1.9. Reversible phosphorylation and the Wnt phospho-proteome

The spectrum of cellular responses is far more diverse than the number of signaling pathways that are implemented to direct these responses. As discussed earlier, a cell's response to signaling varies depending on the spatial and temporal developmental context (Barolo and Posakony, 2002). Another means of creating diversity in a cell's response is by regulating the qualitative state (i.e. the expression versus repression of target genes) and quantitative threshold (i.e. the level of expression of target genes) of signaling. Cells utilize the reversible phosphorylation of proteins to manipulate the state and threshold of signaling pathways (Cohen, 1992; Hunter, 1995). Reversible phosphorylation refers to the covalent attachment and removal of one or more phosphate groups (monoadducts) from a substrate in a process that is mediated by enzymes called kinases and phosphatases respectively. A phosphorylation reaction is catalyzed by a kinase and involves the transfer of the terminal phosphate group from adenosine triphosphate (ATP) to a hydroxyl group of a residue on the protein substrate. ATP is converted to adenosine diphosphate (ADP) by the loss of the phosphate group. This modification is dynamic and is reversed by a phosphatase-mediated hydrolysis reaction (dephosphorylation) that removes the phosphate group to reconstitute the original residue (Fischer and Krebs, 1955; Krebs and Fischer, 1956; Krebs et al., 1959; Sutherland and Wosilait, 1955). Phosphorylation predominantly occurs on serine, threonine, and tyrosine residues of eukaryotic proteins, although in some cases histidine, arginine, aspartate, and lysine residues may also be phosphorylated (Olsen et al., 2006). Due to the size of its ionic shell and charge, a phosphate group confers unique properties on a residue and provides a means of diversifying the chemical nature of the protein surface. The phosphorylation state of a protein thus determines its (local) conformation which influences its functional properties including its activity, stability, interacting partners, or subcellular localization (Hunter, 2012). Reversible phosphorylation is the most frequently employed post-translational protein modification, the importance of which is demonstrated by the presence of large classes of kinases and phosphatases in humans, encoding 2.25% and 1% respectively of the genome (Cohen, 2002; Manning et al., 2002; Shi, 2009).

The Wnt pathway is comprised of numerous phospho-proteins including the effector, co-receptor, intermediaries, and transcription factor amongst others. Accordingly, a number of kinases and phosphatases have been implicated in the phospho-regulation of this pathway from the signal-producing cell to the signal-receiving cell (Verheyen and Gottardi, 2010). However, recent differential phospho-proteome analyses from *Drosophila* and mammalian cell lines have identified novel pathway components and novel phosphorylation sites on known pathway components (Bodenmiller et al., 2007; Tang et al., 2007). It is therefore likely that our current understanding of enzymes that regulate the Wnt pathway through reversible phosphorylation is incomplete.

1.10. Aims of thesis

Preliminary evidence from our laboratory has implicated *Drosophila* Hipk as a novel kinase component of the Wnt pathway. The first aim of this thesis is to uncover the mechanistic role of Hipk in the regulation of Wnt signaling using genetic and biochemical analyses. The second aim of this thesis is to implement an *in vivo* kinome and phosphatome loss-of-function screen in *Drosophila* to identify a more complete list of enzymes that regulate Wnt signaling through reversible phosphorylation.

2. Hipks promote the Wnt pathway at multiple levels of signaling

The experimental data from this chapter have been published in the following journals: Lee, W., Swarup, S., et al. (2009). Development *136*, 241–251. Swarup, S., and Verheyen, E.M. (2011). PNAS *108*, 9887–9892. Verheyen, E.M., Swarup, S., and Lee, W. (2012). Fly *6*, 126-131. Wendy Lee performed the experiments for Figures 2.2.1 A-D, 2.3.1 A-E, 2.4.1 A-H, 2.5.1 A-E. I performed the experiments for all other figures.

2.1. Homeodomain-interacting protein kinases

Homeodomain-interacting protein kinases (Hipks) are an evolutionarily conserved subfamily of enzymes (Kim et al., 1998). Based on the taxonomic classification of the catalytic domain of eukaryotic protein kinases, Hipks are grouped within the Dual-specificity tyrosine phosphorylation-regulated kinase (DYRK) family, which in turn is part of the Cyclin-dependent kinase (CDK), Mitogen-activated protein kinase, Glycogen synthase kinase, CDK-like kinase (CMGC) group of protein kinases (Hanks and Hunter, 1995; Hofmann et al., 2000). The Dyrk and Pre-mRNA processing protein 4 kinase (Prp4) subfamilies are the other classifications included within the DYRK family (Aranda et al., 2011) (Figure 2.1.1). Vertebrates possess four members of the Hipk subfamily, Hipk1-4, while only a single member exists in the invertebrate model organisms *Drosophila* and *Caenorhabditis*, Hipk and Hpk-1 respectively (Aranda et al., 2011; Kim et al., 1998) (Figure 2.1.1). With the exception of the more distantly related Hipk4, members of this family share a common protein structure characterized by a N-terminus kinase domain, a homeoprotein-interaction domain (HID), a PEST domain rich in proline (P), glutamic acid (E), serine (S), threonine (T) residues, and an auto-inhibitory domain (AID) at the C-terminus (Kim et al., 1998; Rinaldo et al., 2007a; Sombroek and Hofmann, 2009) (Figure 2.1.2). Our knowledge of the stability, activity, subcellular localization, interacting partners, and functional roles in developmental processes of

Hipks is a result of biochemical, cell biological, and genetic analyses performed predominantly using vertebrate Hipk2.

Characteristic of other members of the DYRK family, Hipks are dual-specificity kinases and have the capacity to phosphorylate serine, threonine, and tyrosine residues (Aranda et al., 2011). The tyrosine phosphorylation activity of members of this family is however restricted to auto-phosphorylation of the activation loop within the kinase domain and is mediated by a *cis* intramolecular mechanism in the case of the Hipk subfamily (Han et al., 2012; Kentrup et al., 1996; Lochhead et al., 2005; Saul et al., 2013; Siepi et al., 2013). Hipks therefore phosphorylate substrates on only serine and threonine residues. A comparison of the kinase domains of Hipks between vertebrates and invertebrates shows over 90% conservation in protein sequence (Figure 2.1.3). The kinase domain of Hipk2 comprises the phosphate-anchor and the activation loop that are indispensable for catalytic activity. The phosphate-anchor lysine residue interacts with the donor ATP to position it in the correct orientation while the activation loop is required to stabilize the kinase in an active conformation. The HID is approximately 70% conserved in protein sequence across diverse species and facilitates the interaction of Hipks with homeodomain transcription factors of the Nkx and Hox families (Choi et al., 1999; Kim et al., 2006, 1998) (Figure 2.1.3). The PEST domain regulates the interaction of Hipks with numerous non-homeodomain proteins including C-terminal binding protein (CtBP), p300, Groucho, Nemo-like kinase, and High mobility group protein (Rinaldo et al., 2007a). The Hipk2 PEST domain contains nuclear localization sequences that are required by members of this family to localize to sub-nuclear structures (de la Vega et al., 2011) (Figure 2.1.2). Hipk2 predominantly localizes to nuclear speckles and the nucleoplasm (Moller et al., 2003), but a minor population is also present in the cytoplasm (Pierantoni et al., 2007). The AID has been proposed to partially inhibit the catalytic activity of the kinase domain and yields a hyperactive form of the kinase when deleted or cleaved through the action of caspases (Choi et al., 2005; Gresko et al., 2006; Rui et al., 2004). Aside from phosphorylation within the putative activation loop of the kinase domain, Hipk2 is regulated by post-translational modifications at lysine residues located at the termini of the protein. Sumoylation at lysine 25 of Hipk2 regulates its activity and interaction partners (Gresko et al., 2005; Hofmann et al., 2005; Roscic et al., 2006),

while poly-ubiquitination of Hipk2 at lysine 1182 regulates its stability and levels within the cell (Rinaldo et al., 2007b) (Figure 2.1.2).

Hipks are essential for viability and have pleiotropic functions during development such as the regulation of hematopoietic differentiation (Aikawa et al., 2006; Hattangadi et al., 2010; Wee et al., 2008), sensory neuron survival (Doxakis et al., 2004; Wiggins et al., 2004; Zhang et al., 2007), and cell cycle arrest or apoptosis via its interaction with p53 (D'Orazi et al., 2002; Gresko et al., 2006; Hofmann et al., 2002; Mayo et al., 2005; Pistritto et al., 2007; Puca et al., 2009; Rui et al., 2004).

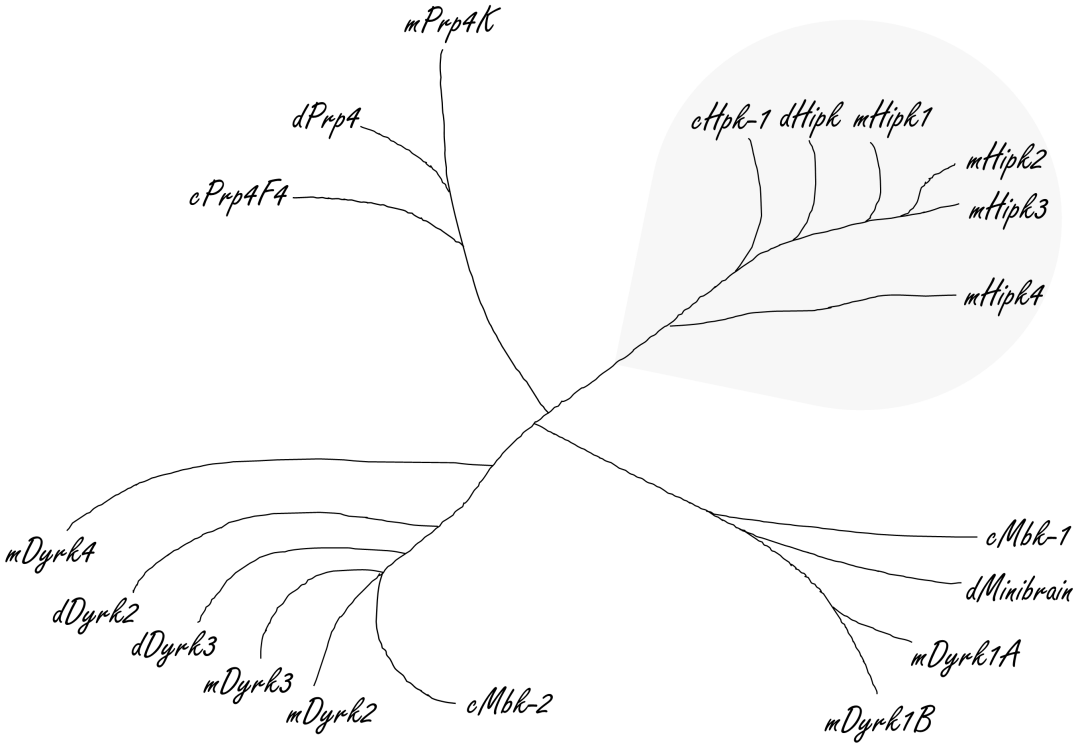


Figure 2.1.1 DYRK family of enzymes

An unrooted phylogenetic classification of the DRYK family of enzymes based on homology of the kinase domain. Members of the Hipk, Prp4, and Dyrk subfamilies are present in invertebrates (*Drosophila* = d, *Caenorhabditis* = c) and vertebrates (*Mus* = m). The Hipk subfamily is shaded in grey. (Figure based on Aranda et al., 2011)

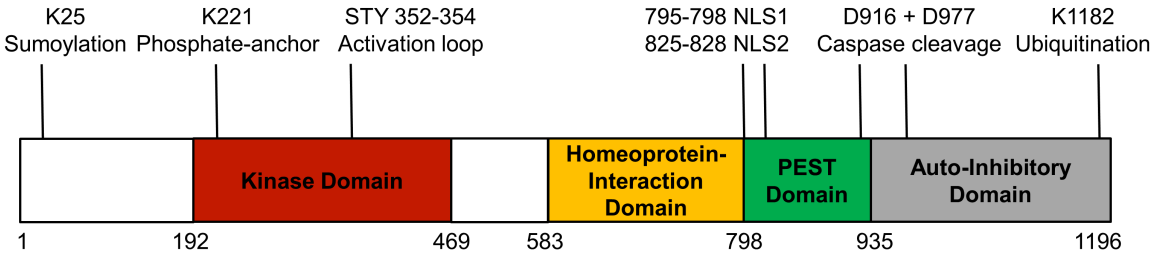


Figure 2.1.2 Schematic representation of vertebrate Hipk2

Members of the Hipk subfamily are characterized by a catalytic kinase domain, a HID domain used to interact with homeoproteins, a PEST domain used to interact with non-homeoproteins, and an auto-inhibitory domain. (Figure based on Rinaldo et al., 2007a)

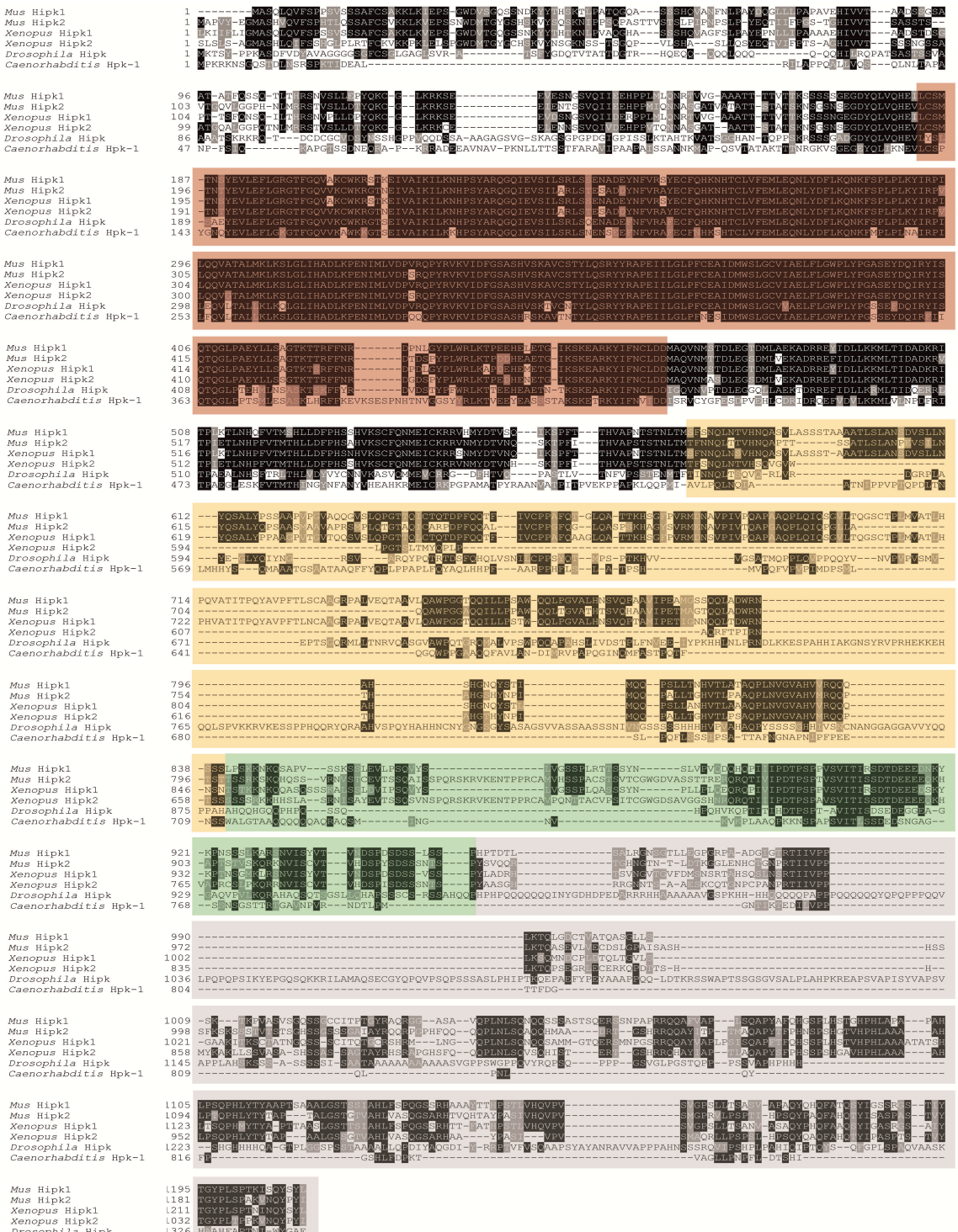


Figure 2.1.3 Comparison of the Hipk subfamily across species

A comparison of the protein sequences of Hipks between vertebrates and invertebrates including the kinase domain (red), HID (orange), PEST domain (green), and AID (grey). Boxes shaded in black or grey represent identical or similar residues respectively.

2.2. Modulation of *hipk* function phenocopies Wg signaling in multiple developmental contexts

The elimination (somatic clones) or reduction (RNAi) of *hipk* function resulted in the loss of parts of the *Drosophila* wing margin (Figure 2.2.1 A-C), a phenotype reminiscent of decreased Wg signaling (Couso et al., 1994; Phillips and Whittle, 1993; Rulifson et al., 1996). Conversely, over-expression of a *hipk* transgene in the wing field mimicked an increase in Wg signaling to induce the formation of an ectopic wing margin (Diaz-Benjumea and Cohen, 1995) (Figure 2.2.1 D). In the *Drosophila* eye, the ectopic expression of *wg* under the control of the *sevenless* (*sev*) promoter disturbs the normal arrangement of ommatidial facets and gives rise to a reduced surface (Figure 2.2.1 E). This phenotype can be suppressed by concomitantly inhibiting Wg signaling through the down regulation of positive regulators or over-expression of negative regulators of the pathway (Bänziger et al., 2006; Brunner et al., 1997; Cadigan and Nusse, 1996; Port et al., 2011). Knock-down of *hipk* function in this genetic background partially suppressed the *sev>wg* phenotype to restore the size of the eye (Figure 2.2.1 F). The results of these phenotypic analyses suggested that Hipk functions as a positive regulator of Wg signaling in multiple tissues in *Drosophila*.

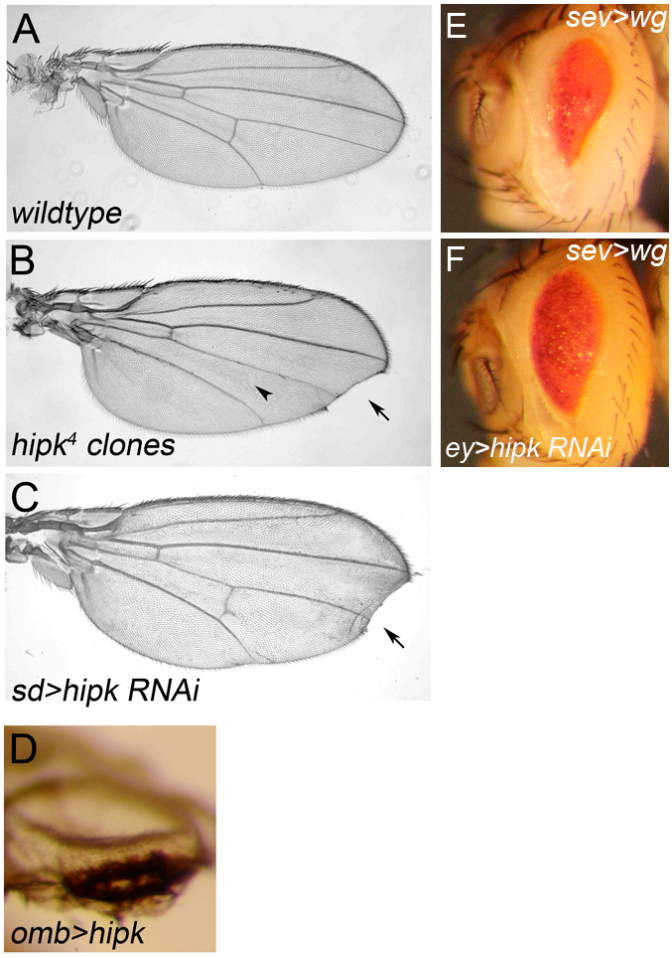


Figure 2.2.1 Modulation of *hipk* phenocopies Wg signaling

(A) A *wild type* wing. (B) *hipk*^{4} somatic loss-of-function clones or a (C) *UAS-hipk RNAi* transgene regulated by the *sd-Gal4* driver resulted in loss of part of the wing margin (arrow heads). (D) Over-expression of *UAS-hipk* using the *omb-Gal4* driver specified an additional wing margin. (E) Ectopic expression of *wg* from the *sev* promoter generated a reduced eye surface. (F) This effect was suppressed to partially restore the size of the eye through the simultaneous knock-down of *hipk* function using the *ey-Gal4* driver.

2.3. Hipk acts downstream of the Wg-Fz-Arr complex to promote signaling in the *Drosophila* wing

As Wg signaling is required to specify the wing field during development, the use of a $wg^{1}/wg^{1\text{-}17}$ loss-of-function allelic combination induced a wing-to-notum transformation with a normally patterned wing occurring in only 14% of individuals (n=72) (Morata and Lawrence, 1977; Sharma and Chopra, 1976) (Figure 2.3.1 B). Over-expression of *hipk* in this genetic background restored the wing structure and suppressed its transformation to notal tissue in 41% of individuals (n=39) (Figure 2.3.1 C). The over-expression of a truncated dominant-negative form of the Fz receptor, *fz2N33*, inhibited Wg signaling and caused loss of the wing margin in all individuals examined (n=41) (Zhang and Carthew, 1998) (Figure 2.3.1 D). Elevating the function of *hipk* in this background restored the wing margin structure with a penetrance of 100% (n=47) (Figure 2.3.1 E). A chimeric protein of Fz and Arr (*Drosophila* LRP) has been previously shown to act as a constitutively active receptor complex capable of autonomously signaling to downstream components in the presence or absence of the Wg ligand (Baig-Lewis et al., 2007; Tolwinski et al., 2003). The over-expression of a *fz2-arr* transgene in the wing field produced ectopic sensory bristles on the wing blade in all individuals tested (n=21) (Baig-Lewis et al., 2007) (Figure 2.3.1 F). Sensory bristles on the wing margin require robust levels of pathway activity to be specified (Couso et al., 1995; Phillips and Whittle, 1993), and ectopic Wg signaling induces the formation of bristles in a wider field around the wing margin (Axelrod et al., 1996; Cadigan et al., 1998; Neumann and Cohen, 1996). The reduction of *hipk* function in combination with the *fz2-arr* transgene suppressed the formation of ectopic bristles in 30% of individuals (n=22) (Figure 2.3.1 G). These genetic interaction studies in the *Drosophila* wing suggested that enhanced Hipk function can compensate for loss or inhibition of Wg signaling at the level of the ligand-receptor complex. Conversely, reduction of Hipk levels can dampen signaling initiated from a constitutively active form of the receptor complex. Therefore, Hipk promotes Wg signaling downstream of the Wg-Fz-Arr complex.

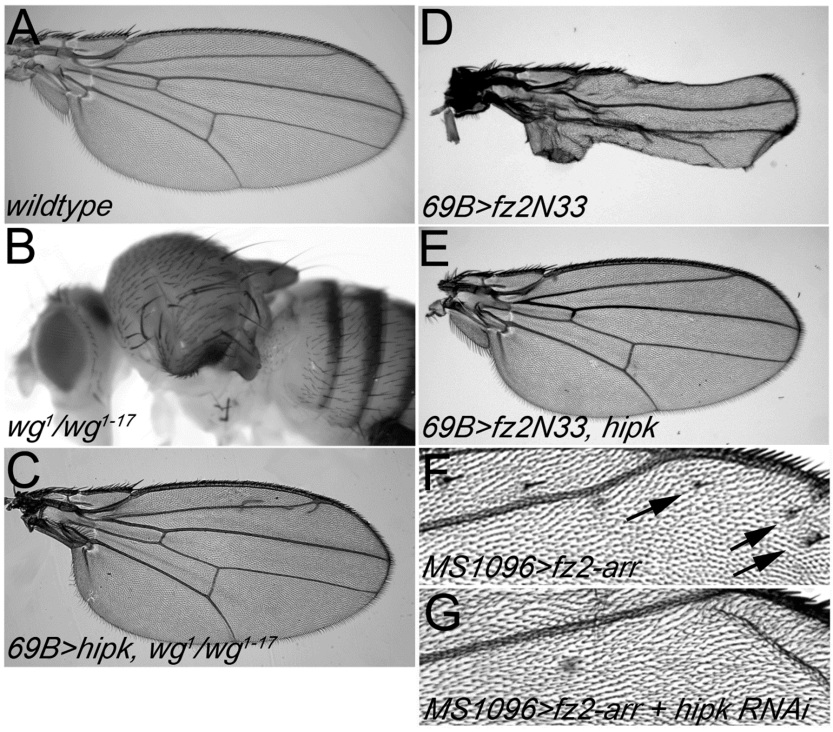


Figure 2.3.1 Hipk promotes Wg signaling downstream of ligand-receptor complex

(A) A *wild type* wing. (B-C) The wing-to-notum transformation generated through the wg^{1}/wg^{1-17} allelic combination was suppressed through the over-expression of *UAS-hipk* using the *69B-Gal4* driver. (D-E) Over-expression of dominant negative *UAS-fz2N33* using the *69B-Gal4* driver resulted in the loss of the wing margin which was restored by the simultaneous over-expression of *UAS-hipk*. (F-G) Expression of a constitutively active *UAS-fz2-arr* transgene produced ectopic sensory bristles on the wing blade (arrows), an effect which was suppressed by knock-down of *hipk* function using the *MS1096-Gal4* driver.

2.4. Hipk regulates Arm stability to promote pathway target gene expression

In response to Wg signaling, Arm (*Drosophila* β-catenin) is stabilized in two stripes flanking the dorsal/ventral compartment boundary of the wing disc (Peifer et al., 1991) (Figure 2.4.1 A, A'). Loss-of-function clones of $hipk^4$ autonomously displayed reduced levels of stabilized Arm in cells that receive the Wg signal (Figure 2.4.1 B-D, B'-D'). $hipk^4$ is a null allele with no gene function (Lee et al., 2009a). Conversely, over-expression of *hipk* in the wing field increased the levels of stabilized Arm (relative to *wild type*), as detected *in vivo* (Figure 2.4.1 E-F) and through western blot analysis (Figure 2.4.1 I). Over-expression of *hipk* also enhanced the expression of the pathway target gene *dll* (Figure 2.4.1 G-H). Hipk therefore functions in the signal-receiving cell to regulate the stability of the pathway effector Arm and direct target gene expression.

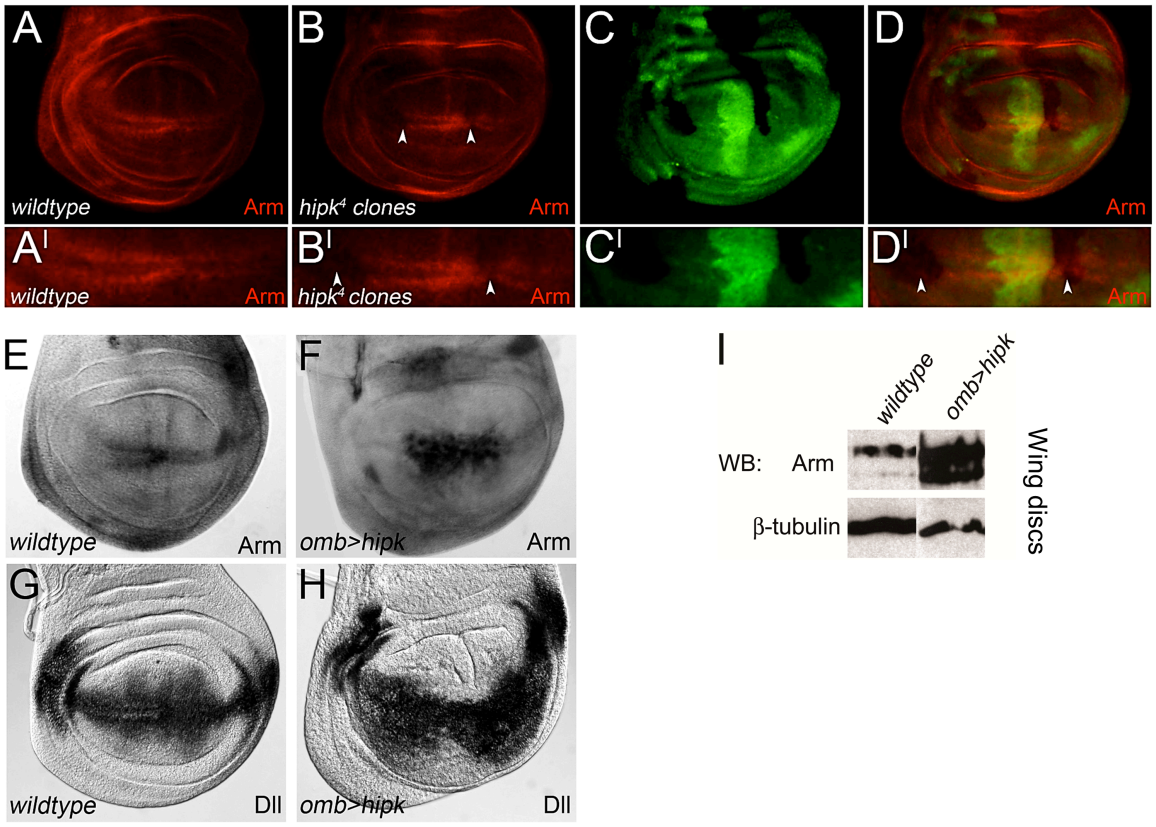


Figure 2.4.1 Hipk stabilizes Arm to promote the Wg pathway

(A, A') Arm is stabilized in two stripes adjacent to the dorsal/ventral compartment boundary in response to Wg signaling in the wing disc. (B-D, B'-D') The levels of stabilized Arm were decreased in *hipk*^{4} somatic clones (loss of GFP) (arrow heads). (E-H) The over-expression of *UAS-hipk* using the *omb-Gal4* driver enhanced the levels of stabilized Arm and the target gene *dll* in the wing disc relative to the *wild type* control. (I) Protein lysates from wing discs with elevated *hipk* function had higher levels of stabilized Arm relative to the *wild type* control.

2.5. Hipk stabilizes Arm independent of the Wg signal

To further elucidate Hipk's effect on the levels of stabilized Arm, the consequence of reducing *hipk* function on the stability of exogenous forms of Arm was evaluated in the *Drosophila* wing disc. The arm^{S2} and arm^{S10} transgenes encode epitope-tagged regular and degradation-resistant forms of the Arm protein respectively (Pai et al., 1997). The over-expression of exogenous arm^{S2} in the wing field resulted in its accumulation at the dorsal/ventral compartment boundary within the Wg signaling domain and in the dorsal hinge primordium away from the Wg signaling domain (Figure 2.5.1 A-B). Although ectopically expressed throughout the wing field, exogenous Arm accumulated to detectable levels in only certain regions as most of it was degraded by the constitutively operational destruction complex within the cell (Kimelman and Xu, 2006). When over-expressed in $hipk^{3}/hipk^{4}$ mutant discs, there was a significant reduction in the levels of exogenous Arm and only a fraction was observed at the dorsal/ventral compartment boundary but none away from the Wg signaling domain (Figure 2.5.1 C). $hipk^{3}$ is a hypomorphic allele of the gene (Lee et al., 2009a). The over-expression of arm^{S10} resulted in the accumulation of the degradation-resistant protein throughout the wing field (Figure 2.5.1 D). As expected, the levels of this form of the protein were unaffected in discs reduced for *hipk* function (Figure 2.5.1 E). Our data suggested that Hipk can stabilize exogenous Arm within and away from the Wg signaling domain while constitutively stable exogenous Arm bypasses the need for Hipk function. The levels of endogenous stabilized Arm away from the Wg signaling domain are negligible and not easily detectable in the wing disc. The morphogenetic furrow in the eye disc is a physical indentation in the epithelial surface generated through the constriction of cells (Ready et al., 1976; Treisman and Rubin, 1995). Due to the constriction of large numbers of these cells in the morphogenetic furrow, the levels of endogenous stabilized Arm in the absence of Wg signaling can be detected and assayed in this tissue. Somatic clones of $hipk^{4}$ in the morphogenetic furrow displayed reduced levels of endogenous stabilized Arm (Figure 2.5.1 F-G). Our results indicated that Hipk can promote the stability of endogenous or exogenous Arm independent of the Wg signal *in vivo*.

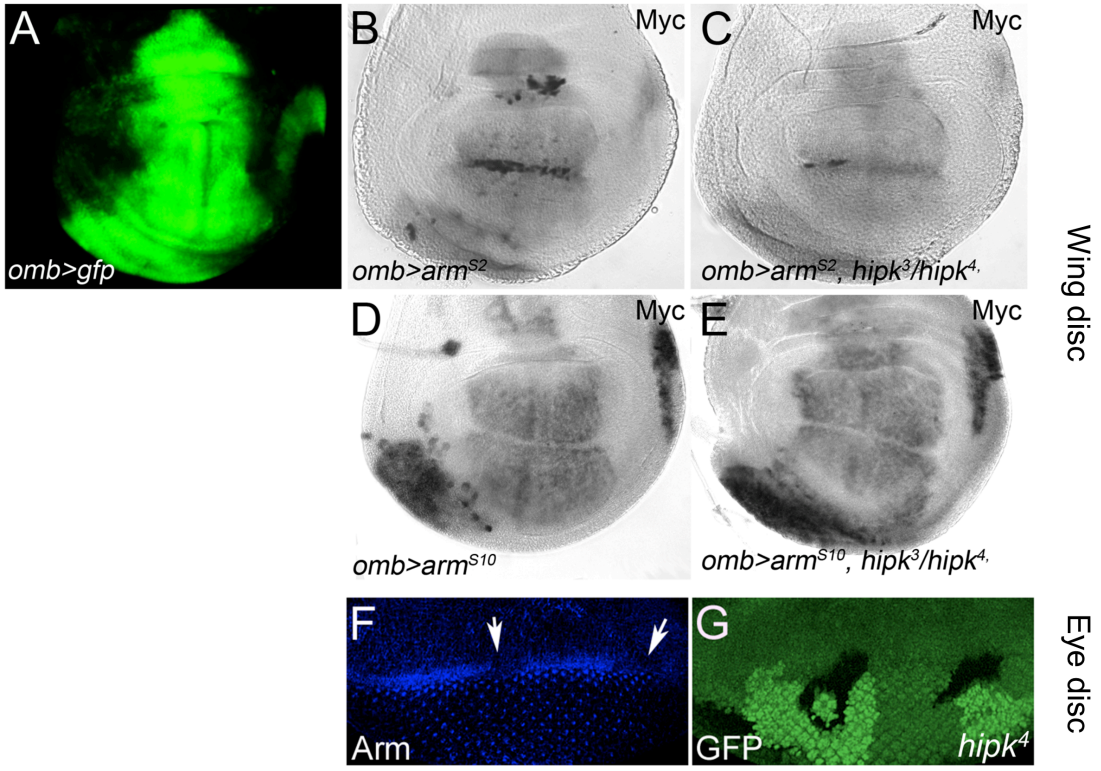


Figure 2.5.1 Hipk stabilizes Arm independent of Wg signaling

(A) The *omb-Gal4* driver expression domain in the wing field. (B) Exogenous Myc-tagged Arm^{S2} that is over-expressed using the *omb-Gal4* driver accumulated in a *wild type* wing disc (C) but not in a $hipk^3/hipk^4$ mutant disc. (D-E) The levels of over-expressed exogenous Myc-tagged degradation-resistant Arm^{S10} were equivalent in *wild type* and $hipk^3/hipk^4$ mutant discs. (F-G) $hipk^4$ loss-of-function clones (absence of GFP) (arrows) had reduced levels of endogenous stabilized Arm in the morphogenetic furrow of the eye disc.

2.6. Hipk promotes Wg signaling and Arm stability in a kinase-dependent process

The ability of Hipk to promote Wg signaling by stabilizing the levels of cytosolic Arm is dependent on its catalytic kinase activity. Transcriptional reporter assays performed in *Drosophila* S2R+ cells (Yanagawa et al., 1998) in the absence or presence of the Wg ligand suggested that only Hipk WT (wild type) but not Hipk KD (a kinase-inactive version of the protein that has the lysine residue of the phosphate-anchor mutated to alanine) can promote signaling. Hipk KD in fact inhibited signaling by acting as a dominant-negative version of the protein (Figure 2.6.1 A). These assays were performed using the Topflash reporter that responds to Wg pathway activity. The Topflash reporter has multiple TCF-binding sites cloned upstream of a luciferase gene. The Fopflash reporter has mutated TCF-binding sites and serves as a negative control. The requirement of the kinase activity of Hipk to stabilize Arm levels was also evaluated in a protein stability assay in HEK293T cells. We found that Hipk WT but not Hipk KD prolonged the stability of exogenous Arm in cell culture. For this assay HEK293T cells were treated with the protein synthesis inhibitor cycloheximide (CHX) to prevent the translation of nascent protein (Figure 2.6.1 B).

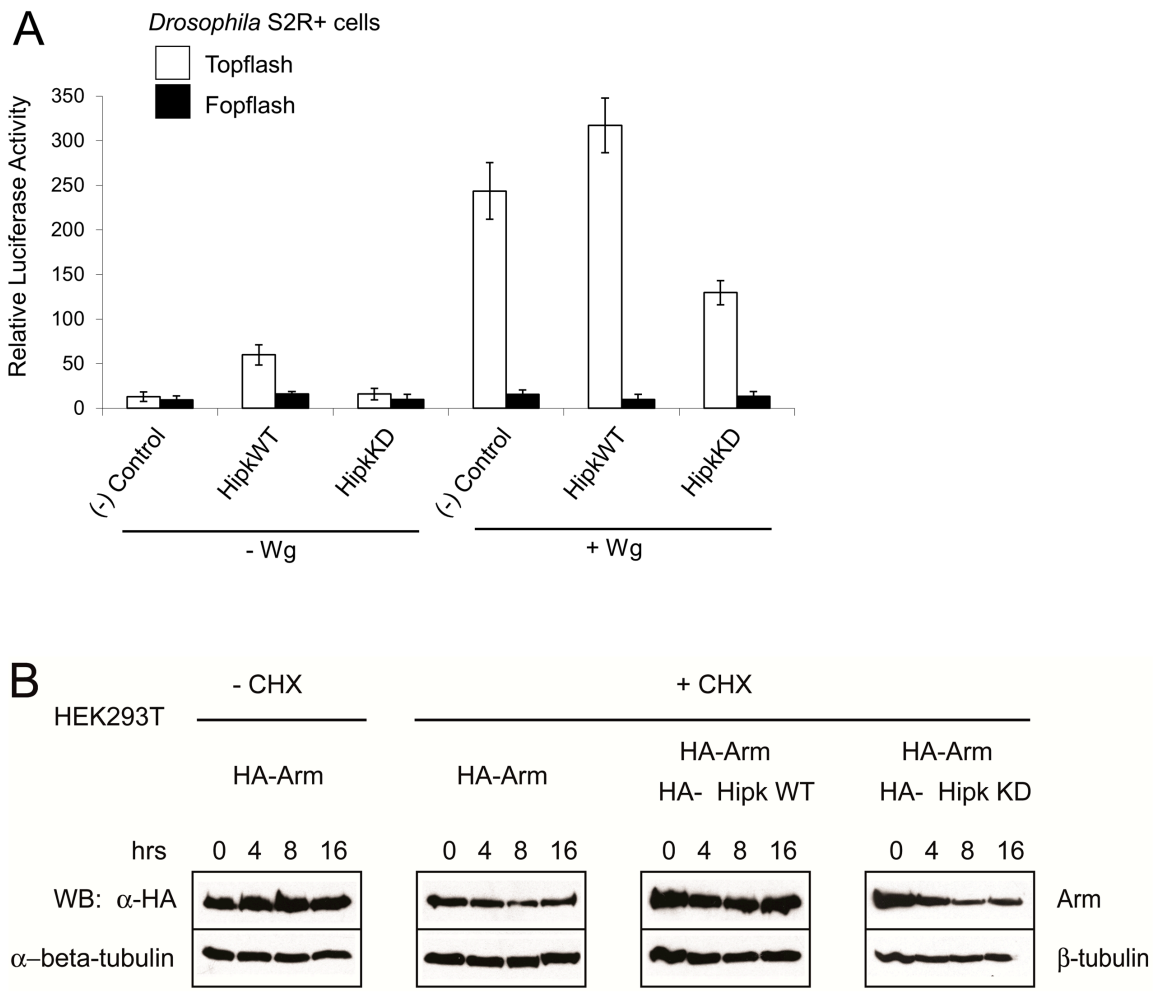


Figure 2.6.1 Hipk's kinase activity is required to promote Wg signaling

(A) Hipk WT (wild type) but not kinase-inactive Hipk KD promoted the transcriptional response from a TCF-regulated reporter (Topflash) but not a mutated reporter (Fopflash) both in the absence and presence of the Wg ligand in *Drosophila* S2R+ cells. (B) The presence of Hipk WT but not Hipk KD prolonged the stability of exogenous Arm in HEK293T cells treated with the protein synthesis inhibitor cycloheximide.

2.7. Hipk genetically interacts with components of the destruction complex and ubiquitination machinery

As Hipk enhanced the levels of stabilized Arm both in the presence and absence of Wg signaling, it must inherently function to inhibit the destruction complex and/or ubiquitination machinery that control Arm phosphorylation and poly-ubiquitination respectively. To test this hypothesis *axin*, *cul1,* and *supernumerary limbs* (*slimb*) (*Drosophila* β-TrCP) were ectopically expressed individually and in combination with *hipk* to evaluate their effect on the levels of stabilized Arm. Over-expression of *axin* in the wing disc reduced stabilized Arm levels adjacent to the dorsal/ventral compartment boundary (Figure 2.7.1 D). Co-expression of *hipk* suppressed this effect of *axin* (Figure 2.7.1 E). Similarly, reduced levels of stabilized Arm as a consequence of over-expression of *cul1* (Figure 2.7.1 F) and *slimb* (Figure 2.7.1 H) were restored by the simultaneous over-expression of *hipk* (Figure 2.7.1 G, I). These results suggested that *hipk* genetically interacts with and inhibits components of the destruction complex and ubiquitination machinery. But as Arm phosphorylation is a prerequisite for its poly-ubiquitination (Liu et al., 1999, 2002), our genetic interaction studies do not distinguish between these possibilities.

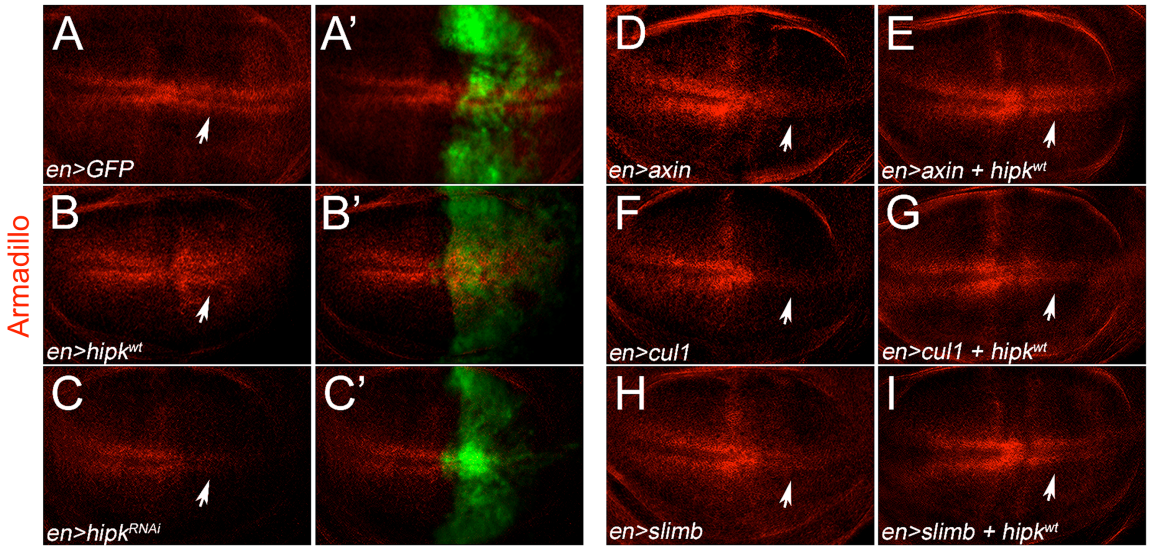


Figure 2.7.1 Hipk inhibits the function of negative regulators of Arm stability

(A, A') Arm is stabilized in two stripes of cells adjacent to the dorsal/ventral compartment boundary in response to Wg signaling in the wing disc (arrows). *en-Gal4* expression domain in the posterior compartment (presence of GFP) of the wing disc. (B, B') Over-expression of *hipk* using *en-Gal4* enhanced the levels of stabilized Arm (arrows) whereas (C, C') reduction of *hipk* function through RNAi decreased the levels of stabilized Arm (arrows). (D) Using *en-Gal4* to over-express *axin* resulted in the loss of stabilized Arm levels (arrows). (E) This effect was suppressed by the co-expression of *hipk* (arrows). (F-I) Similarly, the effects of over-expression of *cul1* and *slimb* were suppressed by the co-expression of *hipk* to restore the levels of stabilized Arm (arrows).

2.8. Hipk inhibits Arm poly-ubiquitination but not phosphorylation *in vitro*

Our genetic analyses in the wing disc suggested that Hipk suppresses the phosphorylation and/or poly-ubiquitination of Arm to stabilize its levels in the cytosol. We thus investigated whether Hipk could inhibit one or both of these processes through the use of biochemical assays. In an *in vitro* kinase assay, Arm was phosphorylated by CK1α and Sgg (*Drosophila* GSK3) as detected with a phospho-β-catenin (S33/S37) antibody (Figure 2.8.1 A). As the consensus phosphorylation sites (along with the region surrounding them) at the N-terminus of β-catenin are perfectly conserved in Arm, the antibody that detects β-catenin phosphorylated at serine 33 and serine 37 also recognizes the corresponding serine 44 and serine 48 phospho-epitope in Arm (Liu et al., 2002; Matsubayashi et al., 2004). We found that the presence of Hipk does not inhibit the ability of CK1α and Sgg to phosphorylate Arm *in vitro* (Figure 2.8.1 A). To evaluate whether Hipk had any effect on the poly-ubiquitination of Arm, an *in vitro* ubiquitination assay was performed with an E1 activating enzyme, a specific E2 conjugating enzyme (UbcH5), Slimb (and other components of the E3 ligase complex), and the substrate. As our earlier analyses suggested that Hipk stabilizes Arm in a process that is dependent on its catalytic activity, Slimb and Hipk were first subjected to an *in vitro* kinase assay prior to the ubiquitination assay with the substrate. Arm was abundantly poly-ubiquitinated in the presence of Slimb as detected by a poly-Ubiquitin antibody (Figure 2.8.1 B). Pre-incubation with Hipk in a kinase assay reduced the ability of Slimb to poly-ubiquitinate Arm. This ability of Hipk to inhibit Slimb function is kinase-dependent as pre-incubation with Hipk in the absence of ATP did not impede the subsequent Slimb-mediated poly-ubiquitination of Arm (Figure 2.8.1 B). Hipk's ability to reduce Arm poly-ubiquitination is a consequence of its phosphorylation of Slimb and not Arm, as pre-incubation of Hipk and Arm in a kinase assay prior to the addition of Slimb did not inhibit Arm poly-ubiquitination (Figure 2.8.1 B). Therefore, Hipk inhibits Arm poly-ubiquitination with no effect on its phosphorylation *in vitro*.

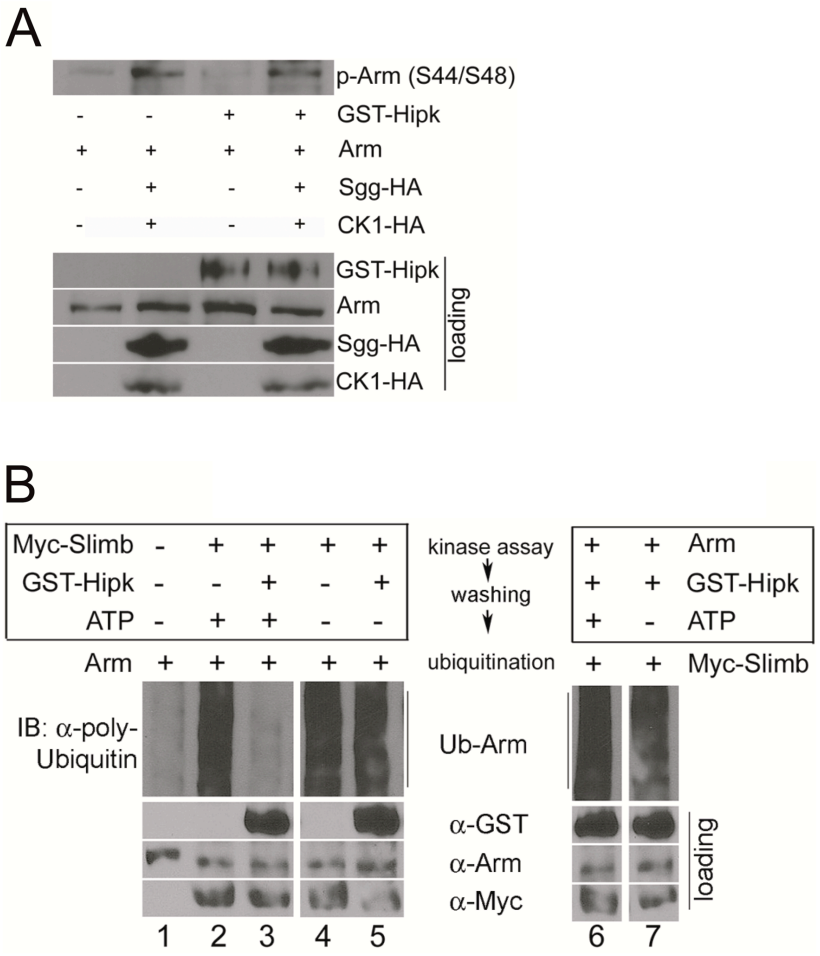


Figure 2.8.1 Hipk inhibits Arm poly-ubiquitination and not phosphorylation

(A) In an *in vitro* kinase assay, Arm was sequentially phosphorylated in the presence of CK1α and Sgg as detected by a phospho-specific Arm (S44/S48) antibody. The presence of GST-Hipk had no effect on the phosphorylation of Arm by the destruction complex kinases. (B) In an *in vitro* ubiquitination assay, Arm was poly-ubiquitinated in the presence of Slimb but not in its absence. Pre-incubation of GST-Hipk with Slimb in a kinase assay inhibited its ability to poly-ubiquitinate Arm. Pre-incubation of GST-Hipk with Slimb in the absence of ATP did not inhibit its ability to poly-ubiquitination Arm. Pre-incubation of GST-Hipk with Arm in a kinase assay did not inhibit its Slimb-mediated poly-ubiquitination.

2.9. Hipk interacts with and phosphorylates Slimb

The results of our biochemical assays suggested that Hipk phosphorylates and inhibits Slimb function to prevent the poly-ubiquitination of Arm. As pre-incubation in the kinase assay and the subsequent ubiquitination assay was performed with all components of the E3 ligase complex, we wanted to confirm that Hipk interacts with and phosphorylates Slimb and not Roc1, Skp1, or Cul1. GST-Hipk interacted with Myc-Slimb protein from *Drosophila* adults in a co-immunoprecipitation assay. This interaction is not a consequence of the GST moiety (Figure 2.9.1 A). Moreover, GST-Hipk also phosphorylated Myc-Slimb *in vitro* (Figure 2.9.1 B). It is likely that the association of Hipk with the substrate recognition component Slimb of the E3 ligase complex is direct.

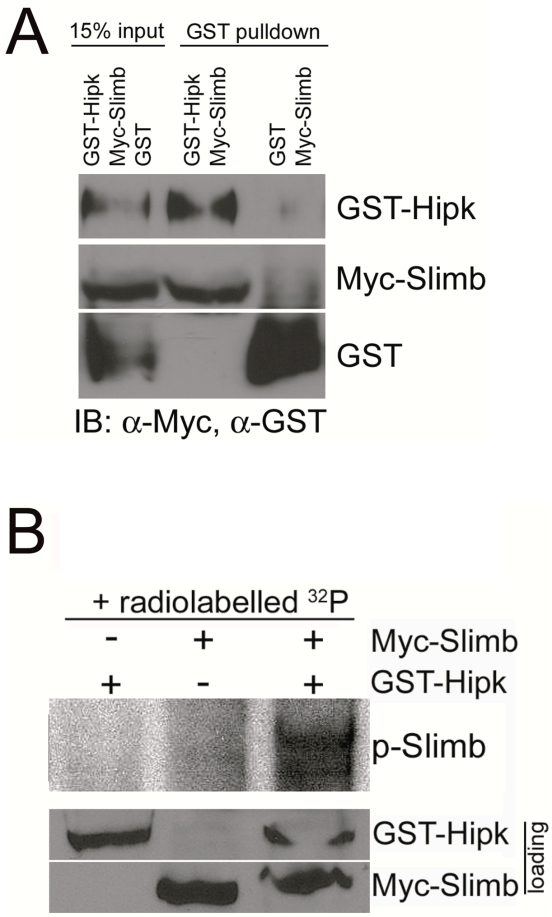


Figure 2.9.1 Hipk interacts with and phosphorylates Slimb

(A) In a co-immunoprecipitation assay, GST-Hipk formed a complex with Myc-Slimb from *Drosophila* adults. The GST moiety alone did not bind Myc-Slimb. (B) In an *in vitro* kinase assay using radiolabeled ATP (^{32}P), purified Myc-Slimb was phosphorylated in the presence of GST-Hipk but not in its absence.

2.10. Hipk regulates poly-ubiquitinated Arm levels *in vivo*

Based on our experimental evidence thus far, we inferred that catalytically active Hipk functions to stabilize the levels of cytosolic Arm *in vivo* and inhibits its poly-ubiquitination *in vitro.* When we quantitated the levels of poly-ubiquitinated Arm from *Drosophila* wing discs, there was less poly-ubiquitinated Arm in the presence of ectopic expression of *hipk* and more poly-ubiquitinated Arm when *hipk* function was reduced (relative to the *wild type* control) (Figure 2.10.1 A). Poly-ubiquitination of a substrate and subsequent proteolysis by the proteasome is a rapid process. Wing discs were therefore treated with the proteasome inhibitor MG132 to be able to accurately quantitate the levels of poly-ubiquitinated Arm (Figure 2.10.1 A). As Hipk inhibits the poly-ubiquitination of phosphorylation-primed Arm, transfection of Hipk into *Drosophila* S2 cells not only increased the levels of total Arm in the cytosol but also resulted in an increase in the levels of phospho-Arm (S44/S48). We observed a similar effect when *Drosophila* S2 cells were treated with the proteasome inhibitor MG132 (Figure 2.10.1 B). The levels of total Arm detected in the cytosol was greater than the levels of phospho-Arm (S44/S48) as presumably on inhibition of its proteolysis it is dephosphorylated. Hipk therefore regulates the levels of poly-ubiquitinated Arm *in vivo*.

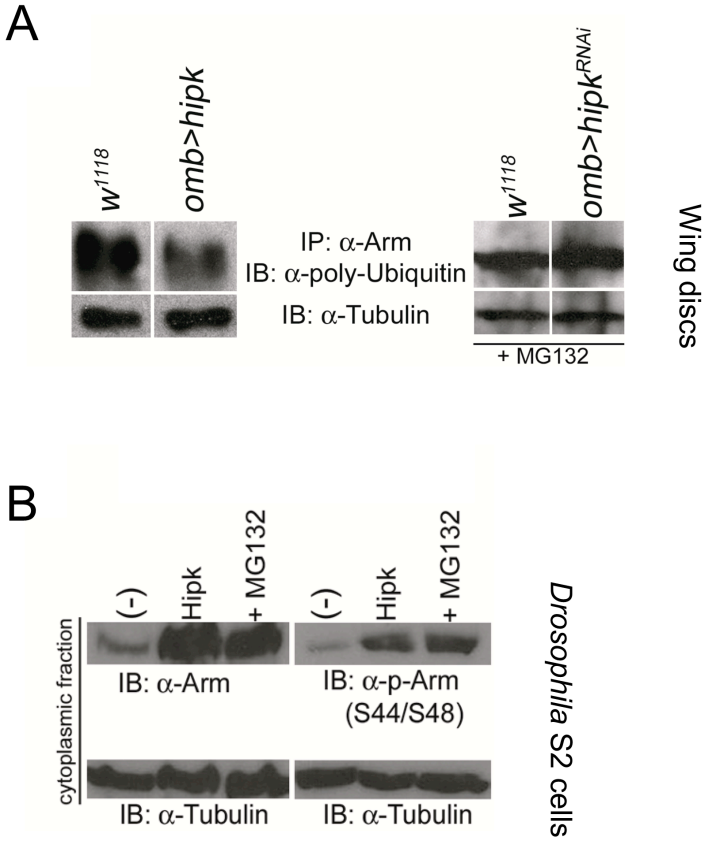


Figure 2.10.1 Hipk regulates the levels of poly-ubiquitinated Arm *in vivo*

(A) Protein lysates from *Drosophila* wing discs were assayed for levels of poly-ubiquitinated Arm. Using *omb-Gal4* to over-express *hipk* resulted in lower levels of poly-ubiquitinated Arm while knock-down of *hipk* function enhanced the levels of poly-ubiquitinated Arm, relative to *wild type* control discs. Wing discs were treated with the proteasome inhibitor MG132 to block proteolysis of poly-ubiquitinated Arm. (B) In *Drosophila* S2 cells, Hipk enhanced the levels of both total Arm and phospho-Arm in the cytosol. This effect was also observed on treating *Drosophila* S2 cells with MG132.

2.11. Hipk enhances the stability of the Hh effector Ci

In addition to its regulation of the Wg pathway, SCF^{Slimb} is a component of Hh signaling where it directs the poly-ubiquitination of the pathway effector Cubitus interruptus (Ci) (Jiang and Struhl, 1998; Smelkinson and Kalderon, 2006; Wang and Li, 2006). In the absence of pathway activity, Ci is poly-ubiquitinated and incompletely degraded via the proteasome to yield a truncated protein. In the presence of pathway activity, the partial degradation of Ci is impeded to yield a full-length protein that can direct the expression of target genes (Hooper and Scott, 2005). As Hipk inhibits the function of Slimb in the Wg pathway, we wanted to test whether it could also block the ability of Slimb to poly-ubiquitinate Ci. In the wing disc, full-length Ci is stabilized along the anterior/posterior compartment boundary in response to Hh signaling where it directs expression of the target gene *patched* (*ptc*) (Aza-Blanc et al., 1997) (Figure 2.11.1 A). Over-expression of *hipk* increased while reducing *hipk* function decreased the levels of full-length Ci and *ptc* expression in the wing disc (Figure 2.11.1 B, C). Hh signaling that induces the formation of full-length Ci is present in the morphogenetic furrow of the eye disc as well. Somatic clones of $hipk^4$ in the morphogenetic furrow displayed reduced levels of full-length Ci and expression of the target gene *dpp* (Figure 2.11.1 D). We also examined Hipk's effect on the Hh pathway in *Drosophila* S2 cells by using a reporter construct (ptcΔ136-Luc) that responds to the levels of full-length Ci (Chen et al., 1999). Transfection of Hipk into *Drosophila* S2 cells increased the response of ptcΔ136-Luc both in the absence and presence of the Hh ligand. A reporter construct with mutated Ci-binding sites (ptcΔ136-mut) did not show any response to Hh signaling and the levels of full-length Ci (Figure 2.11.1 E). Our analyses suggested that Hipk promotes Hh signaling by regulating the levels of full-length Ci *in vivo* and in cell culture, presumably through its inhibition of Slimb function as in the case of Wg signaling.

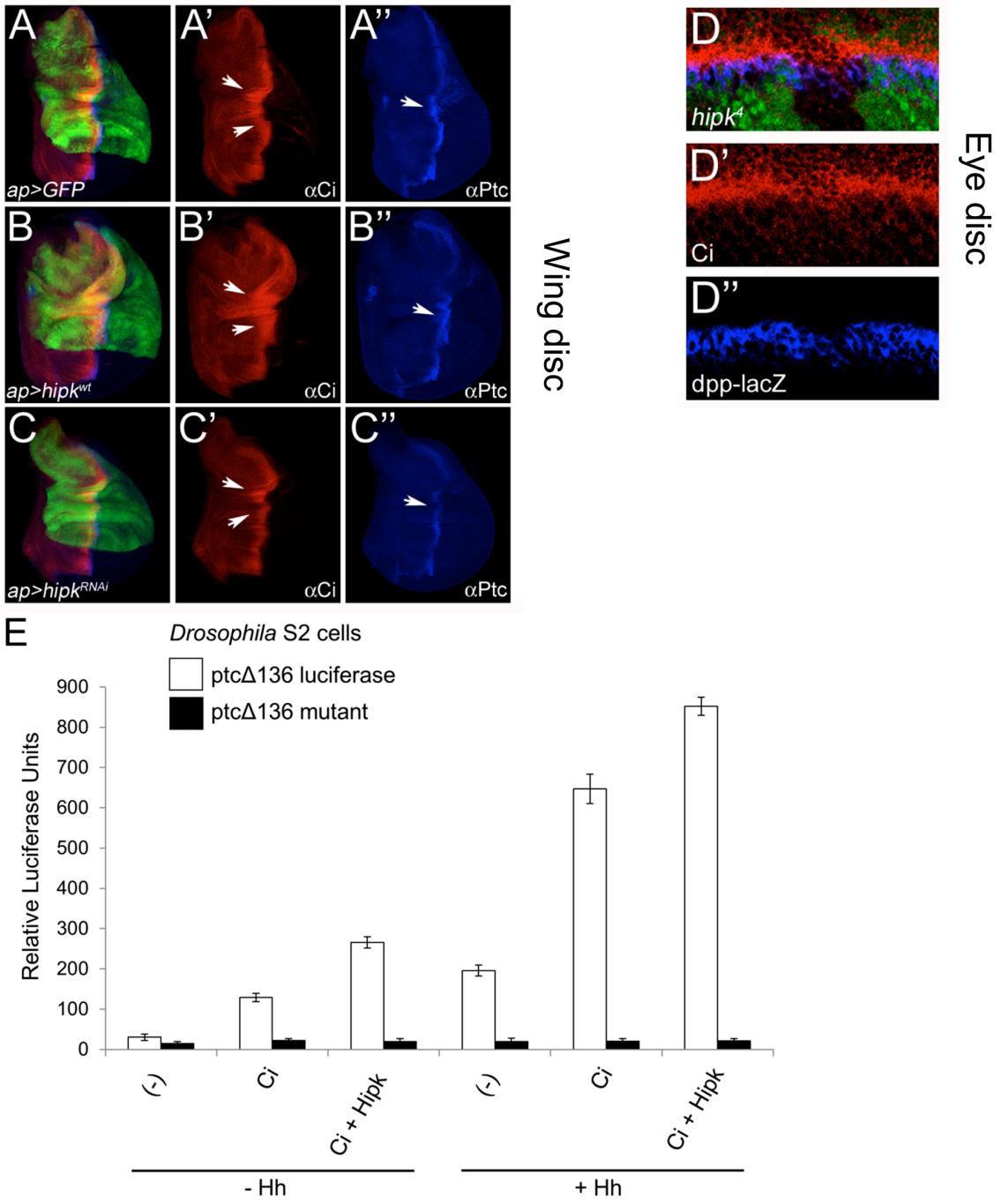


Figure 2.11.1 Hipk promotes the stability of Ci and Hh signaling

(A-C) Over-expression of *hipk* increased while knock-down of *hipk* function decreased the levels of full-length Ci and *ptc* expression relative to the *wild type* control wing disc. (D) $hipk^4$ somatic clones (absence of GFP) in the eye disc displayed reduced levels of full-length Ci and *dpp* expression. (E) In *Drosophila* S2 cells, Hipk enhanced the expression of a Ci-responsive (but not mutant) reporter gene in the absence and presence of Hh pathway activity.

2.12. Hipk promotes Wg signaling independent of its effect on Arm stability

In our analyses of the role of Hipk, we observed that it also has an effect on Wg signaling independent of Arm stabilization. Transcriptional reporter (Topflash) assays were performed in *Drosophila* S2 cells transfected with increasing amounts of Arm (lanes 2-5), increasing amounts of Hipk (lanes 6-9), and a fixed amount of Arm in combination with increasing amounts of Hipk (lanes 10-13) (Figure 2.12.1 A). Increasing amounts of stabilized Arm correlated directly with an increase in reporter activity (lane 2-5). When cells were transfected with increasing amounts of Hipk, we observed an increase in the levels of stabilized Arm and a strong dose-dependent increase in reporter activity (lanes 6-9). The transcriptional response from stabilized Arm in the presence of Hipk was stronger than from an equivalent amount of stabilized Arm in the absence of Hipk (compare lane 2 to lane 9). In addition, lower levels of stabilized Arm in the presence of Hipk had an equivalent transcriptional response to higher levels of stabilized Arm in the absence of Hipk (compare lane 4 to lane 9). Transfection of a fixed liberal amount of Arm with increasing amounts of Hipk into cells resulted in a robust increase in reporter activity even though the increase in stabilized Arm levels was only mild (lanes 10-13) (Figure 2.12.1 A). These results suggested that stabilized Arm is more potent at directing a transcriptional response from a Topflash reporter in the presence of Hipk than in its absence. Hipk therefore has a secondary effect on Wg signaling independent of its ability to stabilize Arm. Accordingly, Hipk WT enhanced the transcriptional response of a degradation-resistant form of Arm (Arm*) that is constitutively stable (Li et al., 2007). Hipk KD functioned as a dominant-negative protein to significantly decrease the transcriptional response of Arm*, suggesting that its catalytic activity is required for its secondary role in the Wg pathway. A Fopflash mutant reporter construct (negative control) did not respond to the presence of any form of Arm or Hipk in these assays (Figure 2.12.1 B).

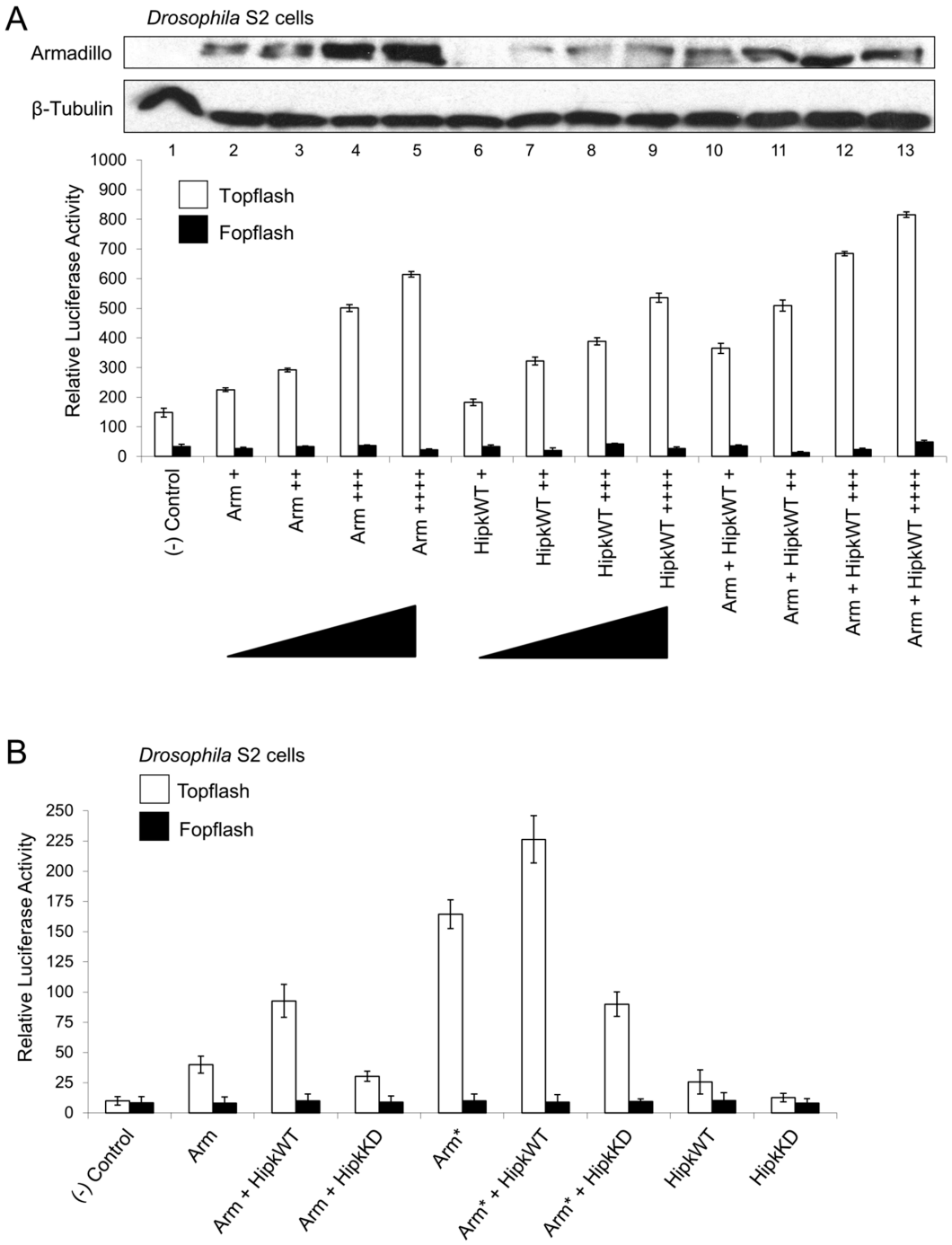


Figure 2.12.1 Hipk promotes signaling independent of its effect on Arm stability

(A) Lower amounts of stabilized Arm in the presence of Hipk induced a stronger response from the Topflash reporter than higher amounts of stabilized Arm in the absence of Hipk in *Drosophila S2* cells. (B) Hipk WT but not Hipk KD enhanced the effect of both regular Arm and degradation-resistant Arm* on the Topflash reporter in *Drosophila* S2 cells.

2.13. Hipk interacts with the Arm/TCF transcriptional complex

As Hipk has a secondary role downstream of the stabilization of Arm, it possibly interacts with the Arm/TCF transcriptional complex to promote Wg signaling. Hipk is abundantly present in the nucleus of cells and has been identified to interact with several transcription factors (Moller et al., 2003; Rinaldo et al., 2007a). From co-immunoprecipitation assays performed with over-expressed epitope-tagged proteins in HEK293T cells, we found Hipk to associate with both TCF (Figure 2.13.1 A) and Arm (Figure 2.13.1 B).

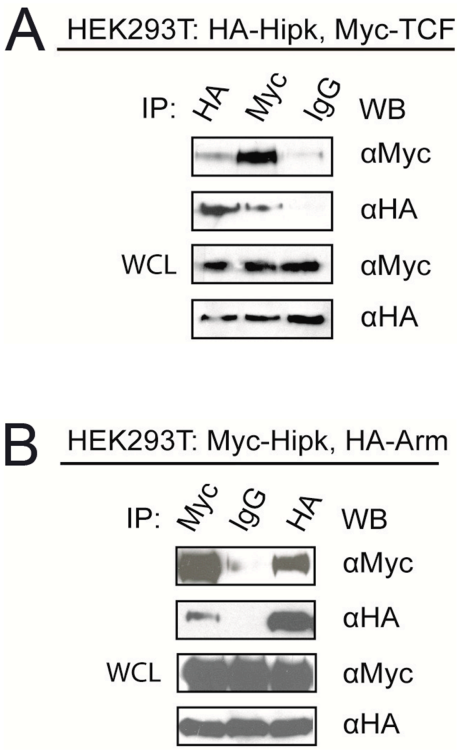


Figure 2.13.1 Hipk interacts with the Arm/TCF transcriptional complex

(A) HEK293T cells were co-transfected with HA-Hipk and Myc-TCF. Proteins were immunoprecipitated using anti-HA, anti-Myc, or IgG (negative control) antibodies. HA-Hipk associated with Myc-TCF regardless of whether anti-HA or anti-Myc was used. Whole cell lysate (WCL) was used as a loading control to indicate that equivalent amounts of proteins were used. (B) Similarly, a co-immunoprecipitation assay performed in HEK293T cells with over-expressed proteins showed an association between Myc-Hipk and HA-Arm regardless of whether anti-HA or anti-Myc was used.

2.14. Hipk phosphorylates the transactivation domains of Arm

As catalytically active Hipk can promote the Wg pathway independent of its effect on Arm stability and associates with the Arm/TCF transcriptional machinery, it likely phosphorylates one or both components of this complex to exert its effect on signaling. We demonstrated that Hipk phosphorylates full-length Arm *in vitro* (Figure 2.14.1 B). To identify the domain(s) of Arm phosphorylated by Hipk, truncations of the N-terminus (Arm N), Arm central repeats (Arm R), and C-terminus (Arm C) were generated (Figure 2.14.1 A). Hipk phosphorylated Arm N and Arm C but not Arm R (Figure 2.14.1 C). The N- and C-termini of Arm are essential for its ability to recruit other coactivators to the basal transcriptional complex and interact indirectly with chromatin factors (Mosimann et al., 2009). Therefore, Hipk likely phosphorylates these transactivation domains of Arm to promote the activity of the Arm/TCF transcriptional complex and expression of pathway target genes.

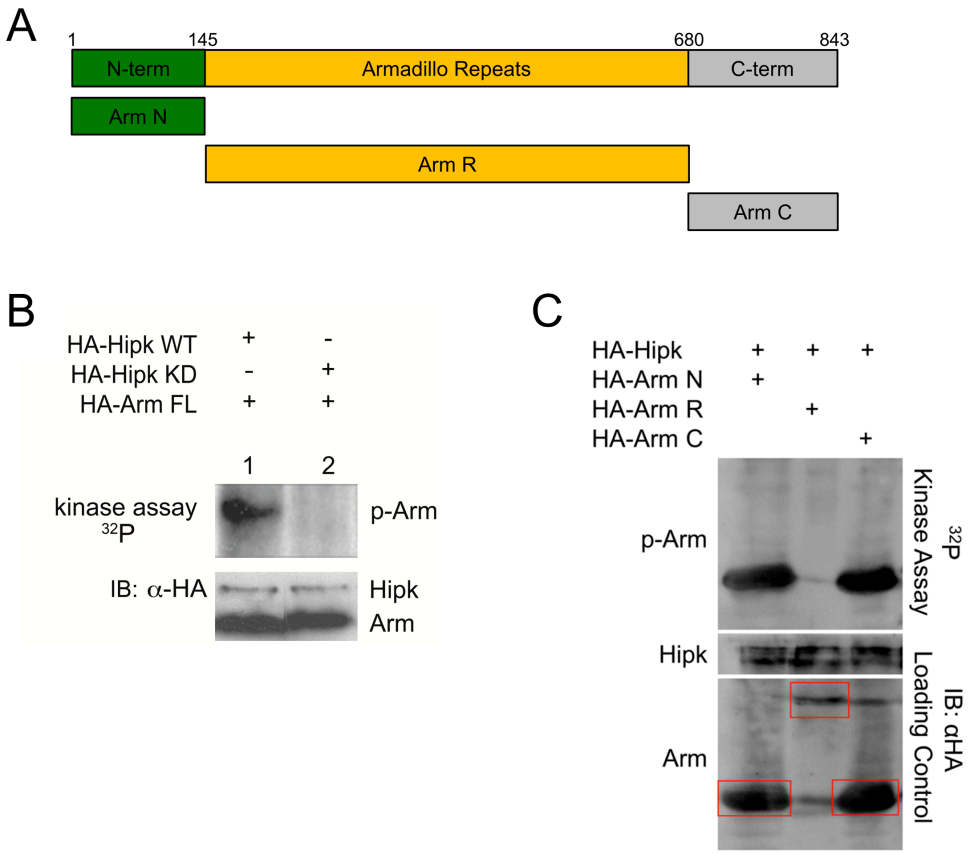


Figure 2.14.1 Hipk phosphorylates the N- and C-terminal domains of Arm

(A) A schematic of the Arm truncations used for the *in vitro* kinase assays. (B) Hipk WT (but not Hipk KD) phosphorylated full-length Arm in a radiolabelled-ATP kinase assay *in vitro*. The loading control had equivalent amounts of substrate and kinase present in both lanes. (C) Hipk phosphorylated the N- and C-termini of Arm but not the central repeats domain *in vitro*. The red boxes in the loading control indicate the relative amounts of proteins used in the experiment.

2.15. Hipk2 promotes Wnt signaling in mammalian cells

We wanted to determine if the regulation of Wg signaling by Hipk in *Drosophila* is functionally conserved by its mammalian counterpart. To test this we performed a transcriptional reporter (Topflash) assay in mammalian HeLa cells. We observed that Hipk2 WT enhances the Lymphoid enhancer factor 1 (LEF1)-mediated response of the Topflash reporter. LEF1 is a member of the TCF family of proteins in mammals that promotes Wnt signaling (Arce et al., 2006). As in the case of *Drosophila*, a kinase-inactive Hipk2 K221R (phosphate-anchor lysine residue mutated to arginine) functioned as a dominant-negative protein to inhibit the transcriptional response (Figure 2.15.1). These assays in HeLa cells were performed in the presence of exogenous Wnt1 and LEF1 to enhance the response from the Topflash reporter which is otherwise relatively weak. Therefore, the ability of the Hipk subfamily of proteins to promote Wnt signaling in a kinase-dependent manner is conserved from *Drosophila* to mammals.

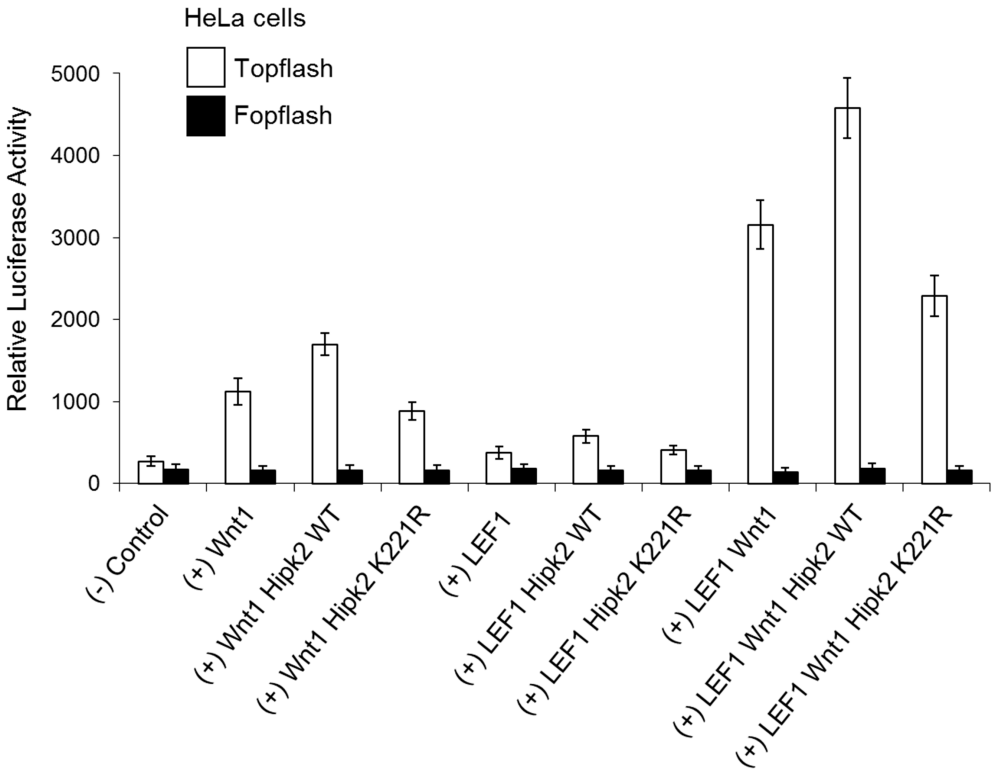


Figure 2.15.1 Hipk2 promotes Wnt signaling in a kinase-dependent process

In HeLa cells, Hipk2 promoted LEF1-mediated transcription of the Topflash reporter in a kinase-dependent manner. Hipk2 K221R functioned as a dominant-negative protein to inhibit the Topflash response. The negative control Fopflash did not respond to the presence of Wnt1, LEF1, or Hipk2.

2.16. Hipk2 interacts with β-TrCP and decreases its affinity for β-catenin in cell culture

To evaluate whether Hipk2 promotes Wnt signaling through the regulation of the stability of the pathway effector as in *Drosophila*, we carried out binding assays in cell culture using the mammalian homologues. In a co-immunoprecipitation assay in HEK293T cells using exogenous proteins, Hipk2 formed a complex with the E3 ligase substrate recognition component β-TrCP (Figure 2.16.1 A). This suggested that Hipk2 might regulate the poly-ubiquitination of β-catenin. Moreover, in the presence of increasing amounts of Hipk2 WT there was progressively less β-catenin that associated with β-TrCP in HEK293T cells. This ability of Hipk2 to reduce the amount of β-catenin bound to β-TrCP is kinase-dependent as increasing amounts of Hipk2 K221R had no effect on this interaction (Figure 2.16.1 B). Therefore, catalytically active Hipk2 interacts with β-TrCP to restrict its ability to sequester β-catenin in mammalian cells.

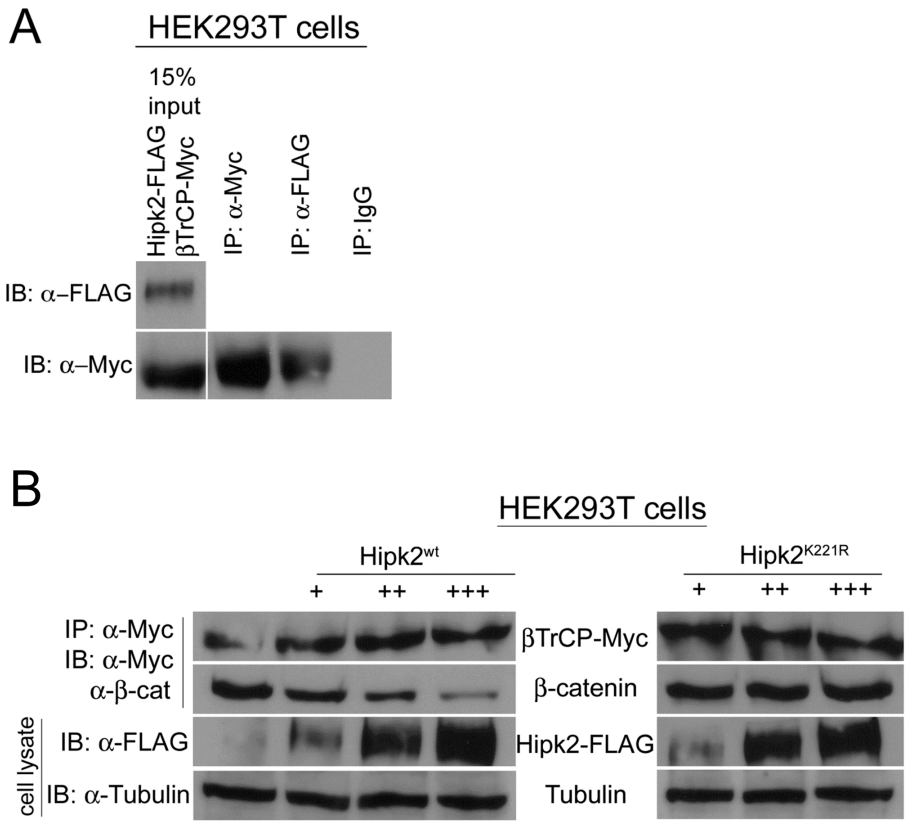


Figure 2.16.1 Hipk2 regulates the amount of β-catenin associated with β-TrCP

(A) In a co-immunoprecipitation assay in HEK293T cells, Hipk2 formed a complex with β-TrCP. IgG was used as a negative control in the assay. (B) In HEK293T cells, increasing levels of Hipk2 WT proportionately reduced the amount of β-catenin bound to β-TrCP. This effect is kinase-dependent as kinase-inactive Hipk2 K221R had no effect on the amount of β-catenin bound to β-TrCP.

2.17. Hipk2 inhibits the β-TrCP-mediated poly-ubiquitination of β-catenin *in vitro*

An *in vitro* biochemical assay was performed to conclusively demonstrate that Hipk2 regulates the poly-ubiquitination of β-catenin, as in the case of its *Drosophila* homologue. β-catenin was poly-ubiquitinated in a process mediated by β-TrCP and other members of the E3 ligase complex but not in its absence (Liu et al., 1999) (Figure 2.17.1). Pre-incubation of β-TrCP with Hipk2 WT (but not Hipk2 K221R) in a kinase assay abrogated its ability to poly-ubiquitinate β-catenin in the subsequent ubiquitination assay (Figure 2.17.1). Therefore, Hipk2 phosphorylates β-TrCP to inhibit its ability to poly-ubiquitinate β-catenin *in vitro*.

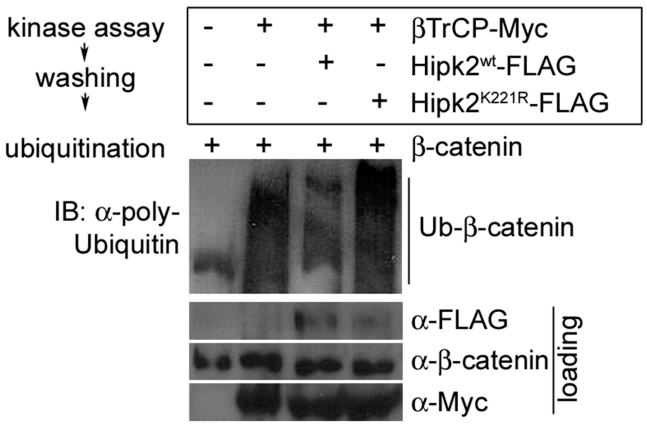


Figure 2.17.1 Hipk2 inhibits β-catenin poly-ubiquitination *in vitro*

In an *in vitro* ubiquitination assay, β-catenin was poly-ubiquitinated in the presence of β-TrCP but not in its absence. Pre-incubation of β-TrCP in a kinase assay with Hipk2 WT but not kinase-inactive Hipk2 K221R reduced the β-TrCP–mediated poly-ubiquitination of β-catenin.

2.18. Hipk2 inhibits β-TrCP-mediated poly-ubiquitination of cellular proteins including GLI

Since *Drosophila* Hipk regulated the stability of multiple substrates of Slimb, in our analyses of the regulation of β-TrCP by Hipk2 we tested whether this effect was unique to β-catenin or whether Hipk2 played a broader role to control the poly-ubiquitination and stability of other proteins regulated by this E3 ligase complex (Cardozo and Pagano, 2004). The transfection of β-TrCP into HEK293T cells mediated the poly-ubiquitination of numerous cellular proteins (Figure 2.18.1 A). We examined the effect of a dosage series of Hipk2 in the presence of β-TrCP and found that as the amount of Hipk2 WT was increased, there was a reduction in overall levels of protein poly-ubiquitination. This suggested that Hipk2 WT may inhibit the β-TrCP–mediated ubiquitination of more than one substrate of the E3 ligase complex. This effect was similar to that observed when cells were treated with the E1 inhibitor PYR41. In contrast, transfection of cells with an increasing dosage of kinase-inactive Hipk2 K221R had no effect on the β-TrCP–mediated poly-ubiquitination of cellular proteins (Figure 2.18.1 A). Additionally, as in the case of *Drosophila* Hipk, the introduction of Hipk2 WT but not Hipk2 K221R into mammalian COS7 cells increased the levels of the full-length Hh effector, Glioma-associated oncogene (GLI) (Figure 2.18.1 B). Therefore, catalytically active Hipk2 inhibits the β-TrCP–mediated poly-ubiquitination of multiple cellular substrates including GLI.

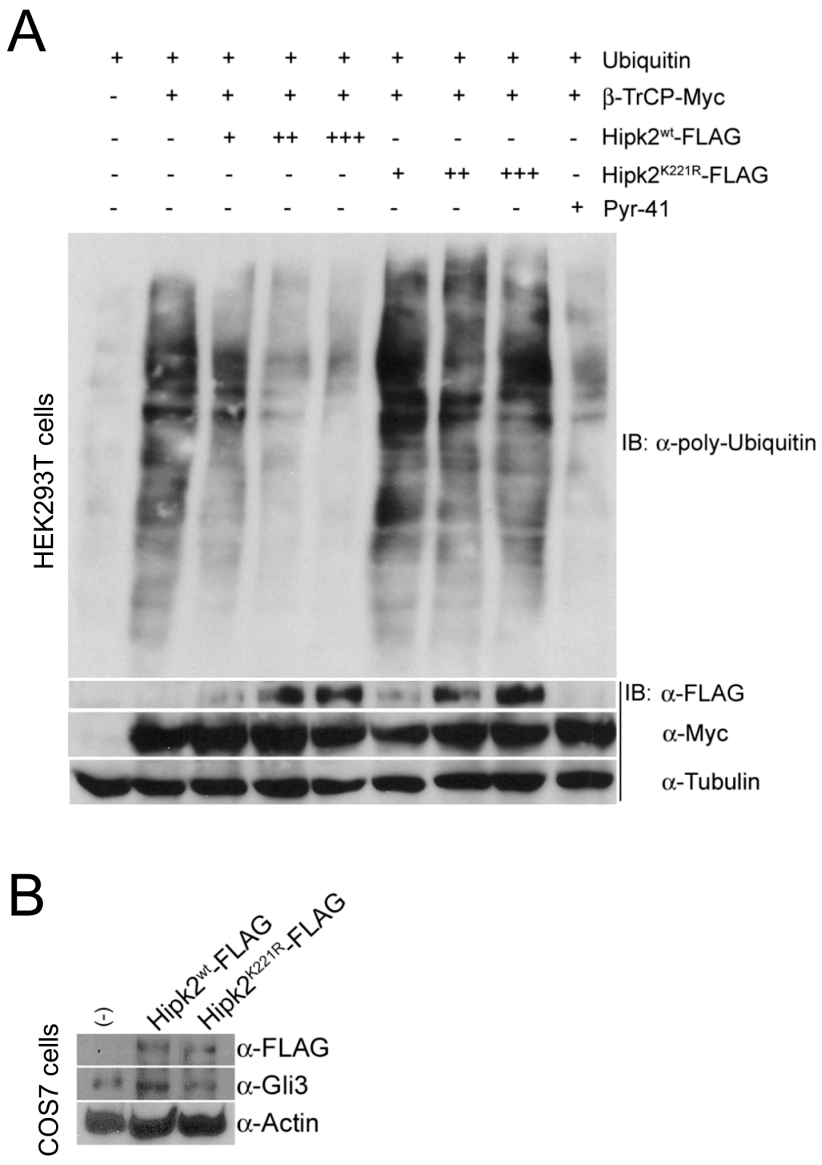


Figure 2.18.1 Hipk2 regulates β-TrCP-mediated ubiquitination of cellular proteins

(A) HEK293T cells transfected with β-TrCP resulted in the poly-ubiquitination of multiple cellular substrates. Increasing amounts of Hipk2 WT in the presence of β-TrCP led to a gradual decline in levels of poly-ubiquitinated cellular proteins, an effect similar to that observed when cells were treated with the E1 inhibitor PYR41. Increasing amounts of Hipk2 K221R had no effect on cellular poly-ubiquitination levels. (B) Hipk2 WT but not Hipk2 K221R stabilized endogenous full-length GLI3 in COS7 cells.

2.19. Hipk2 promotes Wnt signaling independent of its effect on β-catenin stability

Hipk2 enhanced the LEF1-mediated transcriptional response in HeLa cells of exogenous regular β-catenin and a degradation-resistant form of the protein that has a serine to tyrosine substitution at position 33 within the phosphodegron (β-catenin S33Y) (Morin et al., 1997) (Figure 2.19.1). These results were not due to Hipk2's effect on endogenous levels of β-catenin as the combined effect on the Topflash response of Hipk2 in the presence of β-catenin S33Y (lane 7) was greater than the individual effects of Hipk2 (lane 5) and β-catenin S33Y (lane 4) (Figure 2.19.1). We interpret these results to mean that Hipk2 (like *Drosophila* Hipk) has a role independent of its effect on β-catenin stability to promote Wnt signaling.

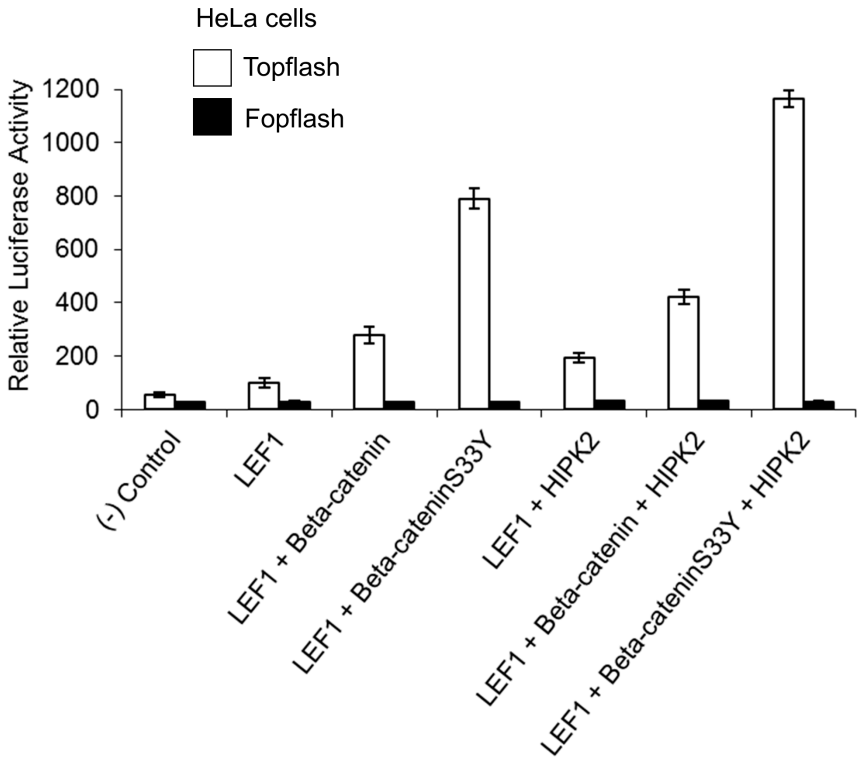


Figure 2.19.1 Hipk2 promotes signaling activity of degradation-resistant β-catenin

Hipk2 enhanced LEF1-mediated Wnt signaling in HeLa cells in the presence of regular β-catenin and degradation-resistant β-catenin S33Y. A mutated form of the pathway transcriptional reporter Fopflash did not respond to the presence of β-catenin or LEF1.

2.20. Summary

2.20.1. Hipk model of function

Our genetic and biochemical analyses revealed that Hipk promotes the Wg pathway at multiple levels of the signaling relay during *Drosophila* development. Hipk functions downstream of the ligand-receptor-co-receptor complex to inherently stabilize the cytosolic levels of the pathway effector Arm, regardless of the status of signaling. Hipk does not affect the phosphorylation of Arm at its consensus N-terminus degradation sites, but rather it phosphorylates and inhibits the E3 ligase component Slimb that controls the poly-ubiquitination and subsequent proteasome-mediated degradation of Arm. As a consequence of Hipk catalytic function, stabilized Arm can translocate to the nucleus to direct the expression of pathway target genes. Stabilized Arm can presumably also be incorporated into the adherens junctions at the plasma membrane (Figure 2.20.1). Additionally, Hipk promotes the Wg pathway downstream and independent of its effect on Arm stability. It exerts this secondary effect on signaling by interacting with the Arm/TCF transcriptional complex and phosphorylates the transactivation domains of Arm to promote pathway target gene expression. Hipk's phosphorylation of the transactivation domains of Arm possibly aid in the recruitment of coactivators and/or deter the binding of corepressors to the transcriptional complex (Figure 2.20.1). We found Hipk2 to have a functionally conserved role to dually promote Wnt signaling in mammalian cells. Hipk2 enhances the transcriptional response from a pathway reporter and prevents the interaction of the E3 ligase component β-TrCP with the pathway effector β-catenin in a kinase- and dose-dependent manner in cell culture. Hipk2 also promotes the transcriptional activity of degradation-resistant β-catenin. Thus, similar to GSK3 and CK1α, Hipk2 acts at two distinct levels of the Wnt pathway, but unlike these kinases that have opposing effects at different levels of the signaling relay, Hipk2 doubly promotes pathway activity.

Future studies would entail the identification of Hipk's one or more phosphorylation sites on its substrates Slimb and Arm in the Wg pathway. Although members of this subfamily of enzymes have not been characterized to have a consensus phosphorylation sequence, Hipks are generally regarded as proline-directed kinases (http://www.phosphosite.org). Therefore, it is likely that the putative

phosphorylation sites on the aforementioned substrates are serine and/or threonine residues with a proline residue at the P+1 position, and that are conserved across species based on sequence homology. However, it should be noted that not all phosphorylation sites on substrates of Hipks identified to date are proline-directed, and other mechanisms such as docking sites outside the kinase domain might direct the specificity of this kinase towards its substrates. Hipk's phosphorylation sites on its substrates can be identified through a combinatorial approach that includes *in vitro* kinase assays, deletion analysis, and site-directed mutagenesis. Once identified, the biological consequences of Hipk phosphorylation can be tested through the generation and expression of phospho-mimetic and phospho-resistant versions of the substrates *in vivo*. Another interesting avenue of research would be to elucidate the functions of the cytoplasmic and nuclear fractions of Hipk with respect to Wg signaling. Hipk presumably inhibits Slimb function in the cytosol and promotes the activity of the Arm/TCF transcriptional complex in the nucleus. However, it is now becoming apparent that all members of the Arm degradation machinery dynamically shuttle between the nuclear and cytoplasmic compartments. So Hipk could potentially exert all of its effect on Wg signaling in only the nuclear compartment. We can test this hypothesis *in vivo* through the generation and over-expression of exogenous Hipk variants that are either exclusively present in the nucleus (through addition of nuclear localization sequences) or the cytosol (through deletion of nuclear localization sequences), and examine their effects on expression of pathway target genes. Lastly, although we and others have demonstrated Hipk to be involved in multiple signaling pathways and developmental processes, how the activity of this kinase is selectively controlled is presently still unclear. Unlike Mitogen Activated Protein Kinases (MAPKs) for which phosphorylation of the activation loop requires an upstream kinase, phosphorylation of the DYRK activation loop is autocatalytic and these kinases have been demonstrated to be constitutively active (Aranda et al., 2011). Thus the catalytic activity of Hipk is not regulated by any upstream signaling event or kinase. Rather, it is likely regulated by phosphorylation outside of the activation loop and other post-translational modifications that directs its diverse cellular activities. By performing mass spectrometry to identify the post-translational modifications of Hipk, we can start to decipher how this promiscuous kinase is regulated by upstream stimuli.

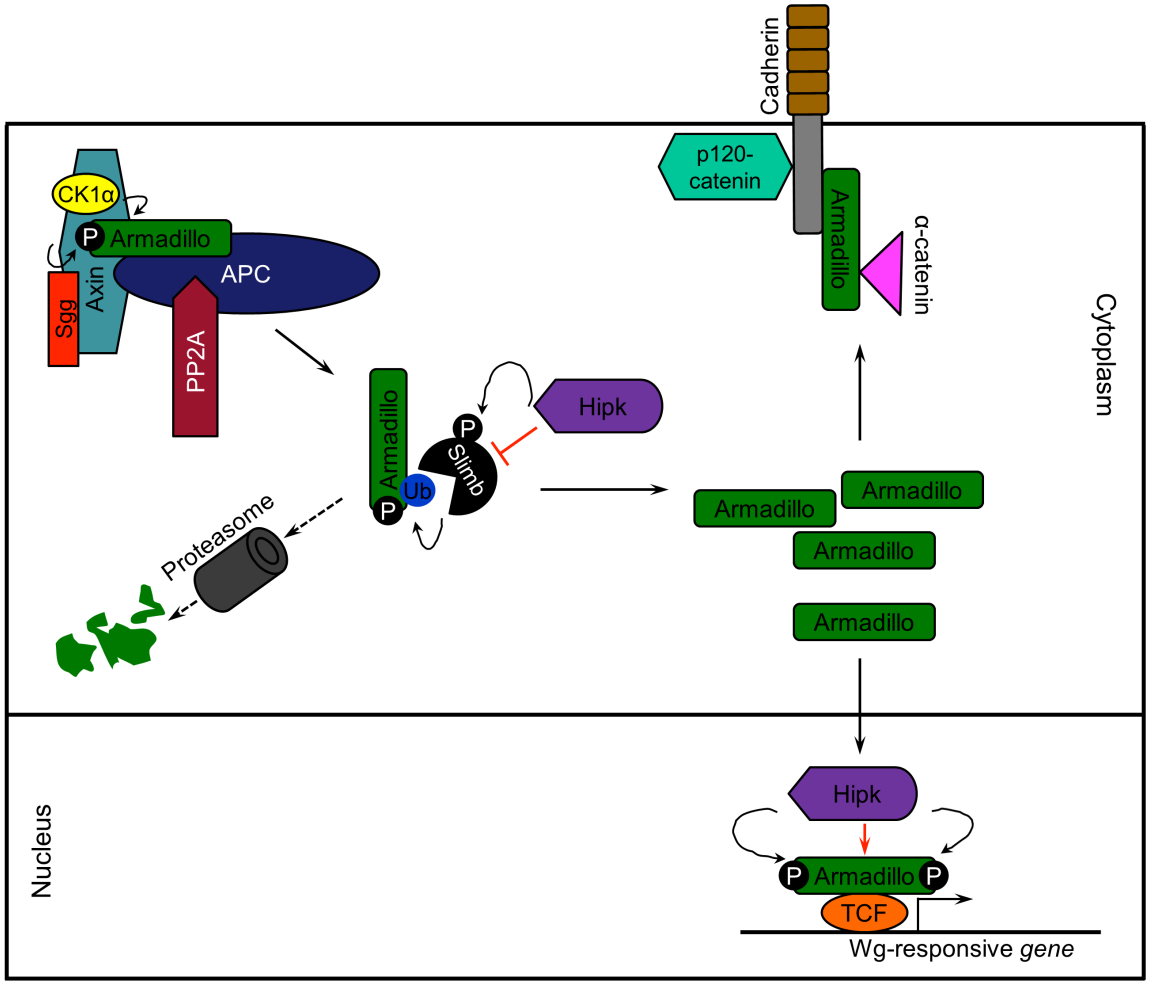


Figure 2.20.1 Hipk dually promotes Wg signaling

Cytosolic Arm is sequentially phosphorylated, poly-ubiquitinated, and degraded. Hipk phosphorylates Slimb to prevent the poly-ubiquitination and degradation of Arm. Stabilized Arm can subsequently relocate to the nucleus to direct the expression of target genes of the Wg pathway. It may also be incorporated into adherens junctions at the cell surface. Hipk additionally interacts with the Arm/TCF transcriptional complex to phosphorylate the termini of Arm and enhance pathway target gene expression.

2.20.2. Hipks, TCFs, and Wnt signaling

The TCF family of DNA-binding proteins are the final gatekeepers of signaling output of the Wnt pathway. According to the current model, in the absence of signaling TCF forms a complex with TLE (and other corepressors) to silence pathway target gene expression, while β-catenin forms a complex with TCF (and other coactivators) in the presence of signaling to direct pathway target gene expression. In contrast to the single TCF gene in *Drosophila* that performs both negative and positive roles in transcriptional regulation, there are four distinct TCF genes in vertebrates, TCF1, LEF1, TCF3, and TCF4, which are specialized to alternatively inhibit or promote expression of pathway target genes depending on the developmental context and cannot usually functionally substitute for one another (Arce et al., 2006). Accumulating evidence suggests that the state of phosphorylation of TCF proteins determines the signaling output of the Wnt pathway. In *Xenopus* embryos, CK1ε and GSK3 phosphorylate TCF3 to have opposing effects on the formation of the transcriptional complex with β-catenin (Lee et al., 2001a). Phosphorylation by CK2 promotes LEF1 binding to β-catenin and chromatin *in vitro* and in HEK293 cells (Wang and Jones, 2006). TCF4 and LEF1 are phosphorylated by Nemo-like kinase (NLK) in mammalian cells and *Xenopus* embryos to inhibit binding of the transcriptional complex to DNA (Ishitani et al., 1999, 2003). Moreover, during *Caenorhabditis* embryogenesis, the TCF homologue is phosphorylated by the NLK homologue that exports it out of the nucleus to determine cell fate specification (Lo et al., 2004; Meneghini et al., 1999; Rocheleau et al., 1999).

Vertebrate members of the Hipk subfamily have been implicated in the regulation of Wnt signaling over the years. In mouse embryonic fibroblasts and keratinocytes, Hipk2 inhibits expression of the pathway target gene *cyclin D1*. As a consequence, *Hipk2*$^{-/-}$ mutants have an expansion of epidermal stem cells due to increased *cyclin D1* expression and proliferation. Hipk2 achieves this suppressive effect on signaling by interacting with the β-catenin/LEF1 transcriptional complex (Wei et al., 2007). Hipk2 has also been shown to inhibit Wnt signaling in a kinase-dependent manner as measured from a pathway reporter in RKO and H1299 cells (Kim et al., 2010; Puca et al., 2008). On the other hand, Hipk1 binds TCF3 and LEF1 and both promotes and inhibits signaling during divergent stages of *Xenopus* embryogenesis (Louie et al., 2009). These studies, some of the results of which are in agreement with ours, suggest

that vertebrate Hipks have complex roles in the regulation of Wnt signaling that differ with respect to the cell line and stage of development. The results of recent studies seem to provide a mechanistic explanation for these discrepancies and resolve the differences. TCF3, TCF4, and LEF1 have been demonstrated to be phosphorylated by Hipk2 in mammalian cell and *Xenopus* embryos. In each case, phosphorylation by Hipk2 results in the removal of the TCF family member from the promoter of pathway target genes (Hikasa and Sokol, 2011; Hikasa et al., 2010). The conservation of the Hipk2 phosphorylation sites across different members of the TCF family provides a possible explanation for the context-dependent function of Hipk2 in Wnt signaling. Based on whether a particular vertebrate TCF protein in a particular context acts as a corepressor or coactivator, its dissociation from DNA in the presence of Hipk2 will result in different outcomes on target gene expression.

2.20.3. More ways to skin a catenin than one

Since its discovery over two decades ago, through a combination of genetic and biochemical approaches, the post-translational regulation of the stability of β-catenin levels has come to be recognized as the central feature of the Wnt signaling pathway. The levels of cytosolic β-catenin are regulated through its sequential CK1α- and GSK3-mediated phosphorylation, $SCF^{\beta\text{-}TrCP}$-mediated poly-ubiquitination, and degradation via the proteasome, a process that is inhibited in the presence of signaling. Shortly after this canonical route of β-catenin degradation was characterized, a second pathway that regulates cytosolic β-catenin levels was discovered (Dimitrova et al., 2010; Liu et al., 2001a; Matsuzawa and Reed, 2001). This pathway is regulated by the Seven in absentia homolog (Siah) E3 ligase. Siah mediates the poly-ubiquitination and subsequent proteasomal degradation of β-catenin in a β-TrCP-independent process. Siah can either act alone or in the context of a SCF-like complex (SCF^{TBL1}). Unlike β-TrCP, Siah or SCF^{TBL1} recognize β-catenin independent of its state of phosphorylation at its consensus N-terminus degradation sites (Dimitrova et al., 2010; Liu et al., 2001a; Matsuzawa and Reed, 2001). Interestingly, Hipk2 has been demonstrated to mediate the phosphorylation and destabilization of Siah under certain conditions (Calzado et al., 2009; Winter et al., 2008). So, it seems that Hipk2 regulates the stability of β-catenin via its interaction with and inhibition of two E3 ligases, β-TrCP and Siah.

2.20.4. DYRKs regulate protein stability

An increasing number of substrates have been identified from different model organisms of the Dyrk subfamily, which along with the Hipk subfamily is part of the larger DYRK family. These substrates have diverse functions such as the regulation of gene transcription, signaling, and the cell cycle. One characteristic feature of many Dyrk members is their ability to act as priming kinases for GSK3, meaning that the phosphorylation of a given residue by a Dyrk is a prerequisite for subsequent phosphorylation of a different residue by GSK3. Another feature of many Dyrk members is their regulation of protein stability. There is accumulating evidence that Dyrks from diverse organisms regulate the turnover of target substrates by either inducing their degradation or stabilizing them (Becker, 2012). We have shown that Hipk2 stabilizes the levels of β-catenin and GLI3 in mammalian cells through phosphorylation and inhibition of β-TrCP. Hipk2 has also been shown to promote the stability of $p27^{Kip1}$ in a process that is kinase-dependent (Pierantoni et al., 2011). There are also substrates that are destabilized by Hipk2, including CtBP, ZBTB4, Siah2, and Cyclin B2 (Calzado et al., 2009; Jaspersen et al., 1998; Yamada et al., 2009; Zhang et al., 2005a). So, characteristic of Dyrks, Hipk2 selectively stabilizes and destabilizes different substrates. It would be interesting to investigate whether Hipk2 functions as a priming kinase for GSK3 for only substrates that it destabilizes but not for those that it stabilizes. This would offer an explanation as to how Hipk2-mediated phosphorylation can have different effects on protein turnover.

3. Screening the *Drosophila* kinome and phosphatome *in vivo* to identify regulators of Wnt signaling

The experimental data from this chapter (and appendices) have been submitted for publication: Swarup, S., Pradhan, T., and Verheyen, E.M. (2013). Tirthadipa Pradhan and I contributed equally to the primary screen. I performed the secondary screen, cross-screen, adult screen, and subsequent analyses.

3.1. Forward versus reverse genetic screens

Drosophila has been traditionally used in forward genetic screens to identify genes that are involved in biological processes. A forward genetic screen encompasses the random mutagenesis of the genome and the selection of mutants displaying a phenotype that differs from that of the control. The mutated locus is subsequently identified through positional cloning or a candidate gene approach (St Johnston, 2002). Several forward genetic screens have been performed in *Drosophila*, including those for zygotic mutations that affect embryonic patterning (Jurgens et al., 1984; Nusslein-Volhard and Wieschaus, 1980; Nusslein-Volhard et al., 1984, 1987; Wieschaus et al., 1984) and modifier screens for genes that regulate photoreceptor specification (Olivier et al., 1993; Rogge et al., 1991; Simon et al., 1991). A forward genetic strategy offers the advantage of potentially isolating a range of alleles, both loss- and gain-of-function, for every gene in the genome. However, this approach generates a set of mutants for which the molecular lesion is not known and identifying the mutated gene is often a laborious process. In addition, depending on the type of screen in certain instances only the earliest function of the gene during development can be assayed due to subsequent lethality.

With the advent of RNA interference (RNAi) (Fire et al., 1998; Lam and Thummel, 2000), a reverse genetic screening strategy has replaced the forward genetic approach to study developmental and cell biological processes in *Drosophila* and other model systems. Using this technique, double-stranded RNA (dsRNA) molecules are introduced into cells to target complementary mRNA molecules for degradation of the corresponding gene that results in partial perturbation of its function. In the case of *Drosophila*, 200-500 bp long dsRNA molecules introduced into cells are cleaved into a pool of 21-23 bp short interfering RNA (siRNA) molecules by the endoribonuclease Dicer. Each siRNA molecule produced associates with the RNA-induced silencing complex (RISC) that unwinds it into two single-stranded RNA (ssRNA) molecules called the passenger strand and the guide strand. The passenger strand is degraded while the guide strand is incorporated into the RISC. The now activated RISC through base pairing complementarity recognizes and cleaves a target mRNA molecule (Hannon, 2002). RNAi is autonomously induced *in vivo* by either injecting long dsRNA molecules into *Drosophila* pre-blastoderm embryos or by the expression of transgenes encoding long dsRNA hairpins cloned as inverted repeats. RNAi induced through injection is restricted to the study of gene function during embryonic development and can be masked by maternally contributed proteins (Yang et al., 2000a). On the contrary, RNAi induced through the use of transgenes (and the *Gal4*/*UAS* system) can be regulated to assay gene function at any stage of development (Brand and Perrimon, 1993; Dietzl et al., 2007).

A reverse genetic screen encompasses the targeted inactivation of genes, the function of each of which is inferred from the resulting phenotype. The advantages of RNAi over a forward genetic strategy are that the identity of every gene is known prior to screening and that gene function can be regulated with spatial and temporal resolution. RNAi screening also has its constraints such as the incomplete knock-down of gene function and non-specific sequence-dependent and sequence-independent effects in the cell (Boutros and Ahringer, 2008). The length and sequence of the siRNA is crucial for specificity and eliminating off-target effects (OTEs). The guide or passenger strands of the siRNA can hybridize with non-target mRNAs to cause transcript degradation or translational inhibition of non-specific genes. Aside from these sequence-dependent

OTEs, the process of RNAi can have sequence-independent OTEs in cells due to the initiation of the interferon or Toll-like response (Kulkarni et al., 2006; Ma et al., 2006).

3.2. *In vivo* Wnt pathway screen

Using transgenic RNAi libraries of *UAS*-inverted repeats and tissue-specific *Gal4* drivers, we spatiotemporally knocked-down the expression of each of 385 kinase and 205 phosphatase genes present in the *Drosophila* genome to assay their effect on Wnt signaling. In addition to protein kinases and phosphatases, we also tested factors that associate with these groups of enzymes as well as non-protein kinases and phosphatases (Figure 3.2.1). At least two non-overlapping inverted repeat sequences per gene were used to minimize positive and negative false discovery. The primary screen was performed using a combination of the *dpp-Gal4* and *hh-Gal4* drivers. The *dpp-Gal4* driver is expressed along the anterior/posterior compartment boundary while the *hh-Gal4* driver is expressed in the posterior compartment of the wing disc. These *Gal4* drivers are expressed both in the signal-producing (boundary) and signal-receiving (non-boundary) cells of the Wnt pathway. A *UAS-dicer-2* transgene was used in combination with the *Gal4* drivers to enhance the efficiency of RNAi-mediated gene knock-down. Wing discs from every genotype were immunostained against Sens, Dll, and Wg to assay for pathway activity and status of the ligand. Cell proliferation and apoptosis are precisely coordinated during the development of imaginal discs, and the perturbation of these processes may induce compensatory mechanisms that maintain tissue homeostasis to produce a close to normal adult organ (Fan and Bergmann, 2008; Huh et al., 2004; Ryoo et al., 2004). We therefore assayed wing discs from every genotype regardless of the presence of an adult phenotype. Any gene that reproducibly modified the wild type domain of Sens and Dll when reduced in function with at least two non-overlapping RNAi transgenes (using any combination of *Gal4* drivers) was considered a candidate regulator of the Wnt pathway (Figure 3.2.1).

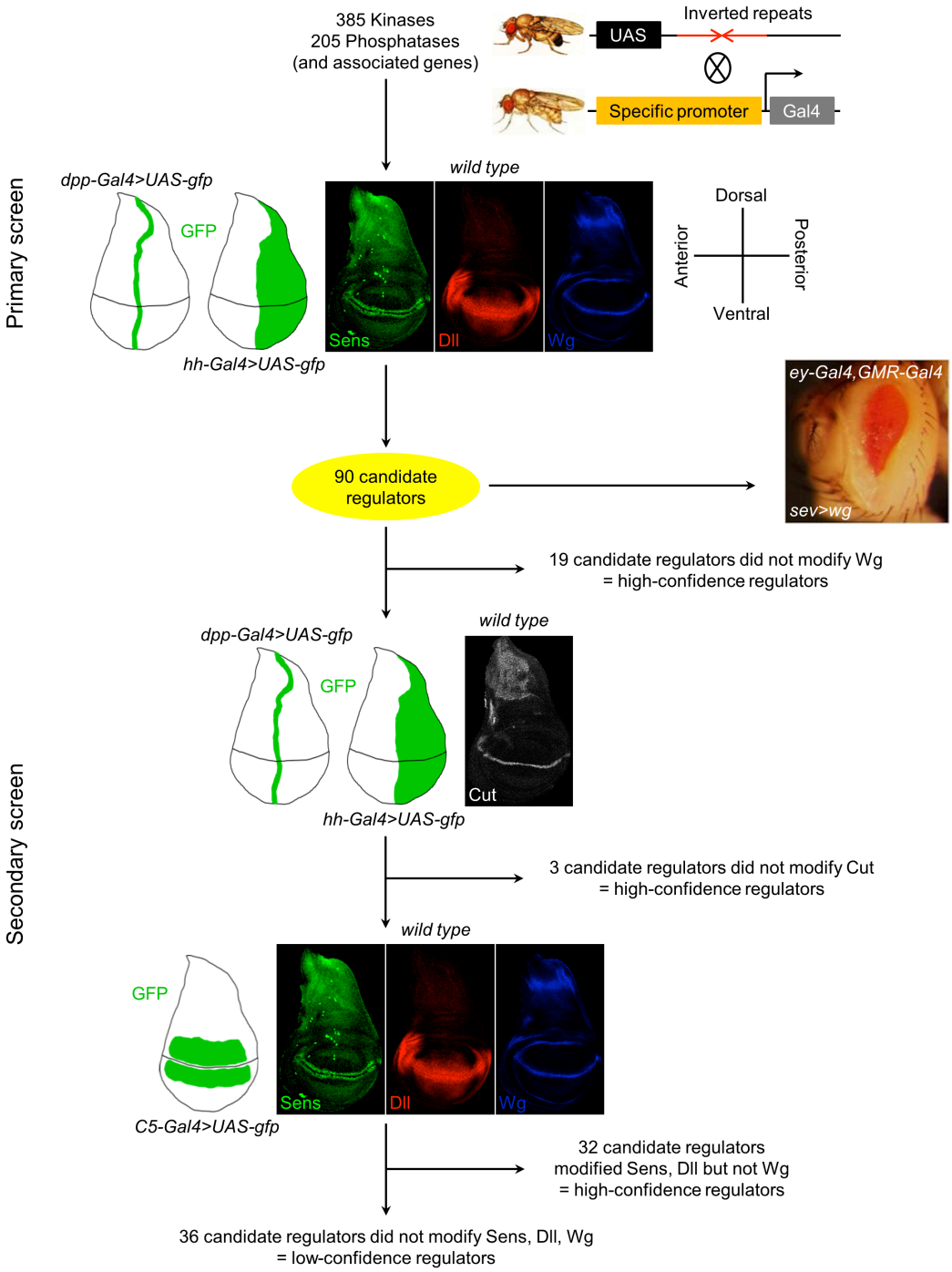


Figure 3.2.1 Wnt pathway screen

The *Drosophila* kinome and phosphatome were screened *in vivo* to identify regulators of Wnt signaling. The 90 candidates from the primary screen were categorized as 54 high confidence regulators and 36 low-confidence regulators of the pathway after secondary analyses. The candidates were tested in the *sev>wg* adult eye for their effect on Wnt signaling.

We identified 90 such candidates in our primary screen (Figure 3.2.1) (Appendix). 19 of these candidates were reclassified as Wnt pathway high-confidence regulators that functioned downstream of the ligand-receptor interaction as they had no effect on Wg protein levels or localization. The Notch pathway signals from non-boundary cells to induce the expression of *wg* in cells at the dorsal/ventral compartment boundary of the wing disc (de Celis et al., 1996; Diaz-Benjumea and Cohen, 1995; Doherty et al., 1996; Rulifson and Blair, 1995) (Figure 3.2.2). The 71 candidates that did modify Wg might have exerted their effect on the Wnt pathway indirectly as a result of their regulation of the Notch pathway, and were therefore analyzed in a secondary screening procedure. These candidates were first tested to determine if they also modified *cut*, another Notch pathway target gene (Micchelli et al., 1997) (Figure 3.2.2). 3 of these candidates had no effect on Cut. We inferred that these candidates had no effect on *wg* expression, but rather affected the ligand post-translationally and were reclassified as Wnt pathway high-confidence regulators that functioned upstream of the ligand-receptor interaction (Figure 3.2.1). The candidates that also affected Cut were inferred to have regulated *wg* expression via the Notch pathway, and were further tested to determine if they independently affected the Wnt pathway. The overwhelming majority of regulators of developmental signaling pathways function in the signal-receiving cell and not the signal-producing cell. So if a gene independently regulated both Wnt and Notch signaling in the wing disc, we reasoned that it would likely do so in different cell types, acting in the non-boundary cells for the former and the boundary cells for the latter. We tested the 68 candidates that we identified as regulators of the Notch pathway (Figure 3.2.3) using a combination of the *C5-Gal4* driver, which is expressed in only non-boundary cells adjacent to the dorsal/ventral compartment boundary, and antibodies against Sens, Dll, and Wg. 32 of these candidates modified Sens and Dll in the non-boundary cells, but did not affect the Notch pathway in these cells to regulate *wg* expression at the dorsal/ventral compartment boundary. These candidates were reclassified as Wnt pathway high-confidence regulators that functioned downstream of the ligand-receptor interaction. The remaining 36 candidates when knocked-down in the non-boundary cells had no effect on Sens, Dll, or Wg, and were reclassified as Wnt pathway low-confidence regulators (Figure 3.2.1). These candidates regulated the Notch pathway in the boundary cells and did not regulate the Wnt pathway in the non-boundary cells. We could not test for candidates that independently had an

effect on both pathways in the boundary cells. False positive results due to cell death, cell proliferation, or non-specific gene transcription were evaluated through testing the ability of candidates to affect different signaling pathways in the wing disc using combinations of *Gal4* drivers (Appendix).

The 90 candidates initially identified were thus classified into 54 high-confidence regulators and 36 low-confidence regulators of the Wnt pathway using unbiased screening analyses (Figure 3.2.4) (Appendix). Of the high-confidence regulators, 33 were kinase (or kinase-associated) genes and 21 were phosphatase (or phosphatase-associated) genes. Approximately a third of the kinases and half of the phosphatases of the high-confidence regulators in the *Drosophila* Wntome, the collection of Wnt pathway regulators in the genome, belonged to the CMGC and PPP groups respectively (Figure 3.2.5). 9 of the 54 high-confidence regulators inhibited signaling while the remaining 45 promoted signaling (Figure 3.2.4). 3 of the 54 high-confidence regulators functioned upstream of the ligand-receptor interaction to regulate the stability, secretion, diffusion, or reception of Wnt, while the remaining 51 functioned downstream of the ligand-receptor interaction (Figure 3.2.4). Remarkably, 32 of the 54 high-confidence regulators had already been described to regulate the Wnt pathway and represented at least twice as many kinases and phosphatases (and associated factors) identified from any previous screen, thereby indicative of the robustness and low false negative error rate of our analyses. Moreover, every known pathway component identified clustered in the category of high-confidence regulators with none in the category of low-confidence regulators. This suggested that many of the 22 novel high-confidence regulators identified are in fact bona fide Wnt pathway components (Figure 3.2.4).

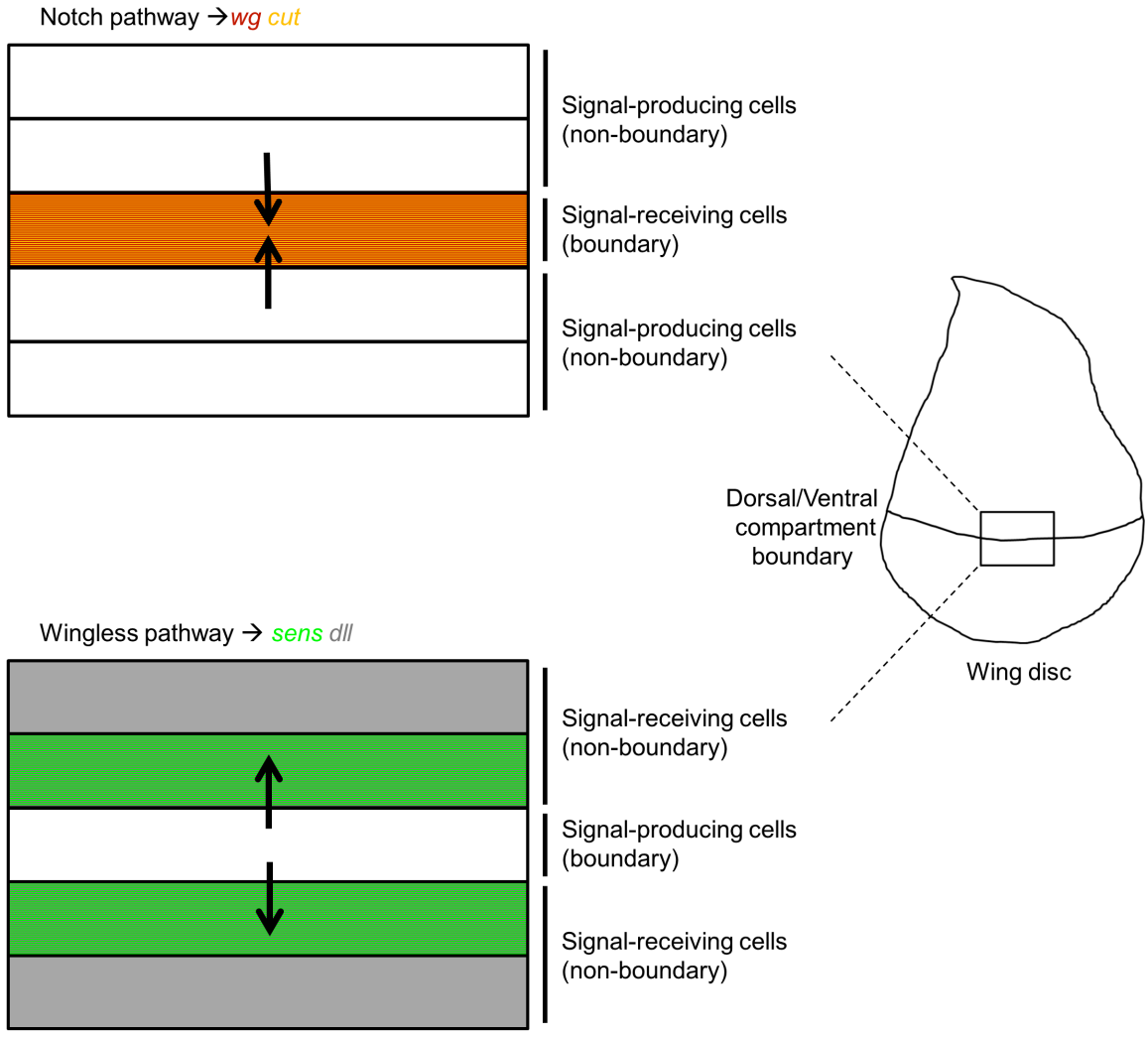


Figure 3.2.2 Wnt and Notch signaling in the *Drosophila* wing disc

The Notch pathway signals from the non-boundary cells to induce expression of the target genes *wg* (red) and *cut* (orange) in cells at the dorsal/ventral compartment boundary. Wg that is subsequently secreted from cells at the dorsal/ventral compartment boundary cells induces the nested expression of its targets genes *sens* (green) and *dll* (grey) in non-boundary cells.

Drosophila kinases and phosphatases that regulate the Notch pathway			
CG10033	for	CG10574	l-2
CG10579	Eip63E	CG11883	
CG10702		CG12217	PpV
CG1107	aux	CG12252	Fcp1
CG11486		CG1395	stg
CG11859		CG14211	MKP-4
CG12306	polo	CG17291	Pp2A-29B
CG13369		CG1810	mRNA-cap
CG14030	Bub1	CG2096	flw
CG14939	CycY	CG2890	PPP4R2r
CG15218	CycK	CG2984	Pp2C1
CG15224	CkIIβ	CG31717	
CG1609	Gcn2	CG32505	Pp4-19C
CG17090	hipk	CG32697	l(1)G0232
CG1725	dlg1	CG33747	primo-2
CG17520	CkIIα	CG42283	5PtaseI
CG17998	Gprk2	CG4733	PR72
CG2028	CkIα	CG5643	wdb
CG2210	awd	CG5650	Pp1-87B
CG2621	sgg	CG5820	Gp150
CG32666	Drak	CG5851	sds22
CG33554	Nipped-A	CG6562	synj
CG34359	IP3K2	CG6593	Pp1α-96A
CG34412	tlk	CG7109	mts
CG4268	PitsIre	CG7115	
CG5179	Cdk9	CG8402	PpD3
CG5363	cdc2	CG8584	
CG6292	CycT	CG9115	mtm
CG6364		CG9156	Pp1-13C
CG6963	gish	CG9351	flfl
CG7028	PRP4	CG9493	Pez
CG7597	Cdk12	CG9554	eya
CG8201	par-1		
CG8485			
CG8878			
CG8967	otk		

Figure 3.2.3 Regulators of the Notch pathway in the *Drosophila* wing disc

36 kinases (and associated-genes) and 32 phosphatases (and associated-genes) in the *Drosophila* genome were identified to regulate the Notch pathway in the wing disc as a result of their effect on the expression of the target genes *wg* and *cut*.

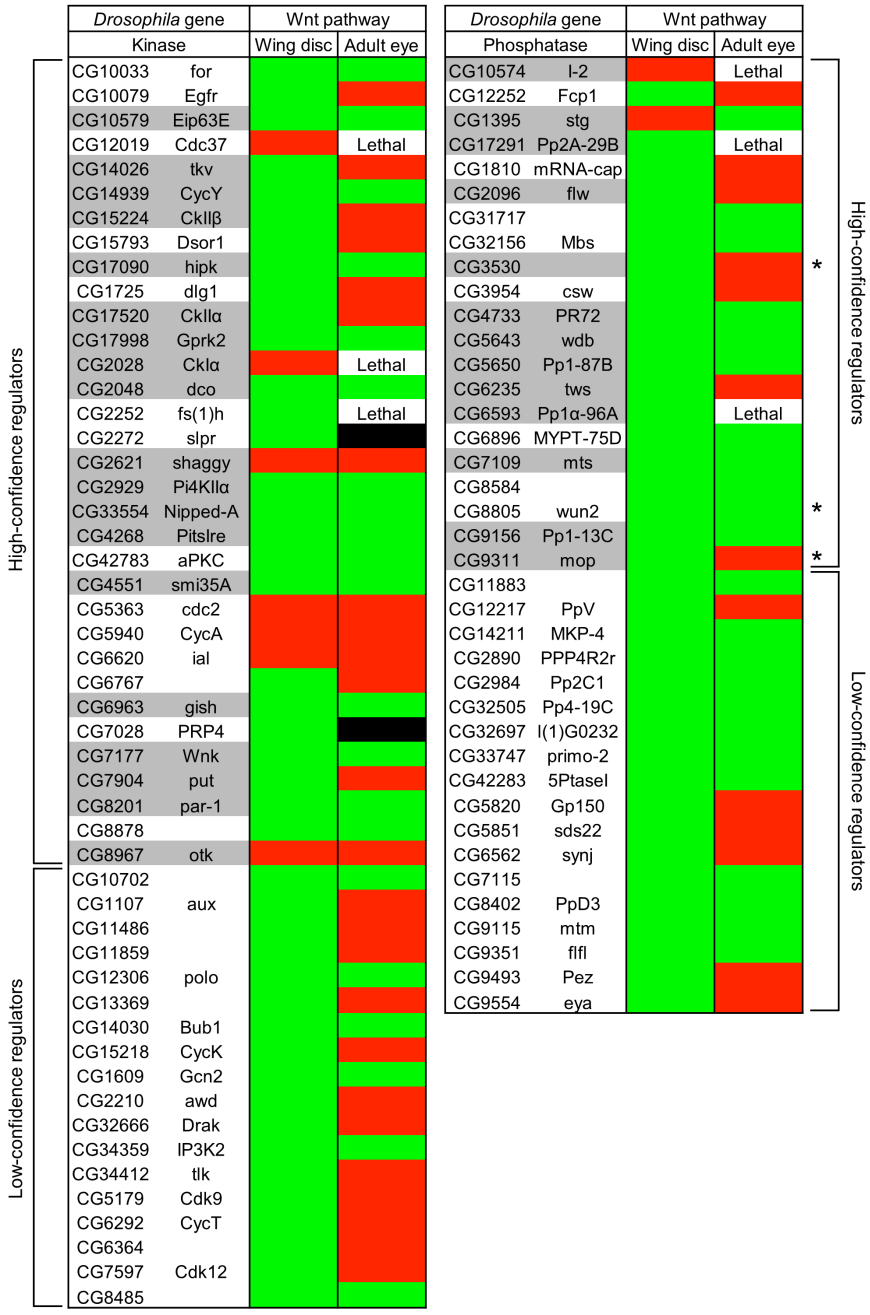
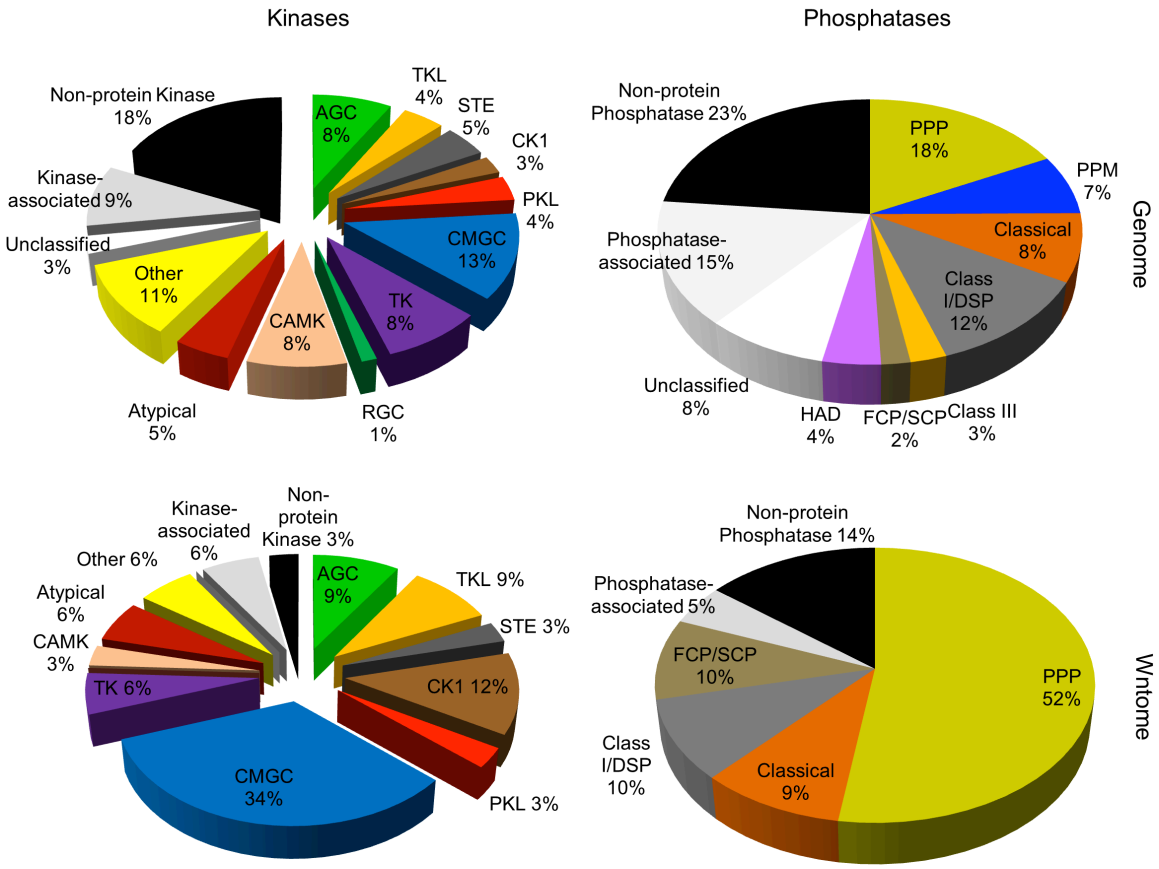


Figure 3.2.4 ***In vivo*** **Wnt pathway regulators**

The *in vivo* screen from the wing disc yielded 54 high-confidence regulators and 36 low confidence regulators of the Wnt pathway. Of the 54 high-confidence regulators, 46 promoted (green) while 9 inhibited (red) the Wnt pathway. 3 high-confidence regulators acted upstream of the ligand-receptor interaction (asterisks) while the remaining acted downstream of the ligand-receptor interaction. 32 of the 54 high-confidence regulators have been previously identified to regulate the Wnt pathway (grey). These regulators when assayed in the adult eye for their effect on Wnt signaling yielded partially discrepant results compared to those of the wing disc, to either promote (green), inhibit (red), or have no effect on signaling (black).



Kinase Group	Drosophila	
	Genome	Wntome
AGC	32	3
Tyrosine Kinase-Like	17	3
STE	18	1
Casein Kinase 1	10	4
Protein Kinase-Like	14	1
CMGC	49	11
Tyrosine Kinase	33	2
Receptor Guanylate Cyclase	5	0
Calcium & Calmodulin-regulated Kinase	32	1
Atypical	18	2
Other	42	2
Unclassified	10	0
Kinase-associated	35	2
Non-protein Kinase	70	1
	385	33

Phosphatase Group	Drosophila	
	Genome	Wntome
Phosphoprotein Phosphatase	36	11
Protein Phosphatase Mg^{+}/Mn^{+}-dependent	15	0
Classical Protein Tyrosine Phosphatase	17	2
Class I/Dual Specificity Phosphatase	24	2
Class III Protein Tyrosine Phosphatase	5	0
Factor IIF-interacting CTD Phosphatase/Small CTD Phosphatase	4	2
Haloacid Dehalogenase-like Hydrolase	8	0
Unclassified	17	0
Phosphatase-associated	31	1
Non-protein Phosphatase	48	3
	205	21

Figure 3.2.5 Kinases and phosphatases in the *Drosophila* Wntome

The distribution of kinase and phosphatase groups in the *Drosophila* genome and wntome is shown. Approximately a third of the kinases and half of the phosphatases in the wntome belong to the CMGC and PPP groups respectively. 33 kinases and 21 phosphatases in the category of high-confidence regulators comprise the wntome.

3.3. Adult eye Wnt pathway screen

The *Drosophila* eye imaginal gives rise to the adult eye and head tissue. During larval development, Wg signaling specifies head formation and inhibits eye formation. Ectopic Wg signaling therefore produces a smaller eye surface (as observed in the *sev>wg* genotype) while a decrease in Wg signaling results in a larger eye surface at the expense of head cuticle (Kumar, 2011). The adult eye has traditionally been used to screen for novel regulators of the Wnt pathway using the *sev>wg* (or equivalent) sensitized genetic background. Any gene that modifies the reduced eye surface genetically interacts with Wnt signaling and might represent a bona fide pathway component. For example, a gene might function as a positive regulator of Wnt signaling if it enhances the *sev>wg* phenotype and a negative regulator of Wnt signaling if it suppresses this phenotype. However, this assay is not specific as several signaling pathways and processes regulate the formation of the eye (Kumar, 2011). We tested the ability of all 90 candidate regulators from the *in vivo* screen to modify the *sev>wg* phenotype in the *Drosophila* eye (Figure 3.2.4) (Appendix). We found that of the high-confidence regulators identified in the wing disc, which included both known and novel components of the Wnt pathway, at least a third displayed different effects on signaling in the adult eye (Figure 3.2.4). These results validated our approach of implementing a specific assay to screen for Wnt pathway regulators *in vivo* rather than relying on a non-specific adult phenotype. There is less discrepancy between the wing disc and adult eye data for the high-confidence regulators (33%) relative to the low-confidence regulators (50%) (Figure 3.2.4), which is further attestation to the authenticity of the regulators in the former category.

3.4. Examples of Wnt pathway regulators

We identified *Cdc37* as a novel high-confidence negative regulator of the Wnt pathway that functioned downstream of the ligand-receptor interaction. Knock-down of *Cdc37* with *dpp-Gal4* resulted in an enhancement of Sens and Dll but did not affect Wg (Figure 3.4.1 B), relative to *wild type* (Figure 3.4.1 A). Knock-down of the phosphatase gene *CG3530* with *dpp-Gal4* resulted in a decrease in Sens and Dll, an increase in Wg, and had no effect on Cut (Figure 3.4.1 C). Thus *CG3530* was classified as a high-

confidence positive regulator of the Wnt pathway that functioned upstream of the ligand-receptor interaction. Knockdown of this component disrupted ligand secretion and/or diffusion, resulting in lowered expression of pathway target genes. Knock-down of *gilgamesh* (*gish*) with *hh-Gal4* resulted in the loss of Sens, Dll, Wg and Cut (Figure 3.4.1 D). When tested with *C5-Gal4*, knock-down of *gish* resulted in a weak decrease in Sens and Dll, but did not affect Wg (Figure 3.4.1 E). *gish* functioned, independently of the Notch pathway, as a high-confidence positive regulator of the Wnt pathway downstream of the ligand-receptor interaction. When we knocked-down the expression of the aforementioned regulators in the *sev>wg* genetic background (Figure 3.4.1 G), in accordance with its role in the wing disc, *gish* functioned as a positive regulator of Wnt signaling to suppress the *sev>wg* phenotype and partially restored the size of the eye (Figure 3.4.1 I) to that of *wild type* (Figure 3.4.1 F). The knock-down of *CG3530* weakly enhanced the *sev>wg* phenotype and suggested that this gene, in opposition to its known effect in the wing disc, behaved as a negative regulator of Wnt signaling in the adult eye (Figure 3.4.1 H). The function of *Cdc37* could not be assayed in the adult eye due to lethality (Figure 3.2.4).

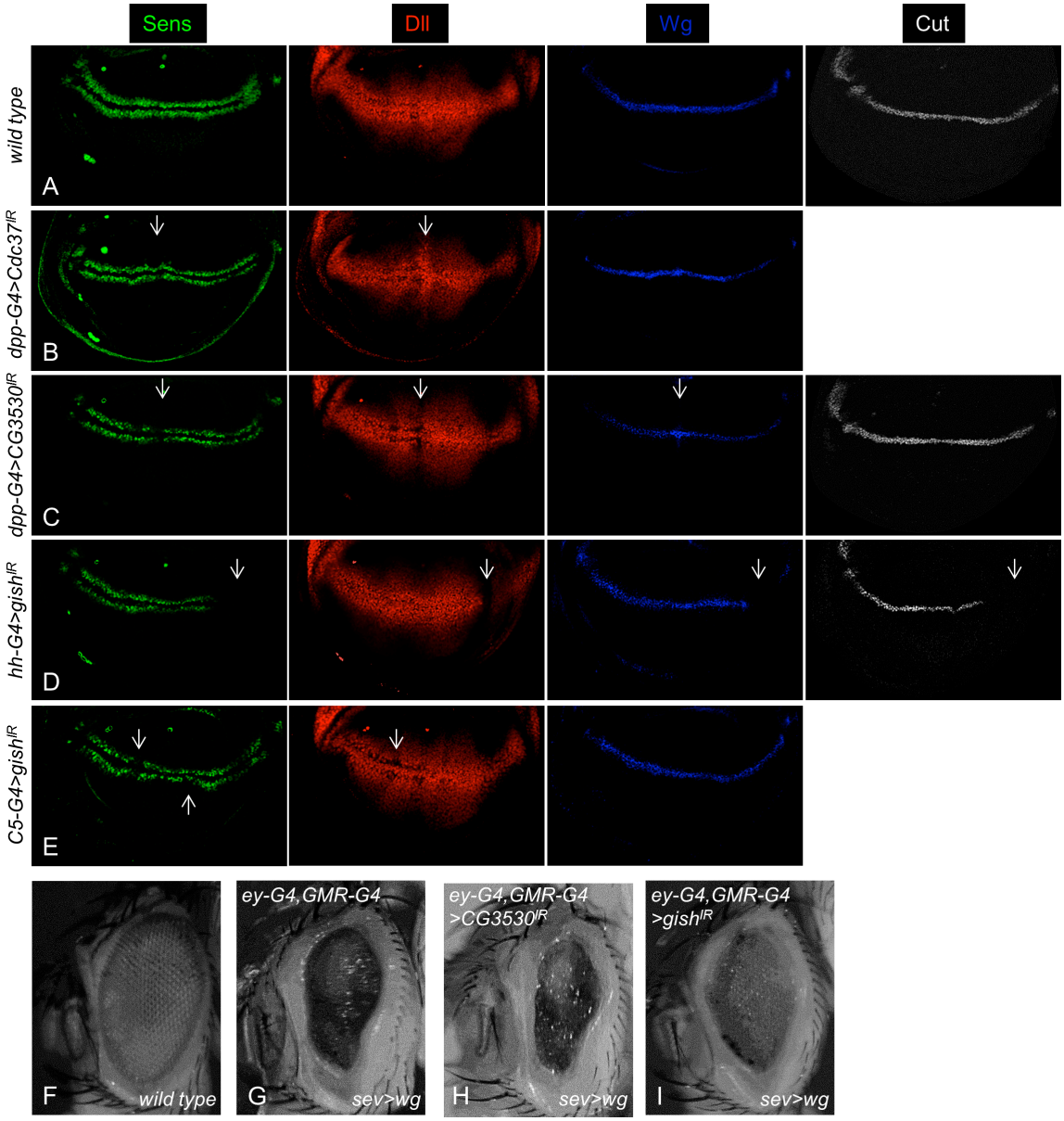


Figure 3.4.1 Examples of Wnt pathway regulators

(A) The expression domains of Sens (green), Dll (red), Wg (blue), and Cut (white) in a *wild type* wing disc. (B) Knock-down of *Cdc37* using *dpp-Gal4* resulted in an increase in Sens and Dll (arrows), but no change in Wg. (C) Knock-down of *CG3530* using *dpp-Gal4* resulted in a decrease in Sens and Dll, an increase in Wg (arrows), and no change in Cut. (D) Knock-down of *gish* using *dpp-Gal4* decreased the expression domains of Sens, Dll, Wg, and Cut (arrows). (E) Knock-down of *gish* using *C5-Gal4* decreased the expression of Sens and Dll (arrows), but not Wg. (F-I) The *sev>wg* genotype produced a reduced eye size relative to that of *wild type*. In this genetic background, knock-down of *CG3530* or *gish* using *ey-Gal4,GMR-Gal4* respectively enhanced or suppressed the reduced eye phenotype.

3.5. Comparison of Wnt and Hh pathways

Hh signaling has diverse essential functions during metazoan development such as the regulation of organogenesis and stem cell homeostasis (Ingham et al., 2011; Varjosalo and Taipale, 2008). As in the case of the Wnt pathway, several components of the Hh pathway were first identified and characterized in *Drosophila*. The Hh ligand, after which the pathway is named, can act at short-range and as a long-range morphogen (Briscoe et al., 2001; Heemskerk and DiNardo, 1994; Johnson and Tabin, 1995; Riddle et al., 1993; Roelink et al., 1995; Strigini and Cohen, 1997). Hh is lipidated by the MBOAT family enzyme Hedgehog Acyltransferase (HHAT) in the ER of the signal-producing cell (Chamoun et al., 2001; Chen et al., 2004; Kohtz et al., 2001; Lee and Treisman, 2001; Lee et al., 2001b; Micchelli et al., 2002), after which it is secreted with the aid of the sorting receptor Dispatched (Amanai and Jiang, 2001; Burke et al., 1999; Caspary et al., 2002; Kawakami et al., 2002; Ma et al., 2002). Outside the cell the transport of Hh is mediated by lipoprotein particles (Eugster et al., 2007; Palm et al., 2013; Panáková et al., 2005), exosomes (Liégeois et al., 2006; Tanaka et al., 2005), oligomerization (Callejo et al., 2006; Chen et al., 2004; Goetz et al., 2006; Vyas et al., 2008), and HSPGs (Desbordes and Sanson, 2003; Eugster et al., 2007; Han et al., 2004; Lum et al., 2003a; Takeo et al., 2005) (Figure 3.5.1).

In the absence of signaling, the transmembrane receptor Ptc inhibits the cell surface accumulation of the signal transducer Smoothened (Smo), a GPCR family member (Alcedo et al., 1996; van den Heuvel and Ingham, 1996; Hooper and Scott, 1989; Ingham et al., 2000; Martın et al., 2001; Stone et al., 1996; Strutt et al., 2001; Taipale et al., 2002). As a result, the pathway effector GLI is partially degraded in the cytosol through its sequential phosphorylation and poly-ubiquitination. GLI is phosphorylated at its C-terminus by Protein Kinase A (PKA), GSK3, and CK1α (Chen et al., 1998; Jia et al., 2002, 2005; Price and Kalderon, 1999, 2002), that are part of a protein complex centred around the scaffolding protein Kinesin family member 7 (Kif7) (Robbins et al., 1997; Sisson et al., 1997; Tay et al., 2005; Zhang et al., 2005b). The phosphorylated residues of GLI serve as a recognition motif for $SCF^{\beta\text{-}TrCP}$ that mediates its poly-ubiquitination and partial proteolysis via the proteasome (Bhatia et al., 2006; Jia et al., 2005; Jiang and Struhl, 1998; Pan and Wang, 2007; Smelkinson and Kalderon,

2006; Smelkinson et al., 2007; Tempé et al., 2006; Wang and Li, 2006). C-terminally truncated GLI functions as a repressor of signaling by binding the pathway response element of target genes to inhibit their expression (Aza-Blanc et al., 1997; Méthot and Basler, 1999; Müller and Basler, 2000; Sasaki et al., 1997) (Figure 3.6.1). Binding of Hh to Ptc abrogates its repression of Smo (Chen and Struhl, 1996; Denef et al., 2000; Fuse et al., 1999; Marigo et al., 1996). The single-pass transmembrane proteins Cell adhesion molecule-related/down-regulated by oncogenes (Cdo) and Brother of Cdo (Boc) act as co-receptors for Hh in this process (McLellan et al., 2006; Tenzen et al., 2006; Yao et al., 2006; Zhang et al., 2006b). The de-repression of Smo results in its accumulation at the cell surface where it is phosphorylated by PKA and CK1α as part of the Kif7 complex (Apionishev et al., 2005; Denef et al., 2000; Jia et al., 2004; Liu et al., 2007; Zhang et al., 2004; Zhu et al., 2003). The association of Kif7 with the phosphorylated C-terminus of Smo in response to the Hh ligand destabilizes the protein complex (Jia et al., 2003; Liu et al., 2007; Lum et al., 2003b; Ogden et al., 2003; Ruel et al., 2003). As a result, the full-length activator form of GLI is stabilized in the cytosol that translocates to the nucleus to direct the expression of target genes of the pathway (Aza-Blanc et al., 1997; Ohlmeyer and Kalderon, 1998; Strigini and Cohen, 1997; Wang and Holmgren, 2000) (Figure 3.5.1).

Although the evolutionary relationship of Wnt and Hh signaling remains unclear, these pathways have a similar molecular mechanism of signal transduction (Kalderon, 2002). In each case the pathway effector is phosphorylated in a cytosolic complex, poly-ubiquitinated, and degraded in the absence of signaling. Whereas in the presence of the ligand, the signal is transduced via a GPCR to inhibit the cytosolic complex through its recruitment to a phosphorylated transmembrane protein at the cell surface and thereby stabilize the levels of the pathway effector. The ligands of both pathways are also lipid-modified by MBOAT proteins and their extracellular movement is regulated in a similar manner (Figure 3.5.1). These pathways share numerous components that exert the same effects on signaling by functioning at analogous levels of the relays, including CK1α, GSK3 (Liu et al., 2002; Price and Kalderon, 2002), $SCF^{\beta\text{-}TrCP}$ (Jiang and Struhl, 1998), PP1 (Kim et al., 2013; Luo et al., 2007; Su et al., 2011), PP2A (Jia et al., 2009; Su et al., 2008), G protein-coupled receptor kinases (Gprks) (Chen et al., 2009, 2010, 2011; Cheng et al., 2010; Molnar et al., 2007), Guanine nucleotide-binding proteins

(Katanaev et al., 2005; Liu et al., 2001b, 2005b; Ogden et al., 2008), Flotillin-2 (Katanaev et al., 2008), Sulfatase-1 (Ai et al., 2003; Kleinschmit et al., 2010; Wojcinski et al., 2011; You et al., 2011), Notum (Ayers et al., 2010; Gerlitz and Basler, 2002; Giráldez et al., 2002), Nedd8 (Ou et al., 2002), phosphatidylinositol kinases (Pan et al., 2008b; Yavari et al., 2010), Hipk2 (Swarup and Verheyen, 2011), CK2 (Gao and Wang, 2006; Jia et al., 2010; Song et al., 2003), and Ubiquitin-specific protease 8 (USP8) (Li et al., 2012; Mukai et al., 2010; Xia et al., 2012). Interestingly, a large proportion of the factors common to both pathways are enzymes that regulate the reversible phosphorylation of signaling components.

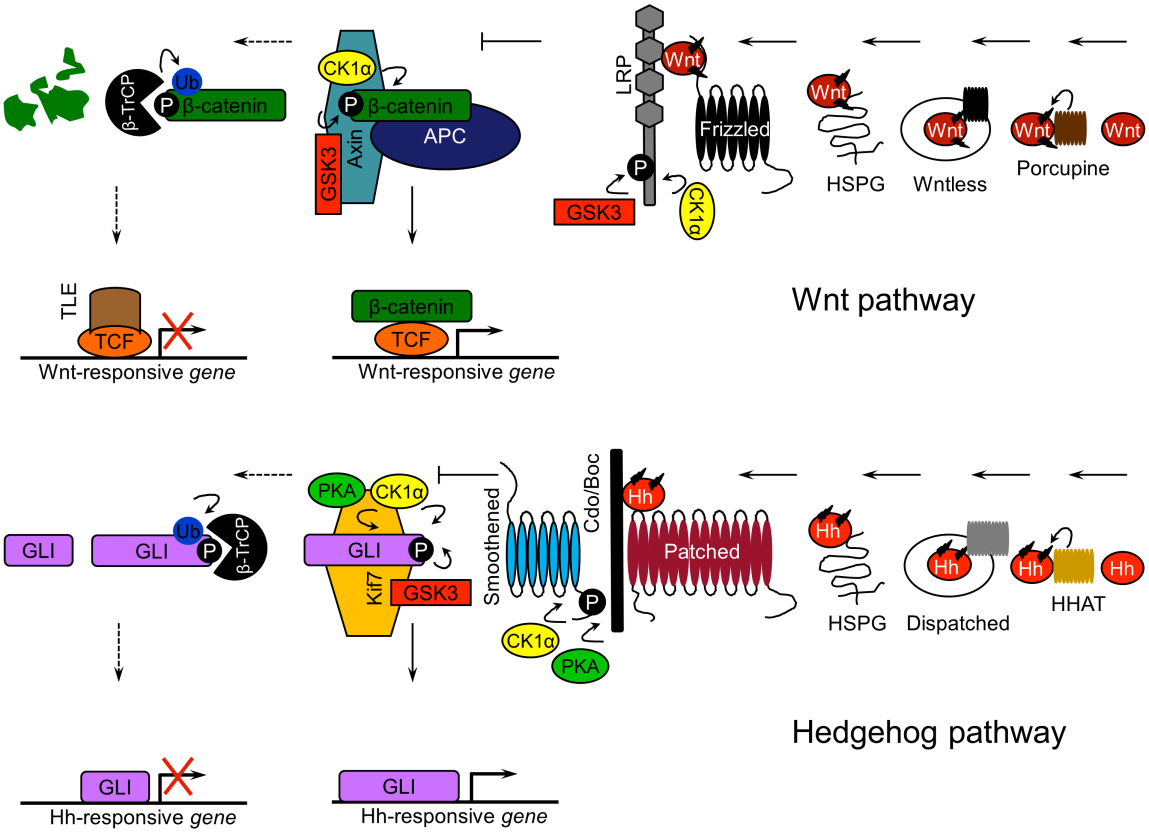


Figure 3.5.1 Comparison of the Wnt and Hh pathways

The Wnt and Hh pathways have a near identical mechanism of signal transduction from the signal-producing cell to the signal-receiving cell. In each case the pathway effector is phosphorylated in a cytosolic complex, poly-ubiquitinated, and degraded in the absence of signaling. The presence of signaling promotes the inhibition of the cytosolic complex via a phosphorylated transmembrane protein at the cell surface to stabilize the levels of the pathway effector. A large proportion of signaling components, especially kinases and phosphatases, are common to both pathways. (Figure based on Kalderon, 2002)

3.6. *In vivo* Hh pathway screen

Due to the mechanistic similarities between the Wnt and Hh pathways (Figure 3.5.1), we tested the ability of all 90 candidates identified from the *in vivo* Wnt pathway screen to regulate the Hh pathway. We performed this cross-screen to identify genes that exert the same effects on both pathways, thereby potentially acting at analogous levels of signaling. In the wing disc, Hh signaling stabilizes full-length Ci to regulate expression of the pathway target gene *ptc* along the anterior/posterior compartment boundary (Strigini and Cohen, 1997). As before, every gene was tested with at least two non-overlapping RNAi transgenes using the *MS1096-Gal4* driver (plus *UAS-dicer-2*) in combination with antibodies against Ci and Ptc (Figure 3.6.1 A). We found that 66 of the 90 candidates of the Wnt pathway also regulated the Hh pathway and modified the domain of Ci (Figure 3.6.2) (Appendix). While the majority of candidates of the Wnt pathway promoted signaling, unexpectedly we found that the majority of Hh regulators inhibited signaling (Figure 3.6.2). Nevertheless, 29 of the 66 Hh regulators exerted the same effects on the Wnt pathway to either promote or inhibit signaling. All but 4 of these 29 regulators of both pathways, which we propose to act at analogous levels of the signaling relays, were part of the category of high-confidence regulators identified from the Wnt pathway screen (Figure 3.6.1 B). We identified *Cdc37* and *gish* as a novel negative and positive regulator respectively of the Hh pathway. Knock-down of *Cdc37* resulted in a strong enhancement of Ci (and Ptc) (Figure 3.6.1 D), while knock-down of *gish* reduced Ci (but not Ptc) (Figure 3.6.1 E), both relative to *wild type* (Figure 3.6.1 C).

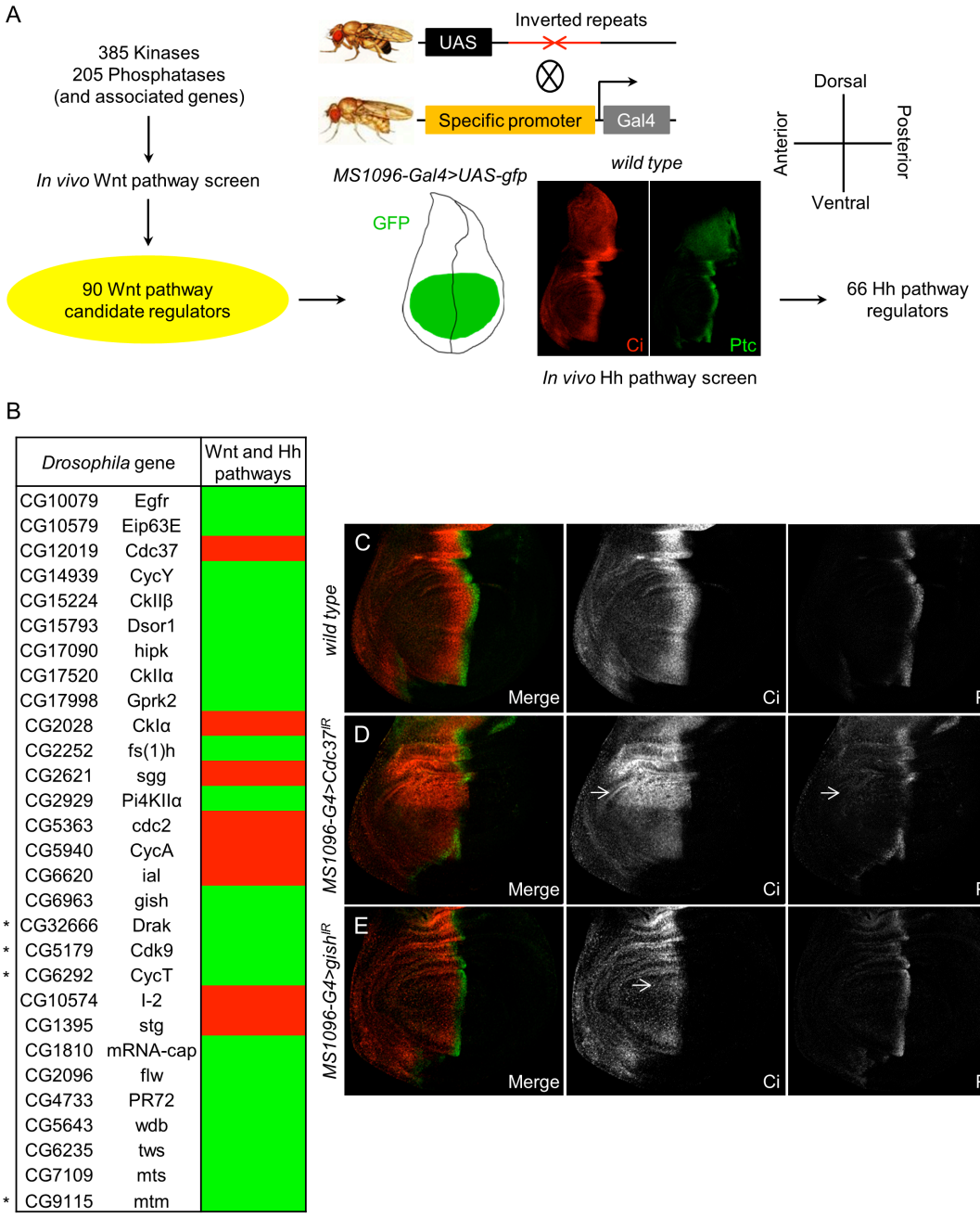


Figure 3.6.1 Hh pathway cross-screen

(A) The candidate regulators of the Wnt pathway were screened in the wing disc using antibodies against Ci, Ptc for their ability to regulate the Hh pathway. 66 of the 90 genes tested affected the Hh pathway. (B) 29 regulators exerted the same effects on the Wnt and Hh pathways and either promoted (green) or inhibited (red) signaling. All but 4 of these 29 genes (asterisks) clustered in the category of high-confidence regulators. (C) The expression domains of Ci, Ptc in a *wild type* wing disc. (D) Knock-down of *Cdc37* using the *MS1096-Gal4* driver resulted in an increase in Ci and Ptc (arrows). (D) Knock-down of *gish* using the *MS1096-Gal4* driver decreased the levels of Ci but not Ptc.

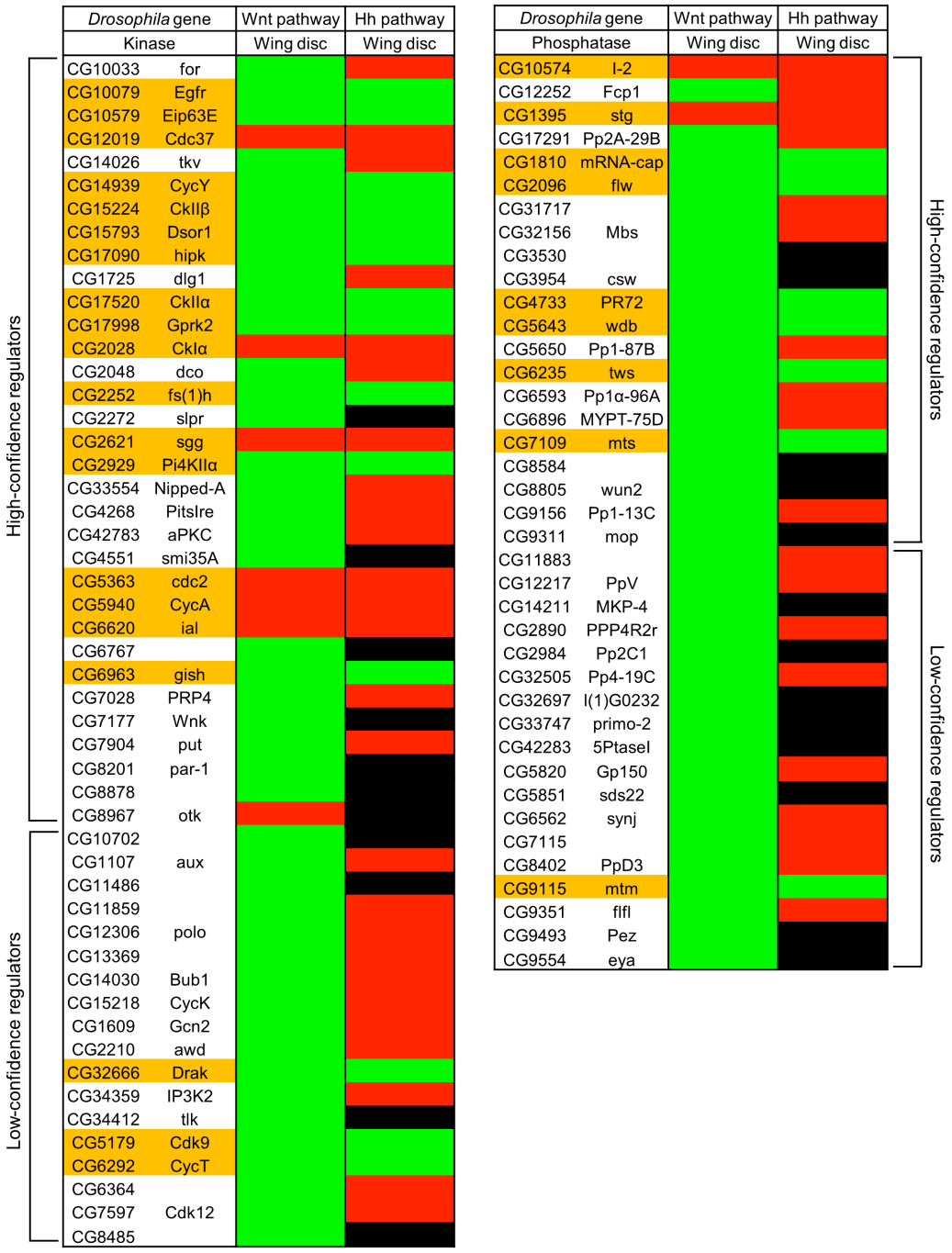


Figure 3.6.2 Comparison of genes that regulate Wnt and Hh signaling *in vivo*

66 of the 90 candidate regulators of the Wnt pathway also regulated the Hh pathway in the *Drosophila* wing disc. 24 candidate regulators of the Wnt pathway did not affect the Hh pathway (black). Of the 66 Hh pathway regulators, 21 promoted (green) signaling and 45 inhibited (red) signaling. 29 genes were identified to have the same effect on both the Wnt and Hh pathways (orange) to either promote or inhibit signaling.

3.7. Proof of concept

As proof of concept for screening *in vivo* for factors that operate at analogous levels of signaling for the Wnt and Hh pathways, we have characterized the functions of Cdc37 and Gish. Cdc37 is a chaperone that functions to regulate the folding and biogenesis of diverse kinases (Caplan et al., 2007). MARCM clones of *Cdc37* resulted in a cell autonomous up-regulation of Wnt and Hh signaling, as indicated by an increase in Dll and Ci respectively (Figure 3.7.1 A-B). These loss-of-function clones were observed away from the domains of the Wnt and Hh pathways, thereby suggesting that Cdc37 inherently functions irrespective of the presence of signaling to negatively regulate the stability of the effectors Arm and Ci respectively. Indeed when we assayed for levels of stabilized Arm, we found more to be present in wing discs reduced for *Cdc37* function compared to *wild type* discs (Figure 3.7.1 C). To validate the functional conservation of Cdc37 across species, we used siRNA to reduce its expression in mammalian 3T3 cells which resulted in a robust increase in the levels of stabilized β-catenin (and signaling activity from the Topflash reporter) and GLI3 (Figure 3.7.1 D-E). As Cdc37 is kinase-associated chaperone, we propose that it functions to promote the folding of CK1α and/or GSK3 that constitutively destabilize the signaling effectors of the Wnt and Hh pathways.

Gish (*Drosophila* CK1γ) is a plasma membrane-associated kinase that positively regulates the Wnt pathway by phosphorylating the co-receptor LRP in response to signaling (Davidson et al., 2005; Zhang et al., 2006a). Thus far no role has been ascribed to this kinase in the Hh pathway. Somatic clones of *gish* displayed a cell autonomous down-regulation of Hh signaling as indicated by a decrease in Ci (Figure 3.7.2 A). We additionally found that *gish* knock-down reduced the phosphorylation of Smo as detected by a mobility shift, both in the case of normal and excess levels of pathway activity. In the former case we observed decreased levels of Smo but not in the latter (Figure 3.7.2 B). Lastly, we found CK1γ to increase the levels of full-length GLI3 in mammalian 3T3 cells (Figure 3.7.2 C). We propose that CK1γ, as in the case of Wnt signaling, functions at the levels of the plasma membrane to phosphorylate the signal transducer Smo and potentiate Hh signaling.

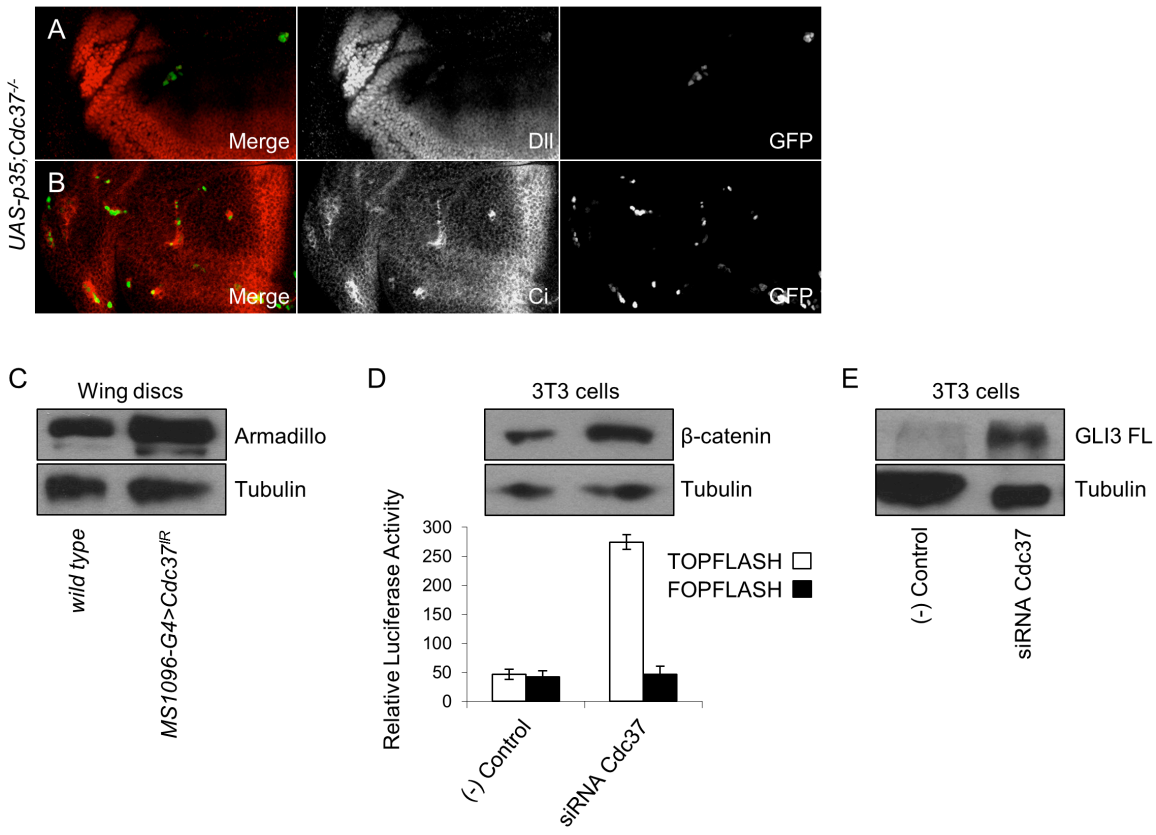


Figure 3.7.1 Cdc37 negatively regulates Wnt and Hh signaling

(A-B) MARCM clones of *Cdc37* (GFP positive) resulted in an increase in Dll and Ci. A *UAS-p35* transgene was overexpressed in these clones to prevent cell death. (C) Protein lysate from wing discs reduced for *Cdc37* function had higher levels of stabilized Arm relative to wild type. (D) Knock-down of Cdc37 levels using siRNA enhanced the levels of stabilized β-catenin in 3T3 cells and Topflash reporter activity. (E) Knock-down of Cdc37 levels using siRNA enhanced the levels of full-length GLI3 in 3T3 cells.

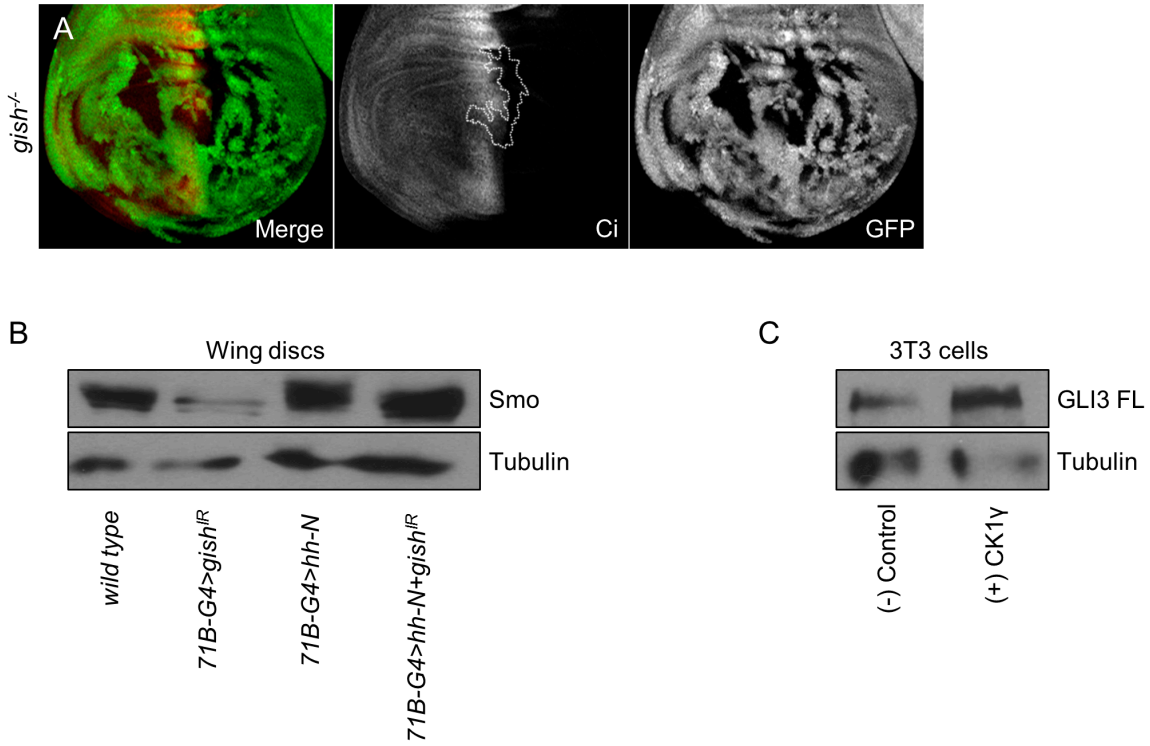


Figure 3.7.2 Gish/CK1γ positively regulates Wnt and Hh signaling

(A) Somatic clones of *gish* (GFP negative) had decreased levels of Ci. (B) Knock-down of *gish* reduced the phosphorylation of Smo (detected by mobility shift) in the presence of normal and excess Hh pathway activity. (C) Over-expression of CK1γ increased the levels of full-length GLI3 in 3T3 cells.

3.8. Summary

3.8.1. Wnt pathway screens: past and present

There have been a number of screens performed *in vivo* and *in vitro* to identify regulators of the Wnt pathway. The *in vivo* studies were performed in *Drosophila* using sensitized genetic backgrounds generated by enhanced levels of pathway activity to evaluate the ability of decreased or increased gene function to modify a non-specific adult phenotype (Cox et al., 2000; Desbordes et al., 2005; Greaves et al., 1999; Schertel et al., 2013). High-throughput *in vitro* screens, both loss-of-function and gain-of-function, have also been carried out in *Drosophila* and mammalian cell lines using a synthetic reporter of the pathway. As with the *in vivo* screens, these cell culture studies employed an artificially enhanced level of pathway activity either through the use of ectopic ligand, a constitutively-active form of the receptor, or a stabilized form of the effector. Moreover, in certain cases due to the specificity of the cell line and screen design there was only a limited ability to identify regulators at every level of the pathway from the signal-producing cell to the signal-receiving cell. (Buechling et al., 2011; Caspi and Rosin-Arbesfeld, 2008; DasGupta et al., 2005, 2007; Firestein et al., 2008; Groenendyk and Michalak, 2011; Jacob et al., 2011; James et al., 2009; Kategaya et al., 2009; Major et al., 2008; Miller et al., 2009; Port et al., 2011; Tang et al., 2008).

We performed a comprehensive *in vivo* loss-of-function RNAi screen to identify regulators of the Wnt pathway in *Drosophila*. We believe that our study is the most robust and stringent to date, offering several advantages over previous studies. The screen was performed entirely *in vivo* as opposed to cell culture, using an animal that has low functional redundancy but high functional conservation of genes with humans (Fortini et al., 2000; Reiter et al., 2001). Rather than relying on non-specific phenotypes in *Drosophila* such as the segmental patterning of the embryonic ectoderm, specification of the wing margin, or the regulation of eye formation, all of which can be attributed to multiple signaling pathways and processes, wing discs were assayed with antibodies against specific targets of the pathway. These targets respond to the entire gradient of signaling as opposed to a synthetic transcriptional reporter that variably responds to the level of pathway activity based on its composition (i.e. the number of high-affinity TCF binding sites) and the availability of signal-independent factors (i.e. cooperative

activation) (Barolo, 2006; DasGupta et al., 2007). Lastly, the study was performed at a physiological and not artificial level of signaling in a tissue that produces and responds to the ligand. Our analyses identified novel Wnt pathway regulators at all levels of the relay from the signal-producing cell to the signal-receiving cell in a biological context.

3.8.2. Screening for regulators of the Wnt pathway *in vivo*

In our analyses, we identified 54 high-confidence regulators of the Wnt pathway, which represent at least twice as many compared to previous studies and 22 of which are novel regulators of signaling. All but 3 of the 54 high-confidence regulators of the Wnt pathway function in the signal-receiving cell downstream of ligand reception and over 80% of the high-confidence regulators identified promote signaling. We also established previously unknown relationships between the mechanistically similar Wnt and Hh pathways by identifying 25 high-confidence regulators of the Wnt pathway that have similar effects on the Hh pathway and thereby potentially function at equivalent levels of signaling. We characterized Cdc37 as a novel dual negative regulator the Wnt and Hh pathways that functions to destabilize Arm and Ci respectively, regardless of the status signaling. Additionally, Gish positively regulates Hh signaling and transduces the signal at the level of the plasma membrane, as in the case of Wnt signaling. The roles of Cdc37 and Gish are functionally conserved in mammalian cells. Our results demonstrate the advantages of implementing a specific *in vivo* assay for identifying regulators of a biological process.

Nevertheless, all novel high-confidence regulators of the Wnt pathway and regulators of the Hh pathway identified need to be validated to confirm their authenticity. Sequence-dependent OTEs can result in false positive results. To limit sequence-dependent OTEs, most design algorithms avoid regions in the target sequences that have 19 or more base pairs of contiguous nucleotide identity to another mature transcript, as a 19-mer is sufficient to induce RNAi knockdown of a target transcript. But shorter perfect matches and imperfect matches to other mRNA sequences may also contribute to OTEs (Birmingham et al., 2006). Sequence-dependent OTEs can be negated through the testing of additional inverted repeat sequences against the gene of interest. It would also be prudent to demonstrate that a phenotype produced as a result of RNAi can be rescued through the over-expression of the corresponding genomic

fragment or cDNA that is inert to knock-down (i.e. has a silent mutation to avoid sequence complementarity and subsequent gene knock-down or does not have 3'-UTR which is frequently targeted using RNAi). Lastly, through the use of existing or generation of novel mutant alleles we can assay a gene's true loss-of-function effect *in vivo*. False negative results are caused by the inefficient knock-down of gene function. Positional effect of the insertion site of the *UAS*-inverted repeat transgene may sometimes cause low levels of dsRNA expression. To avoid this problem, a phiC integrase-mediated transformation system based on site-specific recombination between the *attB* and *attP* recognition sites has been developed. Using this system, transgenes are inserted into defined loci in the *Drosophila* genome that induce strong expression and therefore generate fewer false negative results (Bateman et al., 2006; Bischof et al., 2007; Groth et al., 2004; Venken et al., 2006). Additionally, a *UAS-dicer-2* transgene was incorporated in the screening process to enhance the potency of RNAi-mediated gene knock-down.

Future studies would also entail testing the ability of novel high-confidence regulators to regulate the Wnt pathway in multiple tissues in *Drosophila*. As discussed earlier, synthetic pathway reporters such as Topflash variably respond to the gradient of signaling in mammalian cells and do not work in *Drosophila*. *frizzled3* (*fz3*) is an endogenous universal target gene of the Wnt pathway in all tissues in *Drosophila*. Novel high-confidence regulators that affect *fz3* expression in multiple tissues are more likely to be important regulators of the Wnt pathway than those that differentially or do not regulate *fz3* expression. Also, using commercially available antibodies, novel high-confidence regulators of the Wnt pathway that function in the signal-receiving cell may be further positioned upstream or downstream of stabilized Arm in the wing disc. These assays would allow us to construct a library that provides information on tissue specificity and level of function of each novel high-confidence regulator of the Wnt pathway identified.

3.8.3. Co-evolution of Wnt and Hh signaling

Given the molecular similarities between the Wnt and Hh pathways, it is possible that these signaling relays originated from a common ancestral pathway and were exposed to similar selective pressures to facilitate their co-evolution over time. There

are reasons other than their mechanistic similarities to support this hypothesis. Both Wnt and Hh ligands diversified early during metazoan evolution and subsequently decreased in number or were completely lost in arthropods and nematodes (Bürglin, 2008; Holstein, 2012). As in *Drosophila* embryogenesis, these pathways operate in a feedback loop to regulate segmentation in diverse species (Dray et al., 2010; Farzana and Brown, 2008; Simonnet et al., 2004). Lastly, the Wnt and Hh pathways are fundamental to stem cell renewal and function in tandem in the maintenance of homeostasis of multiple tissues that undergo constant regeneration (Taipale and Beachy, 2001). The comparison of signaling pathways *in vivo* and the identification of specific versus shared regulators will help us elucidate the multistep processes that regulate animal development and disease states.

4. Conclusion

Intercellular signaling pathways regulate all aspects of metazoan development from embryogenesis to adult homeostasis. A precise control of the state and threshold of intercellular signaling is necessary for normal development and is primarily achieved through the reversible phosphorylation of proteins. This post-translational modification of protein function is catalyzed by large numbers of transferases (>500 protein kinases in humans) and reversed by fewer numbers of hydrolases (>140 protein phosphatases in humans). Accordingly, the majority of cellular proteins have been identified to be phosphorylated at one or more sites (Olsen et al., 2006). Not surprisingly, divergent disease states have been attributed to be a cause or consequence of aberrant protein phosphorylation (Reiter et al., 2001).

The Wnt signaling pathway has multiple biological functions during metazoan development such as the regulation of cell proliferation, cell survival, cell motility, and cell fate specification. As a consequence, its deregulation results in a range of human diseases including type II diabetes, terta-amelia, osteoporosis, and different types of cancers. There have been intense efforts in the biotechnology and pharmaceutical sectors over the last two decades to develop effective inhibitors against components of the Wnt pathway. From a therapeutic point of view, this ubiquitous pathway presents several challenges to the development of a targeted drug. In addition to the existence of multiple ligand and receptor isoforms that initiate signaling, specificity of targeting is further complicated by the convergence of downstream events on promiscuous molecules such as Dishevelled and GSK3 that are employed in numerous processes. So, it is not surprising that the drug strategies specifically directed at the Wnt pathway are relatively few in number and not extensively optimized. Currently, aberrant activity of the Wnt pathway is combated through the combinatorial use of non-steroidal anti-inflammatory drugs (NSAIDS), vitamin derivatives, lithium, antisense molecules, neutralizing antibodies, and small molecule inhibitors. Some of these therapies such as NSAIDS and vitamin derivatives are not specific to the Wnt pathway, while others are

specific to target the pathway at all levels of the signaling relay (Barker and Clevers, 2006). Although reversible phosphorylation was identified in the 1950s, it was not until the success of the tyrosine kinase inhibitor Gleevec in the 1990s that researchers realized the importance of treating disease with drugs targeting protein kinases and phosphatases. Since then, research on kinases and phosphatases has accounted for approximately 30% of the drug discovery programs in the pharmaceutical industry, and three kinase inhibitors (fasudil, rapamycin, and Gleevec) and one phosphatase inhibitor (cyclosporine) have so far been approved for clinical use (Cohen and Tcherpakov, 2010).

We have identified Hipk as a novel kinase component of the Wnt pathway in *Drosophila*, and it functions at multiple levels to promote signaling. Hipk functions downstream of the ligand-receptor-co-receptor interaction in the signal-receiving cell to stabilize the pathway effector Arm. It does so phosphorylating and inhibiting the E3 ligase component Slimb that mediates the poly-ubiquitination and subsequent degradation of Arm. In the presence of catalytically active Hipk, stabilized Arm translocates to the nucleus to direct the expression of target genes of the pathway. Moreover, Hipk also has a role in the Wnt pathway independent of its effect on Arm stability. It interacts with the transcriptional complex and phosphorylates the transactivation domains of Arm to promote the expression of target genes of the pathway. We find that Hipk's function is evolutionarily conserved in mammalian cells, where Hipk2 promotes Wnt signaling by regulating both the stability and transcriptional activity of the pathway effector β-catenin. Subsequent to this study, we implemented a loss-of-function RNAi screen to identify additional kinases and phosphatases that regulate the Wnt pathway in *Drosophila*. As opposed to previous studies, ours was performed entirely *in vivo* through the detection of endogenous target genes at physiological levels of signaling. Through our analyses, we identified 22 novel regulators of the Wnt pathway that function at one or more levels of the signaling relay. One of these regulators is a kinase-associated chaperone, Cdc37, which constitutively destabilizes the pathway effector in *Drosophila* and mammalian cells, and likely functions to promote the folding of GSK3 and/or CK1α. We believe that our characterization of Hipk2 as a novel kinase component of signaling and the identification of additional enzymes that regulate the reversible phosphorylation of signaling *in vivo* will

serve as a library of potential therapeutic targets for the future development of inhibitors against the Wnt pathway.

Over the last three decades, the use of genetic analyses in *Drosophila* has elucidated both the function, in various developmental contexts, and the molecular mechanism of the Wnt pathway. Owing to the vast array of genetic techniques available in *Drosophila* and its high degree of functional conservation, no doubt novel pathway components and Wnt-regulated processes identified in future studies using this model system are likely to be directly applicable to human development and disease.

5. Materials and Methods

5.1. *Drosophila* genetics

The following *Drosophila* strains were used: w^{1118} (*wild type*), *Oregon-R* (*wild type*), *omb-Gal4*, *dpp-Gal4/TM6B*, *71B-Gal4*, *C5-Gal4*, *sd-Gal4*, *MS1096-Gal4*, *dpp-lacZ/CyO*, *en-Gal4,UAS-gfp*, *69B-Gal4*, *UAS-lacZ, ap-Gal4/CyO*, *UAS-wg*, *UAS-gfp*, *UAS-dicer-2*, *UAS-flp*, *UAS-p35*, $Cdc37^{eD4}$ *FRT79/TM6B, FRT82,GFP/TM6B, MARCM79, hs-Flp22*;;GFP*,FRT79/TM6B*, wg^{1-17}*/CyO*, wg^{1}*/CyO* (Bloomington *Drosophila* Stock Center), *UAS-hipk*, $hipk^{3}$ *FRT79/TM6B* and $hipk^{4}$ *FRT79/TM6B* (Lee et al., 2009a), $UAS\text{-}arm^{S2}$ and $UAS\text{-}arm^{S10}$ (Pai et al., 1997), *vg-lacZ* (Williams et al., 1994) $UAS\text{-}axin^{A2\text{-}4}$ (Willert et al., 1999b), *UAS-hhN* (Su et al., 2011)*, FRT82* $gish^{e01759}$ (Gault et al., 2012)*, UAS-fz2N33/CyO* (Zhang and Carthew, 1998), *UAS-fz2-arr* (Tolwinski et al., 2003), *UAS-hipk RNAi* (Vienna *Drosophila* RNAi Center), wg^{CX4}*/CyO* (Baker, 1987), *UAS-slimb* (Grima et al., 2002), *UAS-cul1* (Wu et al., 2005), *tub>myc-slimb* (Ko et al., 2002), *hh-Gal4/TM6B* and *eyFlp;ey-Gal4,GMR-Gal4;sev>y+>wg* (Port et al., 2011). All fly strains were maintained according to standard procedures at 25°C. The *UAS-lacZ* transgene was used as a negative control where required to rule out an effect due to titration of the Gal4 protein. The RNAi transgenic lines used for the *in vivo* screen were obtained from the Vienna *Drosophila* RNAi Center (VDRC), National Institute of Genetics (NIG), and Transgenic RNAi Project (TRiP) as indicated in the Appendix. For the *in vivo* screens, wing discs from 20 larvae of each genotype were dissected (30 larvae for genotypes that were non-homozygous with a balancer chromosome). For the eye screen, 20 flies of each genotype were scored. The penetrance of all phenotypes indicated in the Appendix is between 80-100%. The percentage of transgenic RNAi fly strains with predicted sequence-dependent off-target effects are as follows: 65% (0), 27% (1-2), 8% (>2).

5.2. Mosaic analysis

hipk somatic clones were generated by crossing the *hipk*^{4} *FRT79/TM6B* and *hs-Flp22*;;GFP*,FRT79/TM6B* strains and heat-shocking progeny 48 hours AEL for 1.5 hours at 38°C. *Cdc37* MARCM clones were generated by crossing the *MARCM79* and *UAS-p35; Cdc37*^{eD4} *FRT79/TM6B* strains and heat-shocking progeny 48 hours AEL for 2 hours at 38°C. *gish* somatic clones were generated by crossing the *omb-Gal4;;FRT82,GFP/TM6B* and *UAS-flp;FRT82 gish*^{e01759} strains.

5.3. Immunostaining of imaginal discs

Wing and eye discs were dissected from *Drosophila* third stage larvae in 0.1% Triton X-100 PBS (PBST). Discs were fixed in 4% paraformaldehyde at room temperature and washed 3 times for 5 minutes each using PBST. Discs were then blocked (2% BSA in PBST) for 1 hour at room temperature and incubation with primary antibodies (diluted in PBST) was performed overnight at 4°C. The following antibodies were used: anti-Armadillo N27A1 (1:200), anti-Cubitus interruptus 2A1 (1:50), anti-Patched (1:50), anti-Engrailed 4D9 (1:100), anti-Wingless 4D4 (1:100), anti-Cut (1:75) 2B10 (Developmental Studies Hybridoma Bank) anti-β-Galactosidase (1:1500) (Promega), anti-Senseless (1:1000) (Nolo et al., 2000), anti-Distalless (Duncan et al., 1998; Panganiban et al., 1995), anti-MYC (1:500) (SIGMA). After incubation with primary antibodies, discs were washed 5 times for 5 minutes each in PBST. Incubation with fluorescent secondary antibodies was performed at room temperature for 2 hours at a concentration of 1:400 (diluted in PBST) (Jackson Immunolabs). After incubation with secondary antibodies, discs were washed 5 times for 5 minutes each in PBST. Discs were mounted in Vectashield (Vector Laboratories).

5.4. Immunostaining of embryos

Drosophila embryos were collected at the desired stage, dechorionated in 50% bleach for 2 minutes, and rinsed multiple times using distilled water. Embryos were transferred to a glass scintillation vial containing 50% heptane and 50% PEMFA solution

and gently rocked for 15-20 minutes. The lower PEMFA layer was removed and an equal volume of methanol was added to the remaining heptane solution. The vial was then vigorously shaken for 30 seconds and the embryos were allowed to settle to the bottom. The methanol/heptane solution was removed and embryos were washed 3 times with 100% methanol. Embryos were rehydrated in PBST for 1 hour and then incubated in blocking solution (2% BSA in PBST) for 1 hour. Primary antibody staining was performed overnight at 4°C with anti-Engrailed 4D9 (1:100) and anti-Wingless 4D4 (1:100) (Developmental Studies Hybridoma Bank). After incubation with primary antibodies embryos were washed 5 times for 10 minutes each using PBST. Incubation with fluorescent secondary antibodies was performed at room temperature at a concentration of 1:400 (diluted in PBST). All fluorescent secondary antibodies used were from Jackson Immunolabs. After incubation with secondary antibodies embryos were rinsed 5 times for 10 minutes each in PBST. Embryos were mounted in Vectashield (Vector Laboratories).

5.5. Mounting of adult wings

Wings were removed from *Drosophila* adults and dehydrated in 100% ethanol for 5 minutes. The wings were placed onto a glass slide with the dorsal side up and the ethanol was allowed to evaporate. Wings were mounted in Canada balsam and allowed to flatten overnight.

5.6. Cuticle preparations

Embryos were collected 24 hours AEL, dechorionated in 50% bleach for 2 minutes, rinsed multiple times using distilled water, and mounted in Hoyer's medium on glass slides. The mounted embryos were incubated at 65°C for 1-2 days. Embryos were visualized using dark field microscopy.

5.7. Plasmids

The following plasmids were used: pUAST-HipkWT and pUAST-HipkKD (Lee et al., 2009a), pGEX-4T-GST (GE Healthcare), pGEX-GST-Hipk (Choi et al., 2005), pMK33-HA-Sgg (Matsubayashi et al., 2004), pMK33-HA-CK1 (Yanagawa et al., 2002), β-cateninS33Y-pCS2+ and β-TrCP-Myc-pCS2+ (Liu et al., 1999), pCDNA3-Hipk2WT-FLAG and pCDNA3-Hipk2K221R-FLAG (D'Orazi et al., 2002), pCDNA3-Myc-Ubiquitin (Kanei-Ishii et al., 2004), pDA-FLAG-Ci, pDA-FLAG-Hh-N and pDA-Renilla (Fukumoto et al., 2001), ptcΔ136-Luc and ptcΔ136-mut (Chen et al., 1999), pCMV-HA-ArmFL, pCMV-HA-ArmN, pCMV-HA-ArmR, pCMV-HA-ArmC, pCMV-Myc-TCF, pCMV-Myc-HipkWT, pCMV-HA-HipkWT, and pCMV-HA-HipkKD (Lee et al., 2009b), pCDNA-Wnt1, pCDNA-T7-LEF1, TOPFLASH and FOPFLASH (Ishitani et al., 1999), CK1γ-FLAG (Davidson et al., 2005),pAct-Arm-V5 and pAct-Arm*-V5 (Li et al., 2007).

5.8. Cell culture

Drosophila S2, S2R+, and S2-Tubulin-Wg cells (*Drosophila* Genomics Research Center) were maintained at 25°C in Schneider's medium supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) (Invitrogen). Wg-conditioned medium was produced by culturing *Drosophila* S2-Tubulin-Wg cells in Schneider's medium (with 10% FBS) for 2-3 days. Wg-conditioned medium was collected and stored at 4°C. Hh-conditioned medium was similarly produced using *Drosophila* S2 cells transfected with pDA-FLAG-Hh-N. *Drosophila* cells were transfected with plasmids using Effectene according to the manufacturer's instructions (Qiagen). Plasmids containing genes cloned downstream of a metallothionein promoter were induced by the addition of $CuSO_4$ (final concentration 0.5 mM) 12 hours post-transfection. HEK293T, HeLa, NIH-3T3 and COS7 mammalian cells (American Type Culture Collection) were cultured at 37°C in Dulbecco's Modified Eagle Medium supplemented with 10% FBS (Invitrogen). Mammalian cells were transfected with plasmids using Polyfect according to the manufacturer's instructions (Qiagen). All cell types were harvested 48 hours after transient transfection using lysis buffer supplemented with protease inhibitors (Cell Signaling Technology). For stability assays in *Drosophila* S2 cells, MG132 (Calbiochem) was used to treat cells at a concentration of 25 μM for 6 hours. For the cellular

ubiquitination assay in HEK293T cells, PYR41 (Calbiochem) was used to treat cells for 1 hour at a concentration of 50 μM. For cell fractionation assays the Subcellular Protein Fractionation Kit was used according to the manufacturer's instructions to separate the membrane fraction of proteins from the cytosolic fraction (Thermo Fisher). For the examination of protein stability in HEK293T cells, Cycloheximide (SIGMA) was used to treat cells 24 hours post-transfection at a concentration of 25 μg/mL. For siRNA-mediated gene knock-down in NIH-3T3 cells, the Lipofectamine RNAiMAX Transfection Reagent was used according to the manufacturer's instructions (Invitrogen).

5.9. Biochemical assays

In the binding assays, GST-Hipk or GST produced in *Escherichia coli* BL21 cells and purified according to standard procedures (GE Healthcare) was incubated with Slimb lysate and the proteins were immunoprecipitated by using Glutathione Sepharose columns (GE Healthcare) followed by SDS/PAGE/Western blotting. Proteins for biochemical and co-immunoprecipitation assays were purified from *Drosophila* S2 or HEK293T lysates using antibodies and Protein G-Sepharose according to manufacturer's instructions (SIGMA). Kinase assay reactions were performed using purified proteins at 30°C for 30 minutes followed by SDS/PAGE/Western blotting and detection with a phospho-specific β-catenin (S33/S37) antibody (1:750) (Cell Signaling Technology) or autoradiography (in the case of radiolabelled ATP). For the ubiquitination assays, E1, E2 (UbcH5), Mg-ATP solution, ubiquitin, ubiquitination buffer (Enzo Life Sciences) were mixed in reactions with immunoprecipitated proteins and were incubated at 37°C for 1 hour followed by SDS/PAGE/Western blotting and detection with an anti-poly-Ubiquitin antibody (1:1000) (Enzo Life Sciences).

5.10. Transcriptional assays

Transcriptional assays were performed using wild type and mutated (negative control) reporter plasmids. A control reporter plasmid pDA-RL that expresses Renilla luciferase was used for normalizing transfection efficiencies. All plasmids were transiently transfected into cells as mentioned earlier. Luciferase readings were

measured 48 hours after transfection with the Dual Luciferase Reporter Assay System according to manufacturer's instructions (Promega). The value for each data set is the average of three individual experiments. Standard deviation was calculated for each set of values.

5.11. Western blot analyses

Protein lysates were denatured and separated on 10% SDS-PAGE gels using the BioRad Mini-Protean II Electrophoresis Cell and transferred to nitrocellulose membranes using the BioRad Trans-Blot Semi Dry according to the manufacturer's instructions. Membranes were blocked in 0.1% Triton X-100 TBS (TBST) for 1 hour and incubated overnight at 4°C using primary antibodies (diluted in TBST). The following antibodies were used: anti-Tubulin, anti-Actin, anti-FLAG, anti-HA, anti-GST, and anti-MYC (1:1000) (SIGMA), anti-poly-Ubiquitin (1:1000) (Enzo Life Sciences), anti-β-catenin and anti-β-catenin (S33/S37) (1:750) (Cell Signaling Technology), anti-GLI3 (1:400) (Santa Cruz Biotechnology), anti-Armadillo N27A1 (1:750), anti-Smoothened 20C6 (1:10) (Developmental Studies Hybridoma Bank). After incubation with primary antibodies membranes were washed 5 times for 5 minutes each in TBST. HRP-conjugated secondary antibodies (Santa Cruz) at a concentration of 1:5000 (diluted in TBST) were used to probe membranes. After incubation with secondary antibodies membranes were washed 5 times for 5 minutes each. Membranes were visualized using the Enhanced Chemiluminescence System (GE Healthcare).

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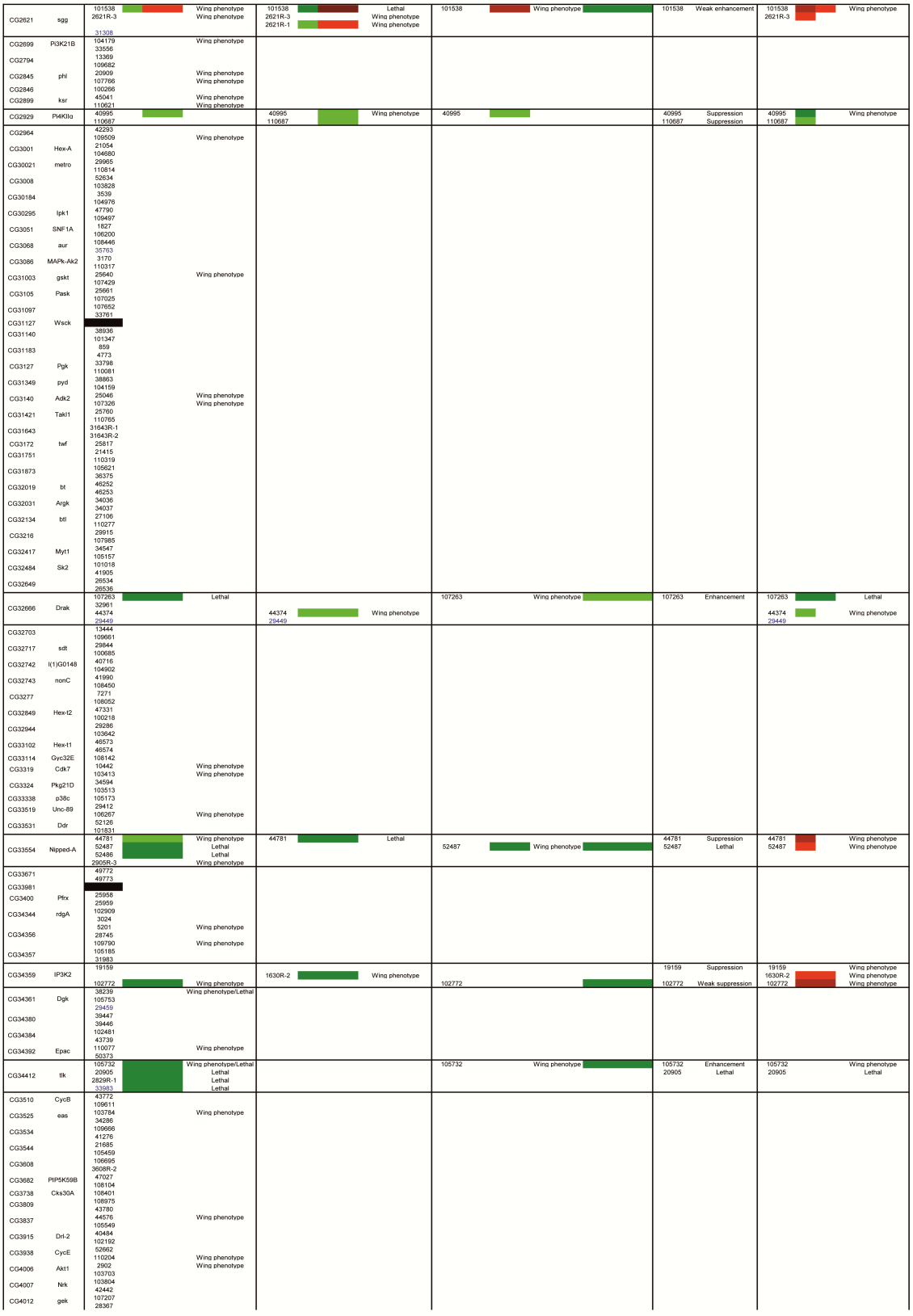
Appendix. Results of *in vivo* Wnt & Hedgehog screens


CG2621 sgg
101538 Wing phenotype
2621R-3 Wing phenotype
31308
101538 Lethal
2621R-3 Wing phenotype
2621R-1 Wing phenotype
101538 Wing phenotype
101538 Weak enhancement
101538 Wing phenotype
2621R-3
CG2699 PI3K21B
104179 Wing phenotype
33556
13369
CG2794
109682
CG2845 phl
20909 Wing phenotype
107766 Wing phenotype
CG2846
100266
CG2899 ksr
45041 Wing phenotype
110621 Wing phenotype
CG2929 PI4KIIα
40995
110687
40995
110687 Wing phenotype
40995
40995 Suppression
110687 Suppression
40995 Wing phenotype
110687
CG2964
42293
109509 Wing phenotype
CG3001 Hex-A
21054
104680
CG30021 metro
29965
110814
CG3008
52634
103828
CG30184
3539
104976
CG30295 Ipk1
47790
109497
CG3051 SNF1A
1827
106200
CG3068 aur
108446
35763
CG3086 MAPk-Ak2
3170
110317
CG31003 gskt
25640 Wing phenotype
107429
CG3105 Pask
25661
107025
CG31097
107652
CG31127 Wsck
33761
CG31140
38936
101347
CG31183
859
4773
CG3127 Pgk
33798
110081
CG31349 pyd
38863
104159
CG3140 Adk2
25046 Wing phenotype
107326 Wing phenotype
CG31421 Taki1
25760
110765
CG31643
31643R-1
31643R-2
CG3172 twf
25817
21415
CG31751
110319
105621
CG31873
36375
46252
CG32019 bt
46253
34036
CG32031 Argk
34037
27106
CG32134 btl
110277
29915
CG3216
107985
34547
CG32417 Myt1
105157
101018
CG32484 Sik2
41905
26534
CG32649
26536
CG32666 Drak
107263 Lethal
32961
44374
29449
44374
29449 Wing phenotype
107263 Wing phenotype
107263 Enhancement
107263 Lethal
44374
29449 Wing phenotype
CG32703
13444
109661
CG32717 sdt
29644
100685
CG32742 l(1)G0148
40716
104902
CG32743 nonC
41990
108450
CG3277
7271
108052
CG32849 Hex-t2
47331
100218
CG32944
29286
103642
CG33102 Hex-t1
46573
46574
CG33114 Gyc32E
108142
CG3319 Cdk7
10442 Wing phenotype
103413 Wing phenotype
CG3324 Pkg21D
34594
103513
CG33338 p38c
105173
CG33519 Unc-89
29412
105267 Wing phenotype
CG3531 Ddr
52126
101831
CG33554 Nipped-A
44781 Wing phenotype
52487 Lethal
52486 Lethal
2905R-3 Wing phenotype
44781
44781 Lethal
52487
52487 Wing phenotype
44781 Suppression
52487 Lethal
44781 Wing phenotype
52487 Wing phenotype
CG33671
49772
49773
CG33981
CG3400 Pfrx
25958
25959
CG34344 rdgA
102909
3024
5201 Wing phenotype
CG34356
28745
109790 Wing phenotype
CG34357
105185
31983
CG34359 IP3K2
19159
102772 Wing phenotype
1630R-2
Wing phenotype
102772
19159 Suppression
102772 Weak suppression
19159 Wing phenotype
1630R-2 Wing phenotype
102772 Wing phenotype
CG34361 Dgk
38239 Wing phenotype/Lethal
105753
29459
CG34380
39447
39446
CG34384
102481
43739
CG34392 Epac
110077 Wing phenotype
50373
CG34412 tlk
105732 Wing phenotype/Lethal
20905 Lethal
2829R-1 Lethal
33983 Lethal
105732
105732 Wing phenotype
105732 Enhancement
20905 Lethal
105732 Wing phenotype
20905 Lethal
CG3510 CycB
43772
109611
CG3525 eas
103784 Wing phenotype
34286
CG3534
109666
41276
CG3544
21685
105459
CG3608
106695
3608R-2
CG3682 PIP5K59B
47027
CG3738 Cks30A
108104
108401
CG3809
108975
43780
CG3837
44576 Wing phenotype
105549
CG3915 Drl-2
40484
102192
CG3938 CycE
52662
110204 Wing phenotype
CG4006 Akt1
2902 Wing phenotype
103703
CG4007 Nrk
103804
42442
CG4012 gek
107207
28367


CG6521 Stam
22497 Wing phenotype
35016 Wing phenotype
CG6535 tefu
22502
108074
CG6551 fu
27662 Wing phenotype
27663 Wing phenotype
CG6612 Adk3
110382
42064
CG6620 ial
104051 Wing phenotype
35107 Lethal
6620R-3 Wing phenotype/Lethal
35107 Wing phenotype
104051 Enhancement/Lethal
35107 Enhancement
104051 Wing phenotype
35107 Wing phenotype
CG6622 Pkc53E
27696
27699
CG6703 CASK
104793
34184
CG6715 KP78a
26722 Wing phenotype
47657
CG6767
35111
35112
109894 Wing phenotype
6767R-1 Wing phenotype
35112
109894 Wing phenotype
109894 Enhancement/Lethal
35112
109894 Lethal
CG6772 Slob
100987
30674
CG6775 rg
107056
36404
CG6800
40394
104255
CG6875 asp
2911 Wing phenotype
110177
CG6963 gish
106826 Wing phenotype
26003 Wing phenotype
28066 Wing phenotype
106826 Wing phenotype
26003 Wing phenotype
106826
26003 Suppression
106826 Wing phenotype
26003 Wing phenotype
CG7001 Pk17E
101951
102179
CG7004 fwd
27786
110159
CG7028 PRP4
27808 Wing phenotype
107042 Wing phenotype
27808 Wing phenotype
27808
107042
27808 Lethal
107042 Wing phenotype/Lethal
CG7069
101116
27834
CG7070 PyK
49533
35165
CG7094
27843
108273
CG7097 hppy
103580
35166
CG7103 Pvf1
6175
102699
CG7125 PKD
22344 Wing phenotype
106255 Wing phenotype
CG7156
28035
28036
CG7177 Wnk
35193 35193
35194 35194 Wing phenotype
106928 Wing phenotype 106928 Lethal
35194 Wing phenotype
35194 Suppression
35193
35194 Wing phenotype
106928 Wing phenotype
CG7186 SAK
27904
105102
CG7207 cert
103563
27914
CG7223 htl
27180
6692
CG7236
27505
CG7281 CycC
27937 Wing phenotype
48834 Wing phenotype
110610
CG7328
27952
7328R-1
CG7335
29035
100062
CG7362
104218
7557
CG7393 p38b
108099
7393R-3
CG7405 CycH
10398
104312 Wing phenotype
CG7470
101476
38955
CG7524 Src64B
35252
7524R-3
CG7525 Tie
27087
26879
CG7551
7551R-1
7551R-3
CG7597 Cdk12
25508 Lethal
25510 Wing phenotype
34838 Lethal
25508 Wing phenotype
25508 Enhancement/Lethal
34838 Enhancement/Lethal
25508 Wing phenotype/Lethal
34838 Wing phenotype
25510 Lethal
CG7616
41408
104663
CG7643 ald
110572
7643R-3 Wing phenotype
CG7693 fray
106919
41719
CG7717 Mekk1
110339
25529
CG7719 gwl
21046
7719R-1 Wing phenotype
CG7766
52572
110184 Wing phenotype
CG7838 BubR1
26109
7838R-1 Wing phenotype
CG7873 Src42A
26019 Wing phenotype
100708 Wing phenotype
CG7892 nmo
3002
101545
25793
CG7904 put
37279 Lethal
107071
7904R-2 Lethal
7904R-3 Lethal
37279 Wing phenotype
37279
107071 Enhancement
Enhancement
37279 Wing phenotype
7904R-2 Wing phenotype
7904R-3 Wing phenotype
CG7995
101869
22652
CG8049 Btk29A
106962
22675
CG8057 alc
104489
8057R-4
CG8094 Hex-C
35338
35337
CG8173
35846 Wing phenotype
105661
CG8174 SRPK
26933
103416 Wing phenotype
CG8201 par-1
52553
52556
32410 Wing phenotype
52553
52556
32410 Lethal
52556 Wing phenotype
52553 Suppression
52556
32410 Wing phenotype
CG8203 Cdk5
104491
35856
CG8222 Pvr
43459
105353
CG8224 babo
3825
106092 Wing phenotype
CG8239
24254
107034
CG8250 Alk
107083
11446
CG8286 P58IPK
109649
14154
CG8298
43541
43539
8298R-3
CG8351 Tcp-1η
108585 Wing phenotype
28895
CG8362 nmdyn-D7
35903
105161
CG8363 Papss
35904
110544
CG8475
2801 Wing phenotype
110591
CG8485
35939 Wing phenotype
35940
35939 Wing phenotype
35939 Suppression
35939 Wing phenotype
35940
CG8565
100449
8565R-2
CG8637 trc
35988 Wing phenotype
107923 Wing phenotype
CG8657 Dgkε
4659 Wing phenotype
8657R-2
109451
CG8726
40719
CG8767 mos
110435
43526
CG8789 wnd
103410
26910

CG8808	Pdk	106641									
		37968									
CG8866		103725									
		44859									
CG8874	Fps85D	36053									
		107266									
CG8878		28971		Wing phenotype				100985		Wing phenotype			28971	Suppression	28971	Wing phenotype
		100985		Wing phenotype											100985	Wing phenotype
CG8914	CK11β2	102633									
		26915									
CG8948	Graf	42165									
		42166									
CG8967	otk	42566			42566						
		30834			30834										30834	
		104688			104688		Wing phenotype		104688				104688	Enhancement	104688	Wing phenotype
		8967R-2			8967R-2											
CG9096	CycD	29024									
		105361									
CG9222		104259									
		27010									
CG9326	vari	104548		Wing phenotype							
		24157									
CG9358	Phk-3	106938									
		49008									
CG9374	lkb1	34362									
		108356		Wing phenotype							
CG9541		49212									
		102912									
CG9738	Mkk4	108561									
		26929									
CG9746	ird1	9746R-1		Wing phenotype							
		110706		Wing phenotype							
CG9774	rok	3793		Wing phenotype							
		104675		Wing phenotype							
CG9961		101702									
		36176									
CG9962		108721									
		36473									
CG9985	sktl	6229		Wing phenotype							
		101624		Wing phenotype							

The Gal4 driver used to test a particular stock in the secondary screen was the same one used with it in the primary screen. If a stock was tested with both the dpp-Gal4 and hh-Gal4 drivers in the primary screen, hh-Gal4 was used in the secondary screen.

The percentage of transgenic RNAi lines with predicted sequence-dependent off-target effects are as follows: 65% (0), 27% (1-2), 8% (>2).

Wing discs from 20 larvae of each genotype were dissected (30 larvae for genotypes that were non-homozygous with a balancer chromosome). 20 flies of each genotype were scored for adult phenotypes.

The penetrance of all phenotypes indicated is between 80-

False positive results due to cell death, cell proliferation, or non-specific gene transcription were evaluated through testing the ability of candidates to affect different signaling pathways in the wing disc.

Wnt pathway = Sens, Dll
Notch pathway = Wg, Cut
Hedgehog pathway = Ci, Ptc

The following kinases affected all 3 pathways in the same way, either positively or negatively: Eip63E, CycY, CKIIβ, hipk, CKIIα, Gprk2, gish

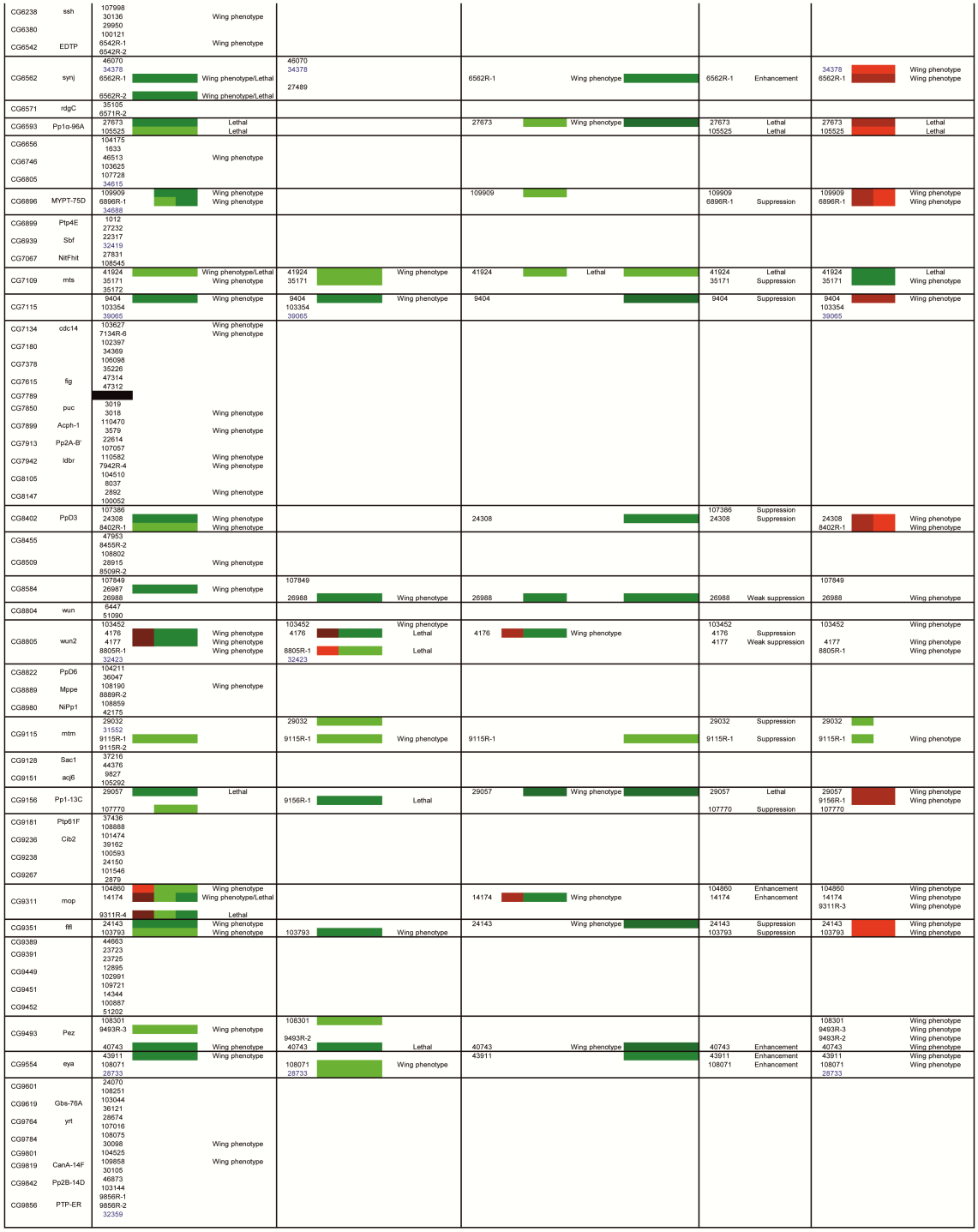
Weak-Medium Decrease
Medium-Strong Decrease

Weak-Medium Increase
Medium-Strong Increase

Transgenic RNAi Project stocks are in blue font
National Institute of Genetics Stocks have the letter R in the stock number
All other stocks are from Vienna Drosophila RNAi Center

Drosophila Phosphatase		PRIMARY SCREEN								SECONDARY SCREEN				WNT EYE SCREEN		HEDGEHOG SCREEN		
Annotation Symbol	Gene Symbol	RNAi Stock	UAS-dcr2;;dpp-Gal4			RNAi Stock	UAS-dcr2;hh-Gal4			RNAi Stock	UAS-dcr2;C5-Gal4		UAS-dcr2; dpp-Gal4 or UAS-dcr2; hh-Gal4	RNAi Stock	eyflp;ey-Gal4,GMR-Gal4;sev>y+>wg	RNAi Stock	MS1096-Gal4;UAS-dcr2	
			Wing disc		Adult		Wing disc		Adult		Wing disc	Adult	Wing disc				Wing disc	Adult
			Wg	Sens	Dll		Wg	Sens	Dll		Wg	Dll	Cut		Adult		Ci	Ptc
CG10089		17991																
		108744																
CG10138	PpD5	18016			Wing phenotype													
		104452			Wing phenotype													
CG10371	Plip	47624			Wing phenotype													
		104774			Wing phenotype													
CG10376		101335			Wing phenotype													
		35475			Wing phenotype													
CG10417		27259																
		106180																
CG10426		16048																
		34037																
		107996																
CG10443	Lar	36270																
		36269																
CG10493	Phlpp	110360																
		45365																
CG10574	I-2	101547			Wing phenotype					101547				101547	Lethal	101547		Wing phenotype
		39054			Wing phenotype											39054		Wing phenotype
CG10592		104767																
		38171																
CG10827		30822																
		100073																
CG10930	PpY-55A	102021																
		16096																
CG10975	Ptp69D	104761																
		40631																
CG11217	CanB2	104370																
		28762																
CG11425		103037																
		8458																
CG11426		42599																
		42600																
CG11437		9452			Wing phenotype													
		31721																
CG11438		11438R-3																
		11438R-2																
CG11440	laza	42594																
		109898																
CG11516	Ptp99A	103457																
		27208																
CG11597		38540																
		104729																
CG11883		106744				106744			Wing phenotype					106744		106744		Wing phenotype
		38590			Wing phenotype					38590				38590	Suppression	38590		Wing phenotype
CG12034		107062																
		12034R-1																
CG12078		101274																
		15288																
CG12091		100800																
		13987																
CG12151	Pdp	31661																
		107271																
CG12169	Ppm1	38631																
		101257																
CG12173		31676																
		31674																
CG12217	PpV	101997			Wing phenotype					101997				101997	Enhancement	101997		Wing phenotype
		31690			Wing phenotype											31690		Wing phenotype
CG12252	Fcp1	38640			Wing phenotype									38640	Enhancement/Lethal	38640		Lethal
		106253			Wing phenotype					106253				106253	Enhancement	106253		Wing phenotype
CG1228	Ptpmeg	38652																
		103740																
CG12538		102895																
		31957																
CG12746		104989																
		33314																
CG13125	TbCMF46	17123																
CG13197		36423																
		105122			Wing phenotype													
CG13311		51574																
		101655			Wing phenotype													
CG13570	spag	103353																
		23895																
CG1395	stg	17760				17760			Wing phenotype	17760		Wing phenotype		17760	Suppression	17760		Wing phenotype
		1395R-1			Wing phenotype													
		1395R-2																
		34831				34831			Lethal					34831	Lethal	34831		Wing phenotype
CG14022		110777																
		31795																
CG14080	Mkp3	45415																
		23911																
CG14211	MKP-4	104884			Wing phenotype					104884				104884	Weak suppression	104884		Wing phenotype
		3146			Lethal													
		3147			Wing phenotype									3147	Suppression	3147		Wing phenotype
		14211R-4																
CG14297		102071																
		14297R-4																
CG14411		17579																
		109622			Wing phenotype													
CG1455	CanA1	32283																
		32284																
CG14616	l(1)G0196	106973																
CG1462	Aph-4	6862																
		110043																
CG14719	I-t	105565																
		32314																
CG14903		49108																
		105752																
CG15035		100241																
		32353																
CG15385		51859																
		110458																
CG15400		7261																
		15400R-2																
CG15528		14865																
		105494																
CG15533		42520																
		102842																
CG15534		108181																
		30187																
CG15743		42686			Wing phenotype													
		42685																
CG1637		101368			Wing phenotype													
		33733																
CG16717		29199																
		105831																
CG16757	Spn	19658																
		105888																
CG16771		6386																
		16771R-2																
CG16870	Acyp	16870R-2																
		16870R-1																
CG16928	mre11	30474																
		30476																
CG1696	Dd	104785																
		12941																
CG17026		32813																
		49199																
CG17027		103270																
		50075																
CG17028		100100																
		32819																
CG17029		32823																
		49565																
CG17124		19078																
		17124R-3																
CG17291	Pp2A-29B	49672			Lethal					49672		Wing phenotype		49672	Lethal	49672		Lethal
														49671	Lethal			
		23886			Wing phenotype									23886	Lethal	23886		Wing phenotype
CG17598		32956			Wing phenotype													
		32955																
CG17746		100178																
		40102																
CG1809		43231																
		1809R-1																
CG1810	mRNA-cap	108809			Wing phenotype					108809		Wing phenotype		108809	Enhancement	108809		Wing phenotype/Lethal
		32847			Wing phenotype	32847			Lethal					32847	Enhancement	32847		Wing phenotype
CG1817	Ptp10D	1102																
		8010																
CG18243	Ptp52F	39175																
		3116																

CG1906	alph	105483 32476			29622 104677 15305R-1	Wing phenotype		104677	Wing phenotype		29622 104677	Weak enhancement		29622 104677	Wing phenotype
CG2096	flw	29622 104677 15305R-1	Wing phenotype												
CG2104		30018 100216													
CG2890	PPP4R2r	105399 25445	Wing phenotype Wing phenotype					25445			105399 25445	Suppression Suppression		105399 25445	Wing phenotype Wing phenotype/Lethal
CG2984	Pp2C1	105249 2984R-4			105249 2984R-3	Wing phenotype Wing phenotype		105249			105249 2984R-4	Suppression Weak suppression		105249 2984R-4	Wing phenotype Wing phenotype
CG30103		105458 4950													
CG30104		10051 106640													
CG3028	lpp	110775 29615													
CG3059	NTPase	110510 7265													
CG31137	twin	13365 104442													
CG31299	cu	45442 109759													
CG31469		102474 23397													
CG31692	fbp	108554 21396													
CG31717		7663 7662 105379 31717R-1	Lethal Wing phenotype/Lethal		105379 31717R-1	Wing phenotype		7663	Wing phenotype		7663	Suppression		7663 7662 105379 31717R-1 31717R-3	Wing phenotype Wing phenotype Wing phenotype
CG31759		106772 47321													
CG3178	Rrp1	108661 19819													
CG31795	IA-2	7560 110595													
CG32156	Mbs	105762 32516	Wing phenotype Wing phenotype		32516			105762	Wing phenotype		32516	Suppression		105762 32516	Wing phenotype Wing phenotype
CG3245	PpN-58A	41901 102060													
CG32487		28154 20778-1													
CG32488		46570 100129													
CG32505	Pp4-19C	25317 27726	Wing phenotype Lethal		38372	Lethal		25317			25317 27726	Suppression Lethal		25317 27726	Lethal Wing phenotype
CG32568		103317 46651													
CG3264		43250 108783													
CG32697	l(1)G0232	104427 21276			21276 3101R-1 33744	Lethal Lethal		3101R-1			104427 3101R-1	Suppression		104427 21276	Wing phenotype Wing phenotype
CG32812		45397 104081													
CG3289	Ptpa	41913 41912													
CG3290		104586 52378													
CG3292		19989 3292R-2													
CG33747	primo-2	23081 23079 39004	Wing phenotype Wing phenotype		23079 39004 50642	Lethal Lethal		50642			23081 23079 39004	Suppression		23081 23079 39004	Wing phenotype/Lethal Lethal
CG33748	primo-1														
CG3376		12226 12227													
CG34099	Mkp	104374 23452	Wing phenotype												
CG34140		23454													
CG3530		110786 26217 26216	Wing phenotype		110786 26216	Wing phenotype Wing phenotype		26216			110786 26216	Weak enhancement		110786 26216	Wing phenotype Wing phenotype
CG3573	Ocrl	34649 110796													
CG3632		110167 26254													
CG3705	aay	23179 110661													
CG3954	csw	108352 21757	Wing phenotype Wing phenotype					21757	Wing phenotype		108352 21757	Enhancement Weak enhancement		108352 21757	Wing phenotype Wing phenotype
CG3980	Cep97	34774 103357													
CG40448	Pp1-Y2	109147 101634													
CG4123	Mipp1	8493 110123													
CG41534	Pp1-Y1	21607 21611													
CG4209	CanB	52390 101200													
CG42249		47397 41672													
CG42271		100176	Wing phenotype												
CG42283	5Ptasel	33768 33769 25030 100802	Wing phenotype Wing phenotype		33768 33769 100802	Lethal Lethal Wing phenotype/Lethal		33769			33769 100802	Suppression		33768 100802	Wing phenotype Wing phenotype
CG42327		106630 47455													
CG4317	Mipp2	14163 108018													
CG4733	PR72	107621 34894			107621 34894	Wing phenotype		34894	Wing phenotype		107621 34894	Weak suppression Weak suppression		107621 34894	Wing phenotype Wing phenotype
CG4827	veil	49359 100050													
CG4965	twe	46064 104147													
CG4993	PRL-1	107836 45518													
CG5026		105674 34916													
CG5150		102646 15298													
CG5171		108686 5171R-3													
CG5177		27367 103024 5177R-1 5177R-2	Wing phenotype												
CG5276		47995 106691													
CG5361		51984 51985	Wing phenotype												
CG5567		44319 106981													
CG5577		22163 5577R-1													
CG5643	wdb	27470 101406 5643R-3			27470 101406 5643R-3	Wing phenotype Wing phenotype/Lethal		101406			27470 101406	Suppression		27470 101406 5643R-3	Wing phenotype Wing phenotype
CG5650	Pp1-87B	35024 35025 32414	Wing phenotype/Lethal Wing phenotype Wing phenotype					32414	Wing phenotype		35025 32414	Weak suppression Lethal		35024 35025 32414	Lethal Lethal
CG5656		110733 18119													
CG5671	Pten	101475 35731	Wing phenotype												
CG5784	Mapmodulin	100283 49385													
CG5820	Gp150	36301 100134	Wing phenotype Wing phenotype					36301			100134	Enhancement		36301 100134	Wing phenotype Wing phenotype
CG5830		101539 5830R-2													
CG5851	sds22	42051 5851R-1	Lethal Wing phenotype/Lethal					42051			42051 5851R-2 5851R-1	Lethal Enhancement Lethal		42051 5851R-1	Wing phenotype Wing phenotype
CG6036		21023 105568													
CG6235	tws	104167 34340	Wing phenotype Wing phenotype					104167	Wing phenotype		104167 34340	Enhancement		104167 6235R-2 34340	Wing phenotype Wing phenotype Wing phenotype


CG6238 ssh
107998
30136
Wing phenotype
CG6380
29950
100121
CG6542 EDTP
6542R-1
Wing phenotype
6542R-2
CG6562 synj
46070
34378
6562R-1
Wing phenotype/Lethal
6562R-2
Wing phenotype/Lethal
46070
34378
27489
6562R-1
Wing phenotype
6562R-1
Enhancement
34378
6562R-1
Wing phenotype
Wing phenotype
CG6571 rdgC
35105
6571R-2
CG6593 Pp1α-96A
27673
105525
Lethal
Lethal
27673
Wing phenotype
27673
105525
Lethal
Lethal
27673
105525
Lethal
Lethal
CG6656
104175
1633
CG6746
46513
Wing phenotype
103625
CG6805
107728
34615
CG6896 MYPT-75D
109909
6896R-1
Wing phenotype
Wing phenotype
34688
109909
109909
6896R-1
Suppression
109909
6896R-1
Wing phenotype
Wing phenotype
CG6899 Ptp4E
1012
27232
CG6939 Sbf
22317
32419
CG7067 NitFhit
27831
108545
CG7109 mts
41924
35171
Wing phenotype/Lethal
Wing phenotype
35172
41924
35171
Wing phenotype
41924
Lethal
41924
35171
Lethal
Suppression
41924
35171
Lethal
Wing phenotype
CG7115
9404
103354
39065
Wing phenotype
Wing phenotype
9404
103354
39065
Wing phenotype
9404
9404
Suppression
9404
103354
39065
Wing phenotype
CG7134 cdc14
103627
Wing phenotype
7134R-6
Wing phenotype
CG7180
102397
34369
CG7378
106098
35226
CG7615 fig
47314
47312
CG7789
CG7850 puc
3019
3018
Wing phenotype
CG7899 Acph-1
110470
3579
Wing phenotype
CG7913 Pp2A-B'
22614
107057
CG7942 Idbr
110582
Wing phenotype
7942R-4
Wing phenotype
CG8105
104510
8037
CG8147
2892
Wing phenotype
100052
CG8402 PpD3
107386
24308
Wing phenotype
8402R-1
Wing phenotype
24308
107386
24308
Suppression
Suppression
24308
8402R-1
Wing phenotype
Wing phenotype
CG8455
47953
8455R-2
CG8509
108802
28915
Wing phenotype
8509R-2
CG8584
107849
26987
Wing phenotype
26988
107849
26988
Wing phenotype
26988
26988
Weak suppression
107849
26988
Wing phenotype
CG8804 wun
6447
51090
CG8805 wun2
103452
4176
Wing phenotype
4177
Wing phenotype
8805R-1
Wing phenotype
32423
103452
4176
Wing phenotype
Lethal
8805R-1
Lethal
32423
4176
Wing phenotype
103452
4176
Suppression
4177
Weak suppression
103452
Wing phenotype
4177
Wing phenotype
8805R-1
Wing phenotype
CG8822 PpD6
104211
36047
CG8889 Mppe
108190
Wing phenotype
8889R-2
CG8980 NiPp1
108859
42175
CG9115 mtm
29032
31552
9115R-1
9115R-2
29032
9115R-1
Wing phenotype
9115R-1
29032
9115R-1
Suppression
29032
9115R-1
Wing phenotype
CG9128 Sac1
37216
44376
CG9151 aqj6
9827
105292
CG9156 Pp1-13C
29057
Lethal
107770
9156R-1
Lethal
29057
Wing phenotype
29057
Lethal
107770
Suppression
29057
9156R-1
107770
Wing phenotype
Wing phenotype
CG9181 Ptp61F
37436
108888
CG9236 Clb2
101474
39162
CG9238
100593
24150
CG9267
101546
2879
CG9311 mop
104860
14174
Wing phenotype
Wing phenotype/Lethal
9311R-4
Lethal
14174
Wing phenotype
104860
14174
Enhancement
Enhancement
104860
14174
9311R-3
Wing phenotype
Wing phenotype
Wing phenotype
CG9351 ffl
24143
103793
Wing phenotype
Wing phenotype
103793
Wing phenotype
24143
Wing phenotype
24143
103793
Suppression
Suppression
24143
103793
Wing phenotype
Wing phenotype
CG9389
44953
CG9391
23723
23725
CG9449
12895
102991
CG9451
109721
14344
CG9452
100887
51202
CG9493 Pez
108301
9493R-3
Wing phenotype
40743
Wing phenotype
108301
9493R-2
40743
Lethal
40743
Wing phenotype
40743
Enhancement
108301
9493R-3
9493R-2
40743
Wing phenotype
Wing phenotype
Wing phenotype
Wing phenotype
CG9554 eya
43911
108071
28733
Wing phenotype
108071
28733
Wing phenotype
43911
43911
Enhancement
Enhancement
43911
108071
28733
Wing phenotype
Wing phenotype
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24070
108251
CG9619 Gbs-76A
103044
36121
CG9764 yrt
28674
107016
CG9784
108075
30098
Wing phenotype
CG9801
104525
CG9819 CanA-14F
109858
Wing phenotype
30105
CG9842 Pp2B-14D
46873
103144
CG9856 PTP-ER
9856R-1
9856R-2
32359
The Gal4 driver used to test a particular stock in the secondary screen was the same one used with it in the primary screen. If a stock was tested with both the dpp-Gal4 and Hh-Gal4 drivers in the primary screen, hh-Gal4 was used in the secondary screen.
The percentage of transgenic RNAi lines with predicted sequence-dependent off-target effects are as follows: 65% (0), 27% (1-2), 8% (>2).
Wing discs from 20 larvae of each genotype were dissected (30 larvae for genotypes that were non-homozygous with a balancer chromosome). 20 flies of each genotype were scored for adult phenotypes.
The penetrance of all phenotypes indicated is between 80-
False positive results due to cell death, cell proliferation, or non-specific gene transcription were evaluated through testing the ability of candidates to affect different signaling pathways in the wing disc.
Wnt pathway = Sens, Dll
Notch pathway = Wg, Cut
Hedgehog pathway = Ci, Ptc
The following phosphatases affected all 3 pathways in the same way, either positively or negatively: mRNA cap, ftw,
Weak-Medium Decrease
Medium-Strong Decrease
Weak-Medium Increase
Medium-Strong Increase
Transgenic RNAi Project stocks are in blue font
National Institute of Genetics Stocks have the letter R in the stock number
All other stocks are from Vienna Drosophila RNAi Center