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RESEARCH ARTICLE

Genome-wide identification of phospho-regulators of Wnt signaling in *Drosophila*

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ABSTRACT

Evolutionarily conserved intercellular signaling pathways regulate embryonic development and adult tissue homeostasis in metazoans. The precise control of the state and amplitude of signaling pathways is achieved in part through the kinase- and phosphatase-mediated reversible phosphorylation of proteins. In this study, we performed a genome-wide *in vivo* RNAi screen for kinases and phosphatases that regulate the Wnt pathway under physiological conditions in the *Drosophila* wing disc. Our analyses have identified 54 high-confidence kinases and phosphatases capable of modulating the Wnt pathway, including 22 novel regulators. These candidates were also assayed for a role in the Notch pathway, and numerous phospho-regulators were identified. Additionally, each regulator of the Wnt pathway was evaluated in the wing disc for its ability to affect the mechanistically similar Hedgehog pathway. We identified 29 dual regulators that have the same effect on the Wnt and Hedgehog pathways. As proof of principle, we established that Cdc37 and Gilgamesh/CK1γ inhibit and promote signaling, respectively, by functioning at analogous levels of these pathways in both *Drosophila* and mammalian cells. The Wnt and Hedgehog pathways function in tandem in multiple developmental contexts, and the identification of several shared phospho-regulators serve as potential nodes of control under conditions of aberrant signaling and disease.

KEY WORDS: Wnt, Wingless, *In vivo* RNAi screen, Hedgehog, Notch

INTRODUCTION

The canonical Wnt signaling pathway is evolutionarily conserved and regulates essential biological processes such as cell fate specification, proliferation and migration during metazoan development. As a consequence, aberrant Wnt signaling can result in diverse human developmental disorders and cancers (MacDonald et al., 2009; Wodarz and Nusse, 1998). Cells use the reversible phosphorylation of proteins to control the state and amplitude of signaling pathways (Cohen, 1992; Hunter, 1995; Salazar and Höfer, 2009). In the silent state of the Wnt pathway, the transcriptional effector β-catenin is phosphorylated within a cytosolic Axin (Axn) complex by Casein Kinase 1α (CK1α) and Glycogen Synthase Kinase 3β (GSK3β). This modification facilitates its poly-ubiquitination and degradation (Aberle et al., 1997; Amit et al., 2002; Kitagawa et al., 1999; Liu et al., 1999, 2002). In the absence of stabilized β-catenin, the DNA-binding protein TCF represses target gene expression (Cavallo et al., 1998; Roose et al., 1998). Binding of secreted Wnt ligand to its transmembrane Frizzled (Fz) receptor and LRP co-receptor initiates pathway activity through the recruitment of the Axn complex to the cell surface (Cliffe et al., 2003; Tamai et al., 2004; Umbhauer et al., 2000; Wong et al., 2003; Yang-Snyder et al., 1996). This induces the phosphorylation of LRP by CK1α, GSK3β and the plasma membrane-associated CK1γ (Davidson et al., 2005; Tamai et al., 2004; Zeng et al., 2005), which then reciprocally facilitates the disassembly of the Axn complex to prevent β-catenin degradation (Cselenyi et al., 2008; Kim et al., 2013; Mi et al., 2006; Willert et al., 1999). Stabilized β-catenin translocates to the nucleus to form a transcriptional complex with TCF to direct expression of target genes (Behrens et al., 1996; Molenaar et al., 1996; van de Wetering et al., 1997). Preceding these events in signal-receiving cells, the secretion, diffusion and reception of Wnt itself is phospho-regulated in ligand-producing cells and in the extracellular environment (Buechling and Boutros, 2011).

Although several kinases and phosphatases are known to regulate Wnt signaling (Gao et al., 2014; Verheyen and Gottardi, 2010), our current knowledge of these enzymes remains incomplete. In fact, differential phospho-proteome analyses have identified both novel phospho-regulators and novel phosphorylation sites on known regulators of the pathway (Bodenmiller et al., 2007; Tang et al., 2007). It is unlikely that the reversible phosphorylation of all of these newly identified phospho-epitopes is catalyzed by the current subset of known kinases and phosphatases of Wnt signaling, thereby suggesting that there are additional unidentified phospho-regulators of the pathway.

Several large-scale *in vitro* screening analyses have been performed in various *Drosophila* and mammalian cell lines to identify regulators of the Wnt pathway. These high-throughput loss- and gain-of-function studies quantitated the response of exogenous Wnt pathway reporters under conditions of elevated signaling (Anton et al., 2011; Buechling et al., 2011; Caspi and Rosin-Arbesfeld, 2008; DasGupta et al., 2005; 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). In this study, we have performed a comprehensive genome-wide *in vivo* RNAi screen for kinases and phosphatases in *Drosophila* to build a phospho-regulatory network of the Wnt pathway. *Drosophila* has significantly contributed to our understanding of the molecular mechanism of the Wnt pathway (Bejsovec, 2006), and has low functional redundancy but high functional conservation of the genes with humans (Fortini et al., 2000; Reiter et al., 2001). The strength of our approach is that we assayed the effects of putative phospho-regulators of the Wnt pathway on endogenous targets under physiological levels of signaling in an intact tissue. Wing discs from third instar larvae were immunostained against direct high- and low-threshold targets of the pathway, allowing us to evaluate the entire ligand-induced gradient of signaling (Barolo, 2006). As the wing disc comprises distinct

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cells that either produce or respond to the ligand, we could identify phospho-regulators at discrete positions in the pathway. Our analyses of the Wnt pathway have yielded a large subset of high-confidence kinases and phosphatases, including 22 previously unidentified regulators of signaling. Among candidate Wnt regulators, we determined which acted indirectly through the Notch pathway to modulate *wg* transcription. Furthermore, we established those shared between the Wnt and Hh pathways. Last, we validated two dual regulators of the Wnt and Hh pathways using biochemical assays to demonstrate that their roles are evolutionarily conserved from *Drosophila* to mammalian cells.

RESULTS

Design of an *in vivo* screen to identify phospho-regulators of the Wnt pathway

In the wing imaginal disc, Wingless (Wg) (*Drosophila* Wnt) produced from cells at the dorsal/ventral (D/V) compartment boundary induces the nested expression of pathway target genes *senseless* (*sens*) (high-threshold) and *Distal-less* (*Dll*) (low-threshold) in adjacent non-boundary cells that receive the ligand (Neumann and Cohen, 1997; Zecca et al., 1996) (Fig. 1). We compiled a list of all protein kinases and phosphatases, non-protein kinases and phosphatases, as well as factors that associate with these enzymes, such as cyclins and regulatory subunits, which we will collectively refer to as the kinome and phosphatome (supplementary material Figs S1, S2). Using transgenic RNAi libraries of UAS-driven inverted repeats (IRs) and tissue-specific Gal4 drivers, we knocked down in a spatially restricted fashion the expression of each of 385 and 205 genes present in the *Drosophila* kinome and phosphatome, respectively, to assay their effect on Wnt signaling (Fig. 1). At least two non-overlapping IRs per gene were independently tested to minimize positive and negative false discovery.

The primary screen was performed using a combination of *decapentaplegic* (*dpp*)-*Gal4* and *hedgehog* (*hh*)-*Gal4* that are expressed along the anterior/posterior compartment boundary and in the posterior compartment of the wing disc, respectively. These *Gal4* drivers were used to knock down gene function in both the ligand-producing and ligand-receiving cells (Fig. 1). A *UAS-dicer-2* transgene was used in combination with the *Gal4* drivers for all screening analyses to enhance the efficiency of RNAi-mediated gene knockdown (Dietzl et al., 2007). Wing discs from every genotype were immunostained against Sens, Dll and Wg to assay for pathway activity and status of the ligand. A subset of known regulators of the Wnt pathway was knocked down in a pilot screen to validate our experimental design (supplementary material Fig. S3). We consistently observed reproducible and expected defects in pathway targets (and Wg protein in certain cases) with known components of signaling, although some effects were subtle, yet highly penetrant and reproducible (e.g. supplementary material Fig. S3).

The wing disc is specified by Wnt and other signaling pathways (Couso et al., 1994; Rulifson et al., 1996), and disruption of this process may induce compensatory mechanisms that form a relatively normal adult wing (Herrera et al., 2013; Ryoo et al., 2004; Wells et al., 2006). Discs were therefore immunostained from every genotype irrespective of the presence of an adult wing or other phenotype. A gene that modified the levels of Sens and/or Dll when knocked down independently with at least two non-overlapping IRs using any combination of *Gal4* drivers was classified as a candidate of the Wnt pathway (examples of modifiers are shown in Fig. 2). We identified 90 candidates from the primary screen (Fig. 3). Scoring of

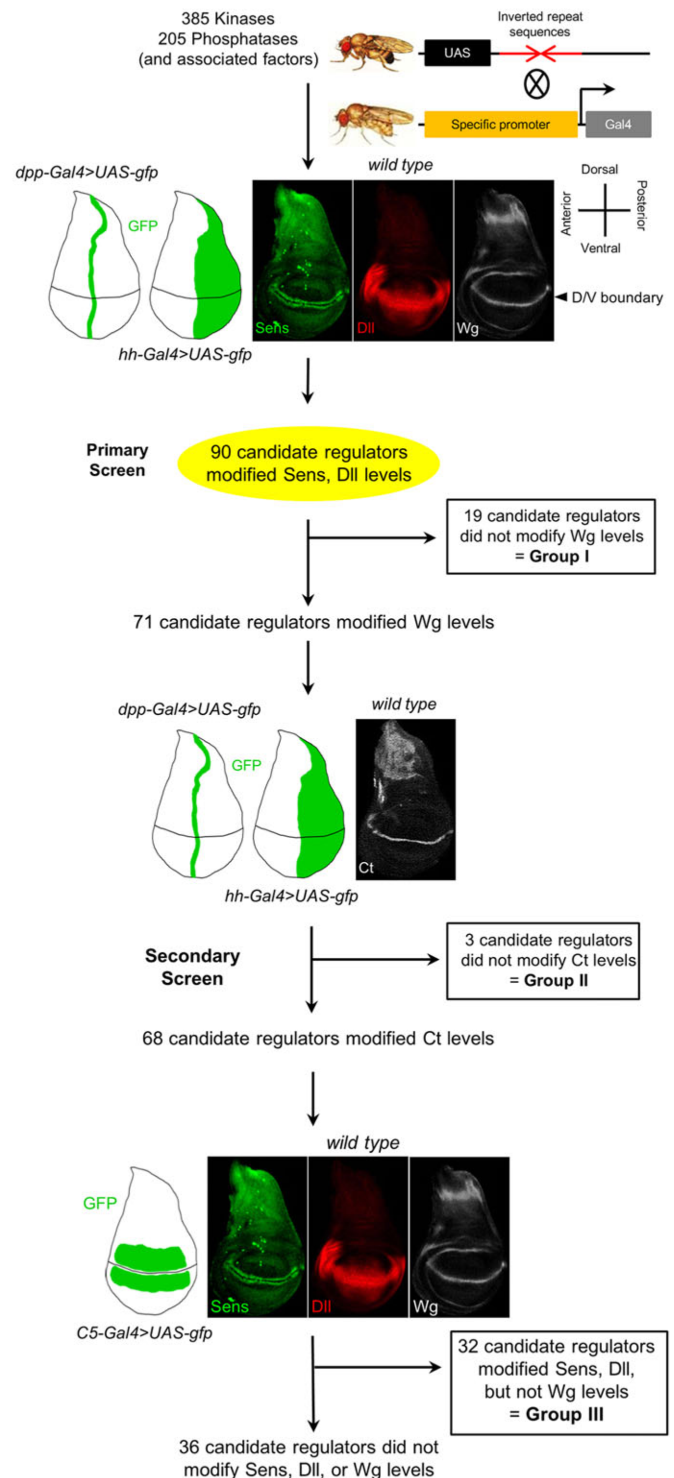

Fig. 1. Design of Wnt pathway screen in the *Drosophila* wing disc. Schematic illustration of *in vivo* Wnt pathway screen. Five-hundred and ninety kinases, phosphatases and associated factors were knocked down in a spatially restricted fashion in the wing disc through the use of *Gal4* drivers/ *UAS*-IRs. Secondary screens were performed to further functionally classify the regulators, as described in the text. Ninety primary screen candidates were classified into 54 high-confidence Group I, Group II and Group III regulators based on secondary screens.

all crosses with multiple RNAi lines is provided in supplementary material Figs S4, S5. No gene, when knocked down, modified the levels of only Sens or Dll, although in some cases one pathway

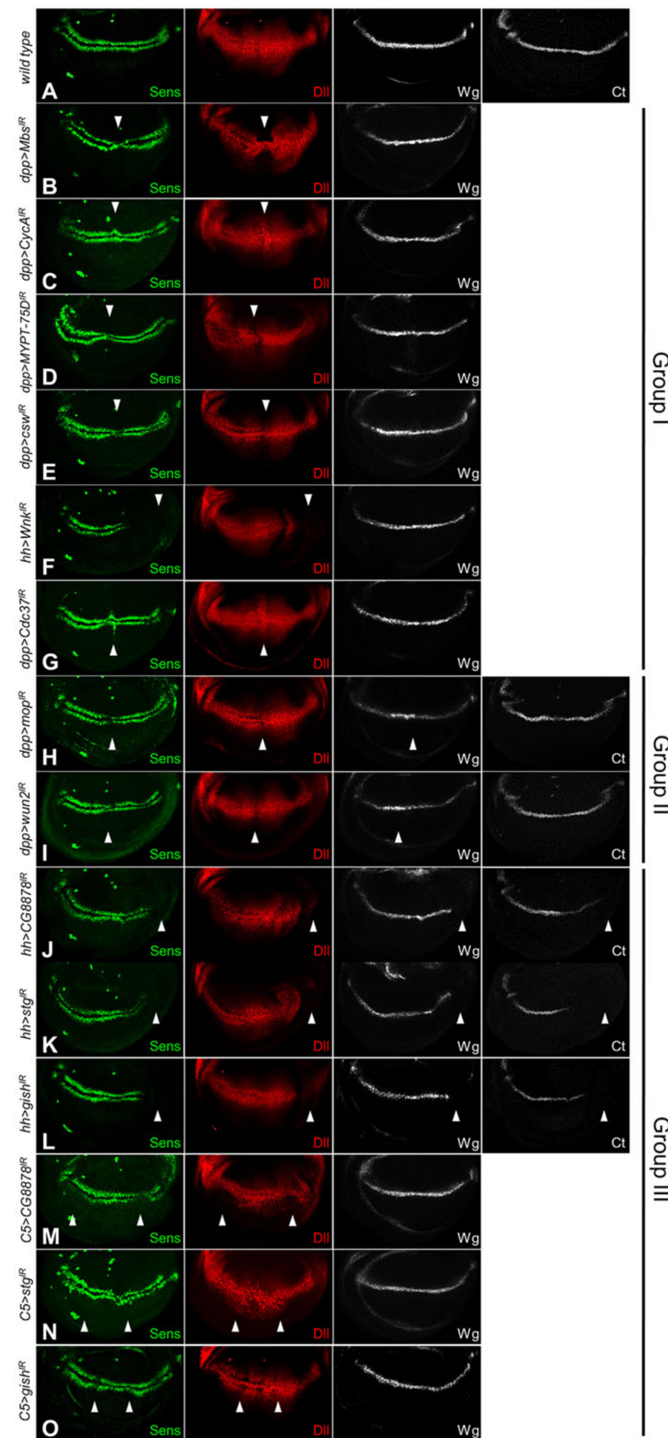

Fig. 2. High-confidence regulators of the Wnt pathway identified in the *Drosophila* wing disc. (A) A wild-type wing disc that displays the levels of Sens, Dll, Wg and Ct. (B-G) Group I regulators knocked down using *dpp-Gal4* or *hh-Gal4* modified the levels of Sens and Dll, but not Wg. Knockdown of *Mbs*, *MYPT-75D*, *csw* and *Wnk* decreased the levels of Sens and Dll (arrowheads), while knockdown of *CycA* and *Cdc37* increased the levels of Sens and Dll (arrowheads). (H,I) Group II regulators modified the levels of Sens, Dll and Wg, but not of Ct. Knockdown of *mop* and *wun2* decreased the levels of Sens and Dll (arrowheads), and increased Wg levels (arrowheads). (J-O) Group III regulators, such as *CG8878*, *stg* and *gish*, modified the levels of Sens, Dll, Wg and Ct (arrowheads). (M-O) Knockdown of these regulators in only the ligand-receiving cells with *C5-Gal4* modified the levels of Sens and Dll (arrowheads), but not Wg.

target was affected to a greater extent than the other (Figs 2, 3). Of note, most candidates had mild to moderate effects on targets, consistent with results obtained in our pilot screen with known Wnt pathway components, suggesting that under these assay conditions relatively subtle effects are valid, as confirmed by further analyses.

Nineteen of the 90 candidates had no observable effect on Wg levels or distribution, and thus do not function upstream of or at the level of the ligand-receptor interaction to affect the secretion, diffusion or reception of Wg. These candidates were classified as Group I high-confidence regulators of the Wnt pathway that function downstream of the ligand-receptor interaction in the ligand-receiving cells (Figs 2, 3). Group I includes known regulators of signaling such as *Wnk* (Serysheva et al., 2013) and novel regulators such as *Myosin binding subunit* (*Mbs*), *Cyclin A* (*CycA*), *MYPT-75D*, *corkscrew* (*csw*) and *Cdc37* (Fig. 2A-G).

Seventy-one of the 90 candidates modified the levels of Wg (Fig. 2; supplementary material Figs S4, S5). The Notch pathway signals from non-boundary cells to induce the expression of *wg* in cells at the D/V boundary of the wing disc (Diaz-Benjumea and Cohen, 1995; Neumann and Cohen, 1996; Rulifson and Blair, 1995; supplementary material Fig. S6). To test whether the candidates that affect Wg levels do so as a result of their regulation of the ligand after translation or by affecting expression of the ligand via the Notch pathway, they were re-analyzed in a secondary screen. To address transcriptional regulation by Notch signaling, we determined whether they also modified the levels of another Notch pathway target, Cut (Ct) (Micchelli et al., 1997; Fig. 1). Only three of the 71 candidates when knocked down with either *dpp-Gal4* or *hh-Gal4* had no effect on Ct levels. We inferred that these three candidates have no effect on the Notch pathway and *wg* expression, but rather modify the secretion, diffusion or reception of Wg, which was detected as a change in its levels. These candidates were classified as Group II high-confidence regulators of the Wnt pathway that function upstream of or at the level of the ligand-receptor interaction in the ligand-producing cells or extracellular environment, respectively (Figs 2, 3). Group II includes a known regulator of signaling, *myopic* (*mop*) (Miura et al., 2008; Pradhan-Sundd and Verheyen, 2014), and a novel regulator, *wunen2* (*wun2*) (Fig. 2H-I). As further evidence of the functional role of Group II candidates, we and others have confirmed that two of the three Group II candidates (CG3530, Mop) have no effect on *wg-lacZ* but do affect the Wg protein (Silhankova et al., 2010; Pradhan-Sundd and Verheyen, 2014).

The remaining 68 of the 71 candidates modified Ct levels and were inferred to regulate *wg* expression as a result of their effect on multiple Notch targets (Fig. 3). These candidates were further tested to determine whether they also affected the Wnt pathway independently of their regulation of *wg* expression. The majority of regulators of developmental pathways function in the ligand-receiving cells and not the ligand-producing cells. Therefore, if a gene independently affects the Wnt and Notch pathways in the wing disc, it would likely do so in the ligand-receiving cells of both pathways, regulating Wnt signaling in the non-boundary cells and Notch signaling in the boundary cells. We knocked down the 68 candidates that we identified as regulators of the Notch pathway using the *C5-Gal4* driver, which is expressed in only non-boundary cells adjacent to the D/V compartment boundary (Fig. 1). By knocking down gene function in only the ligand-receiving cells of the Wnt pathway, which correspond to the ligand-producing cells of the Notch pathway (supplementary material Fig. S6), we could distinguish candidates that independently regulate both Wnt and Notch signaling from those that regulate only Notch signaling in the

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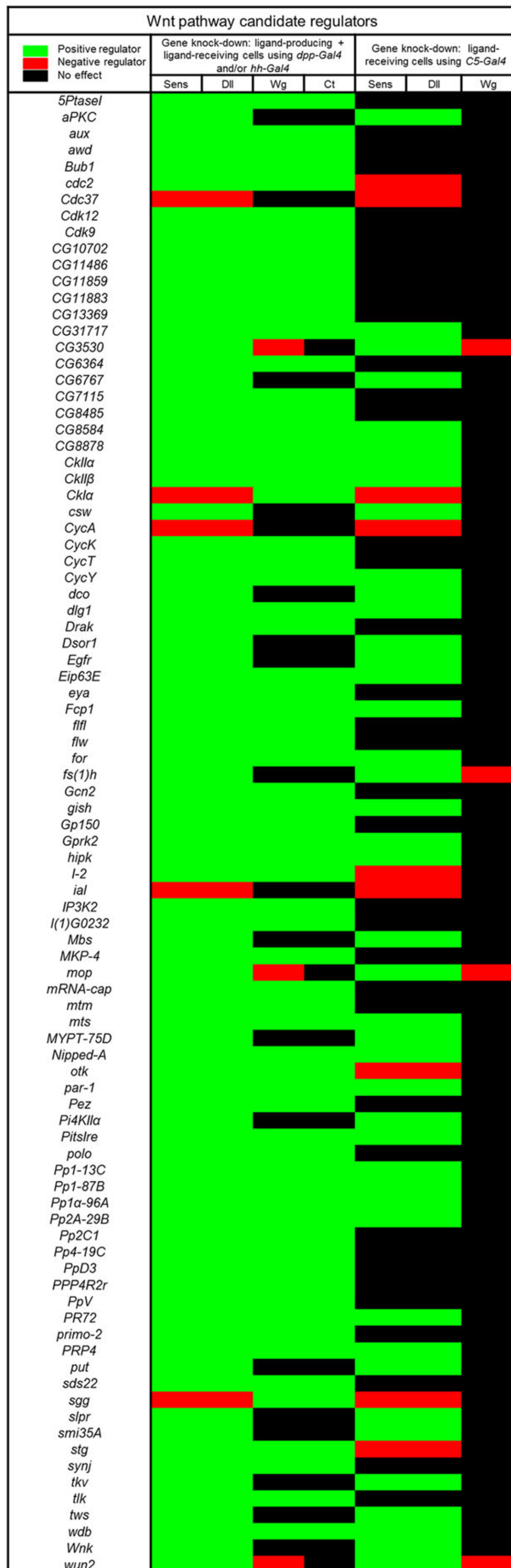

Fig. 3. Results of Wnt pathway screen in the *Drosophila* wing disc. Graphical summary that displays whether knockdown of candidates decreased (green), increased (red) or had no effect (black) on the levels of Sens, Dll, Wg and Ct. Summary reflects results obtained with at least two unique RNAi lines (see supplementary material Figs S4 and S5 for all data) knocked down in signal-producing and signal-receiving cells (using either *dpp-Gal4* or *hh-Gal4*) or in ligand-receiving cells only (using *C5-Gal4*) of the wing disc.

wing disc. When knocked down in the non-boundary cells, 32 of the 68 candidates modified the levels of the Wnt pathway targets Sens and Dll, but not Wg, and thus did not affect the Notch pathway in these cells to regulate *wg* expression at the D/V compartment boundary. These 32 candidates were classified as Group III high-confidence regulators of the Wnt pathway that function downstream of the ligand-receptor interaction in the signal-receiving cells and that affect the Notch pathway independently (Figs 2, 3). Group III includes known regulators of signaling, such as *string* (*stg*) (Davidson et al., 2009) and *gilgamesh* (*gish*) (Davidson et al., 2005; Zhang et al., 2006), and a novel regulator, *CG8878* (Fig. 2J-O). The remaining 36 candidates when knocked down in the non-boundary cells had no effect on the levels of Sens, Dll or Wg, and thus do not regulate the Wnt or Notch pathway in these cells (Fig. 3). Although these 36 candidates (supplementary material Fig. S7) regulate the Notch pathway and *wg* expression in the boundary cells, through our analyses we could not determine whether any of these candidates, albeit unlikely, also independently regulate Wg secretion from these cells to affect the Wnt pathway.

In summary, the 590 phospho-regulators screened in the wing disc yielded 90 candidates that were classified into 54 high-confidence regulators of the Wnt pathway and 36 candidates that regulate *wg* expression to indirectly affect the Wnt pathway. The high-confidence regulators were further classified into three phenotypic categories, referred to as Groups I, II and III, each of which comprise known and novel regulators of signaling (Fig. 4). Of the high-confidence regulators, 33 are kinases (and associated factors) and 21 are phosphatases (and associated factors). Forty-five of the 54 high-confidence regulators promote (blue in Fig. 4A), while the remaining nine inhibit (yellow in Fig. 4A), signaling.

Remarkably, 32 of the 90 candidates are known regulators of the Wnt pathway (indicated by gray shading in Fig. 4). This large subset of known regulators is reflective of the robustness and low false-negative error rate of our screen design. Moreover, based on our unbiased genetic analyses, all 32 of these known regulators cluster together in the category of high-confidence Groups I, II and III regulators. This strongly suggests that at least some of the 22 novel high-confidence regulators identified are bona fide regulators of the Wnt pathway. We classified the high-confidence regulators of the Wnt pathway according to their respective kinase and phosphatase groups. The *Drosophila* kinome comprises 13 groups (defined in supplementary material Fig. S1), of which 12 are represented by the high-confidence regulators of the Wnt pathway (Fig. 4B; supplementary material Fig. S1). The *Drosophila* phosphatome comprises nine groups (defined in supplementary material Fig. S2), of which six are represented by the high-confidence phosphatases (and associated factors) of the Wnt pathway (Fig. 4C; supplementary material Fig. S2).

Hh pathway counterscreen identifies Cdc37 and Gish as dual regulators of signaling

The Hh pathway has diverse functions during metazoan development, such as the regulation of organogenesis and stem

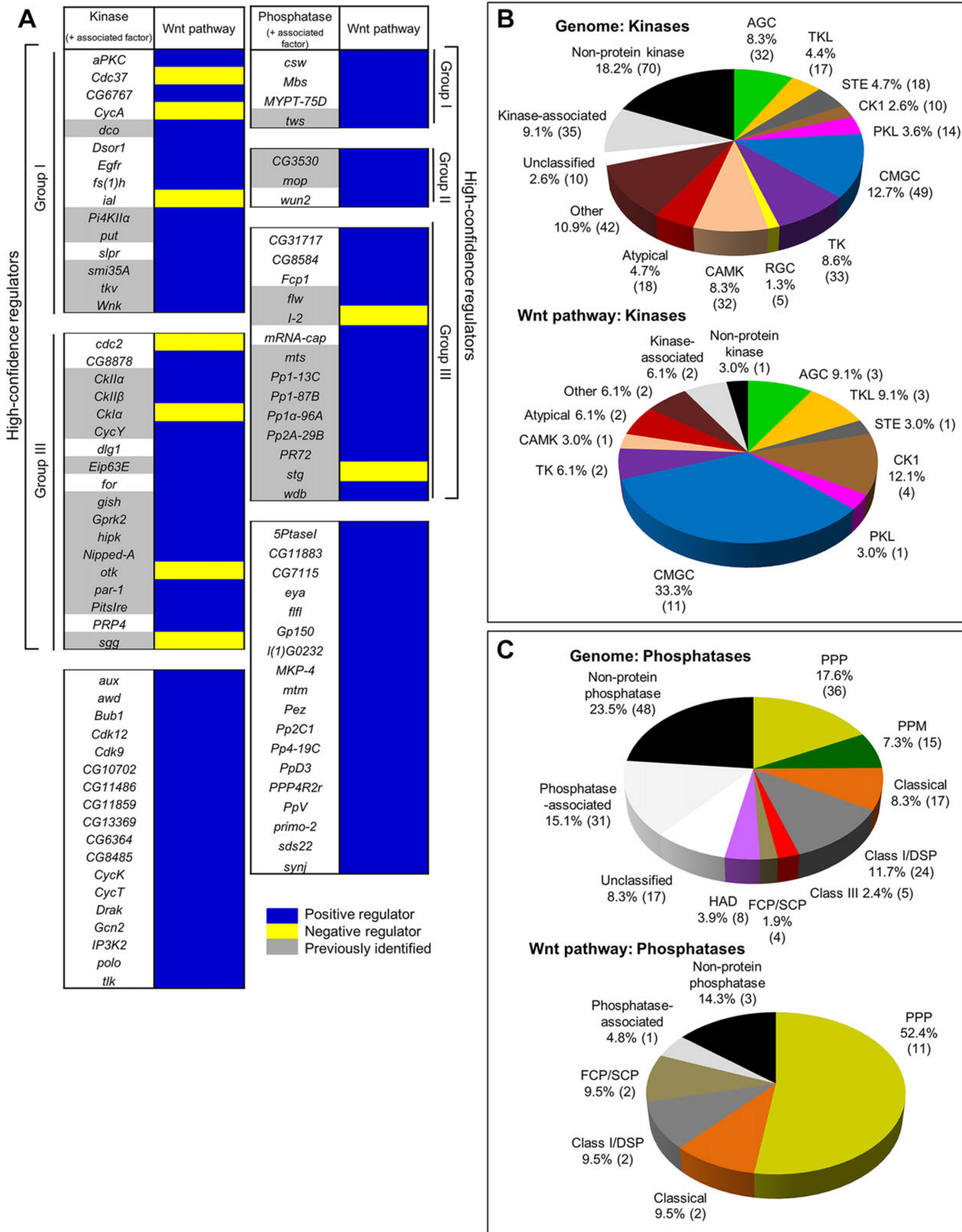

Fig. 4. Summary of phospho-regulators of the Wnt pathway identified in the *Drosophila* wing disc. (A) Ninety candidates of the Wnt pathway were refined into 54 high-confidence regulators (Group I, Group II and Group III). Forty-five high-confidence regulators promote (blue) and nine inhibit (yellow) the Wnt pathway. Three high-confidence regulators function upstream or at the level of the ligand-receptors interaction (Group II), whereas the remaining 51 function downstream of the ligand-receptor interaction (Group I, Group III). Thirty-two of the 90 candidates identified are previously validated regulators of the Wnt pathway (gray). (B,C) Graphical summary of the kinase (B) and phosphatase (C) groups in the *Drosophila* genome, and the subset of these that regulate the Wnt pathway.

cell homeostasis (Varjosalo and Taipale, 2008). Similar to the Wnt pathway, the Hh pathway is also subject to reversible phosphorylation in its silent and active states. In the absence of signaling, the transcriptional effector GLI is phosphorylated by Protein Kinase A (PKA), GSK3β and CK1α within a cytosolic Kif7 complex (Chen et al., 1998; Jia et al., 2002, 2005; Price and

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Kalderon, 1999, 2002; Zhang et al., 2005). The phosphorylation of GLI triggers its poly-ubiquitylation and partial degradation to yield a truncated form of the protein that represses target gene expression (Aza-Blanc et al., 1997; Jia et al., 2005; Jiang and Struhl, 1998; Méthot and Basler, 1999; Smelkinson and Kalderon, 2006; Smelkinson et al., 2007; Tempé et al., 2006). Binding of the Hh ligand to its receptor Patched (Ptc) recruits the Kif7 complex to the transmembrane signal transducer Smoothened (Smo) (Lum et al., 2003; Ogden et al., 2003; Ruel et al., 2003; Zheng et al., 2010). This induces Smo phosphorylation by PKA and CK1α, which promotes its accumulation at the cell surface (Apionishev et al., 2005; Denef et al., 2000; Jia et al., 2004; Zhang et al., 2004; Zhu et al., 2003). The interaction between Kif7 and phospho-Smo disassembles the complex to stabilize full-length GLI that directs target gene expression (Aza-Blanc et al., 1997; Jia et al., 2003; Liu et al., 2007; Ohlmeyer and Kalderon, 1998). Although the evolutionary relationship between Wnt and Hh signaling remains unclear, these pathways have a similar phospho-regulatory mechanism of signal transduction and comprise similar or identical regulators that exert the same effect on signaling by functioning at analogous levels of the relays (Kalderon, 2002).

We evaluated the ability of all 90 candidates of the Wnt pathway to regulate the Hh pathway *in vivo*. We performed this counter-screen to identify shared phospho-regulators that exert the same effect to either promote or inhibit signaling, and thereby potentially function at analogous levels of these pathways. Hh signaling stabilizes full-length Cubitus interruptus (Ci) (*Drosophila* GLI) to regulate expression of the target gene *ptc* along the anterior/posterior (A/P) boundary in the anterior compartment of the wing disc (Strigini and Cohen, 1997) (Fig. 5A). Each candidate was knocked down using *MS1096-Gal4* (with *UAS-dicer-2*) and wing discs were immunostained to detect Ci and Ptc. The *MS1096-Gal4* domain is in the center of the wing disc with stronger expression in the dorsal half relative to the ventral half (supplementary material Fig. S8A). The enhancer of *ptc*, unlike that of other Hh pathway targets, responds in a cooperative manner to both the levels and active state of Ci; thus, a change in the levels of Ci that modulates the amplitude of Hh signaling might not necessarily result in an effect on the expression of *ptc* (Parker et al., 2011). Any gene that modified the levels of Ci, but not necessarily Ptc, when independently knocked down with at least two non-overlapping IRs was classified as a regulator of the Hh pathway (Fig. 5B; supplementary material Fig. S8B). A limitation of this approach is that the counter-screen cannot identify regulators of the Hh pathway that do not affect the levels of Ci, but function downstream of its stabilization to modulate the expression of targets other than *ptc*. Sixty-six of the 90 candidates of the Wnt pathway modified the levels of Ci (and Ptc in most cases) to regulate the Hh pathway (supplementary material Figs S4, S5). The remaining 24 candidates of the Wnt pathway had no effect on the levels of Ci or Ptc (supplementary material Figs S4, S5). While the majority of Wnt regulators promoted signaling, 45 of 66 regulators of the Hh pathway inhibited signaling (supplementary material Fig. S8B). Nevertheless, 29 Wnt candidates exerted the same effect on the Hh pathway to either promote or inhibit signaling in the wing disc (Fig. 5B). Of these 29 dual regulators that we propose function at analogous levels of these pathways, 25 are high-confidence regulators and eight are novel regulators of the Wnt pathway. As proof of principle, we recovered *hipk* in our screen, which we have previously shown to be a dual regulator of Wnt and Hh acting on the E3 ubiquitin ligase Supernumerary limbs (Slimb) at analogous levels of the pathways (Swarup and Verheyen, 2011).

We identified *Cdc37* and *gish* as novel negative and positive regulators, respectively, of the Hh pathway. Knockdown of a negative control, *lacZ* (supplementary material Fig. S8A), displayed normal levels of Ci and Ptc, as seen in wild-type tissue (Fig. 5C). Knockdown of *Cdc37* resulted in the robust enhancement of Ci levels, both within and away from the signaling domain in the anterior compartment of the wing disc (Fig. 5D). Knockdown of *Cdc37* consistently led to the distortion of the morphology of the disc, which precluded an accurate evaluation of Ptc levels. Based on our data, *Cdc37* knockdown does not seem to appreciably alter Ptc levels, but we cannot definitively rule out this possibility. *Cdc37* knockdown phenocopies loss-of-function mutants of other negative regulators of the Hh pathway that strongly enhance the levels of Ci but have minimal or no effect on Ptc levels, such as *sgg* (*Drosophila* GSK3β) (Jia et al., 2002; Price and Kalderon, 2002) and *slimb* (Wang et al., 1999). Knockdown of *gish* resulted in the reduction of Ci and a reduction in the levels of Ptc, as indicated by the width of its expression domain in the central region of the wing disc (Fig. 5E).

In our screening analyses, all 90 candidates of the Wnt pathway were evaluated against Notch (as part of the secondary screen) and Hh (as part of the counter-screen) pathways in the wing disc (Fig. 6A). This allowed us to distinguish between candidates that are specific to the Wnt pathway from those that are shared between the Wnt and other signaling pathways (Fig. 6B; Tabata and Takei, 2004). These analyses also allowed us to evaluate whether a candidate of the Wnt pathway does so only indirectly as a result of its effect on cell death, cell proliferation or non-specific gene transcription. For example, if knockdown of a candidate decreases Wnt signaling but does not affect or increases Notch and/or Hh signaling, we inferred that this candidate does not affect cell death or non-specific gene transcription. However, if knockdown of a candidate decreases Wnt, Notch and Hh signaling, it is possible that it indirectly regulates the Wnt pathway due to an effect on cell death or non-specific gene transcription. Twelve of the 90 candidates of the Wnt pathway when knocked down had the same effect (i.e. positive or negative regulator of both pathways) on both the Notch and Hh pathways (Fig. 6C). This does not necessarily imply that these 12 candidates indirectly regulate the Wnt pathway, but that further analysis of these candidates is required. Coincidentally, 11 of these 12 candidates have been previously described to regulate the Wnt pathway (Fig. 6C).

Cdc37 inhibits the Wnt and Hh pathways by destabilizing their transcriptional effectors

As proof of concept for dual regulators that operate at analogous levels of the Wnt and Hh pathways, we further 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). We identified *cdc37* as a novel negative regulator whose knockdown resulted in increased pathway activity within the signaling domain and ectopic pathway activity outside the signaling domain (Fig. 2G). The *Drosophila* eye has frequently been used to screen for regulators of the Wnt pathway using a *sevenless* (*sev*)>*wg* (or equivalent) gain-of-function genetic background in which eye development is impaired (Fig. 7A; Greaves et al., 1999; Port et al., 2011). Heterozygosity for a loss-of-function allele of *cdc37* caused an enhancement of the *sev*>*wg* phenotype (Fig. 7B), suggesting that reduction of *cdc37* enhances the aberrant eye phenotype caused by Wg signaling. We examined embryos homozygous for the $cdc37^{eD4}$ allele. Wg signaling is required for specification of regions of naked cuticle

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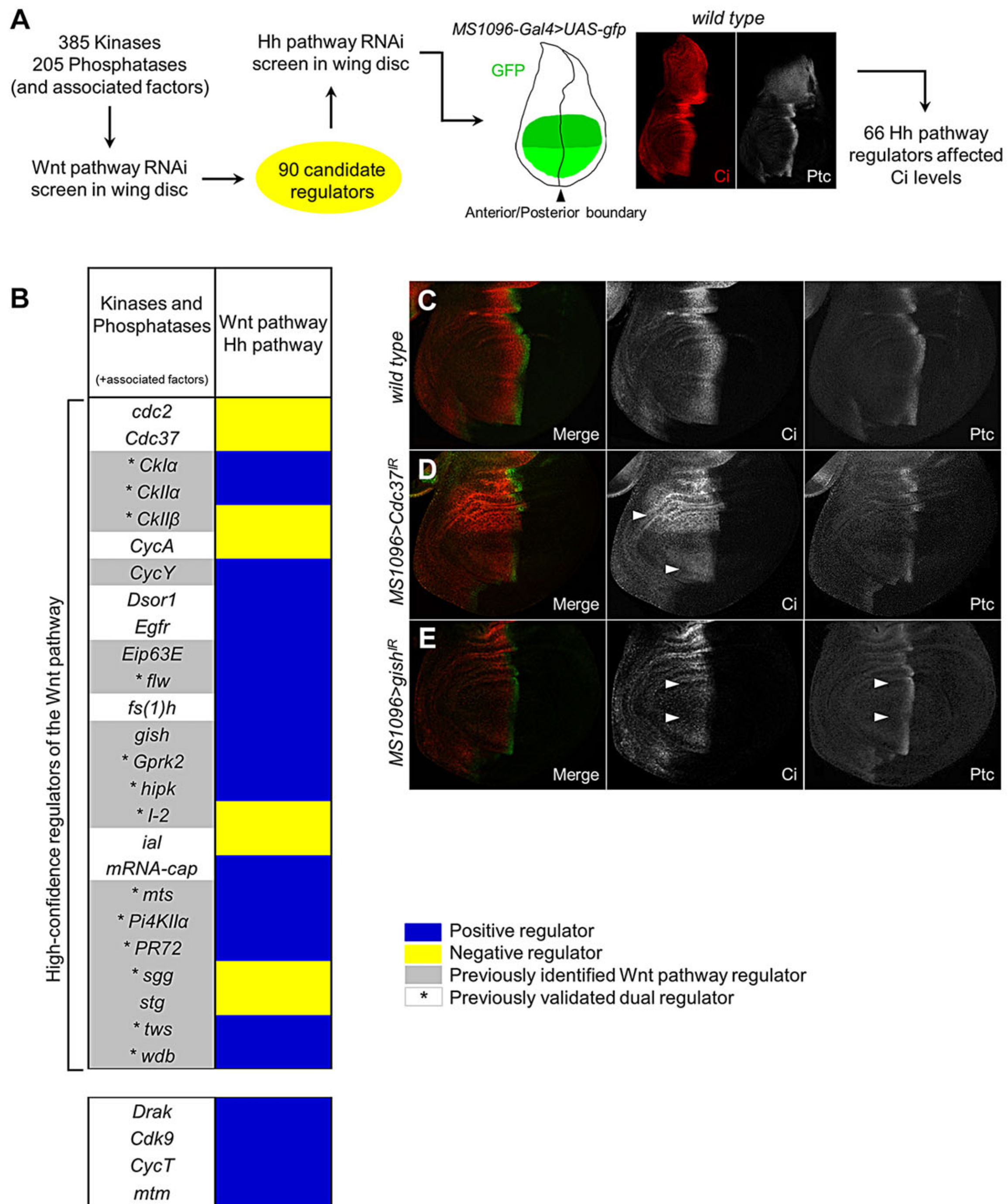

Fig. 5. Hh pathway counter-screen in the *Drosophila* wing disc. (A) Ninety candidates of the Wnt pathway were knocked down throughout the wing pouch using *MS1096-Gal4* and wing discs were immunostained against Ci and Ptc. Sixty-six candidates modified Ci levels to regulate the Hh pathway. (B) Twenty-nine candidates of the Wnt pathway were identified to have the same effect on the Hh pathway to either promote (blue) or inhibit (yellow) signaling. All but four of these 29 candidates are high-confidence regulators and 17 are known regulators (gray) of the Wnt pathway. Thirteen of the 29 candidates have been previously validated as dual regulators (asterisks) of the Wnt and Hh pathways. (C) The levels of Ci and Ptc along the anterior/posterior compartment boundary of a wild-type wing disc. (D) Knockdown of *cdc37* increased the levels of Ci (arrowheads), but did not affect Ptc. (E) Knockdown of *gish* decreased the levels of Ci and Ptc (arrowheads).

between denticle belts in the cuticle (Fig. 7C). Enhanced Wg signaling caused loss of denticle belts, such as was seen in *cdc37* mutant embryos, suggesting loss of *cdc37* promotes Wg signaling (Fig. 7D,E). Somatic loss-of-function clones of *cdc37* do not survive in the wing disc due to its requirement for cell viability (Lange et al., 2002). We therefore generated MARCM loss-of-function clones of *cdc37* (positively marked with nuclear GFP) that overexpress the apoptosis inhibitor *p35*. These MARCM clones, although small in size, displayed a cell-autonomous upregulation of Wnt and Hh signaling, as indicated by an increase in Dll and Ci levels, respectively. Similar to the effect seen with RNAi (Figs 2G, 5D), ectopic Dll (Fig. 7F) and Ci (Fig. 7G) were observed in MARCM clones away from the signaling domains of these pathways. This result suggests that Cdc37 may normally destabilize the effectors to function

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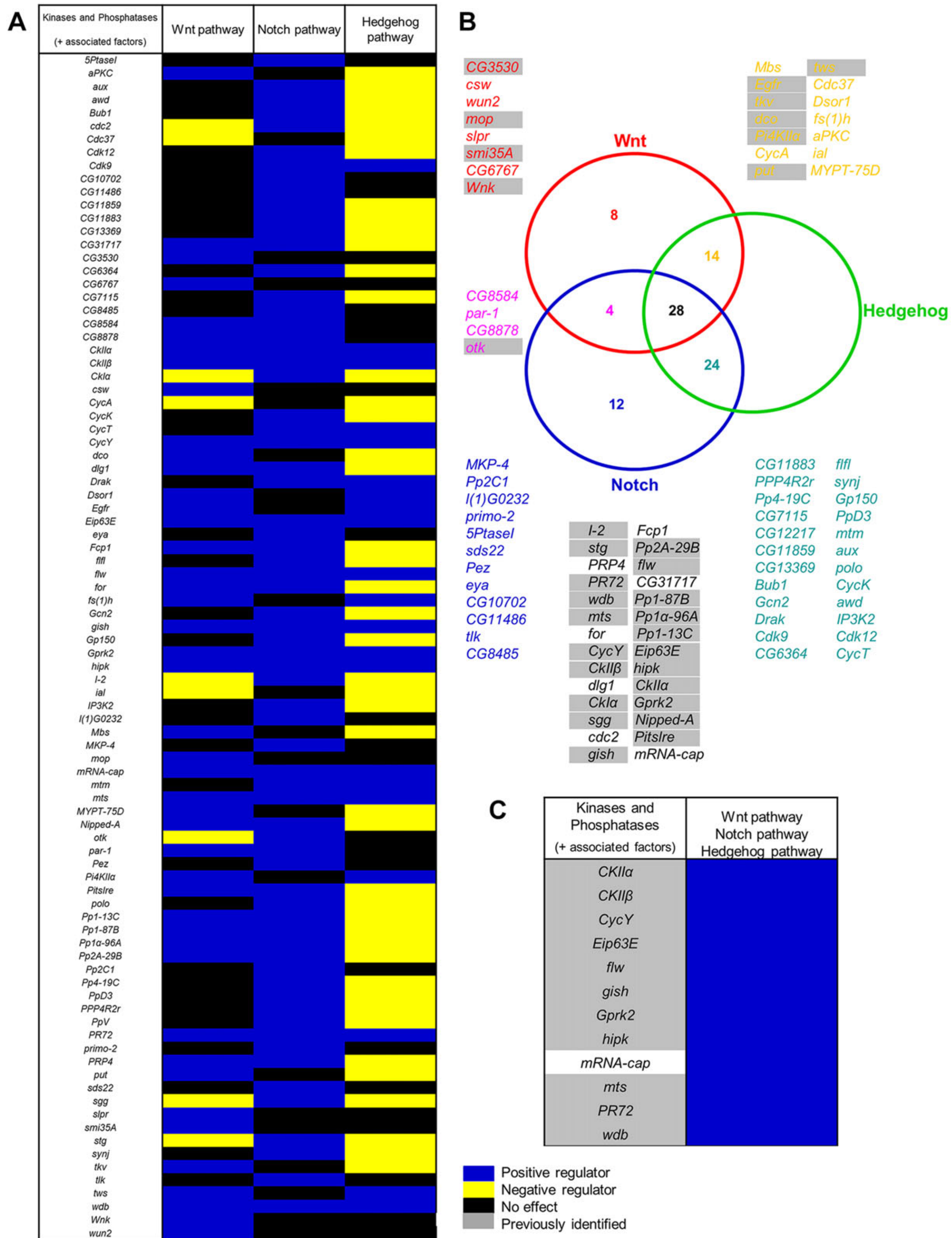

Fig. 6. Specific and shared phospho-regulators of the Wnt, Notch and Hh pathways in *Drosophila*. (A) Graphical summary that displays whether a regulator promotes (blue), inhibits (yellow) or has no effect (black) on the Wnt, Notch and Hh pathways in the wing disc. (B) Venn diagram showing specific or shared regulators of the Wnt, Notch and Hh pathways. Previously known regulators of the Wnt pathway are indicated with gray shading. (C) Twelve of the 90 candidates of the Wnt pathway identified also had the same effect on the Notch and Hh pathways in the wing disc.

as a negative regulator of signaling in the silent and active states of the Wnt and Hh pathways. Indeed, when we assayed for levels of stabilized Armadillo (Arm) (*Drosophila* β-catenin), we found more in wing discs reduced for *cdc37* function compared with wild type (Fig. 7I). Although *cdc37* RNAi did not significantly affect *ptc* expression, it did result in increased expression of another target gene of the Hh pathway, *dpp-lacZ* (supplementary material Fig. S9A). Although loss of *cdc37* clearly stabilizes Ci, studies suggest that there

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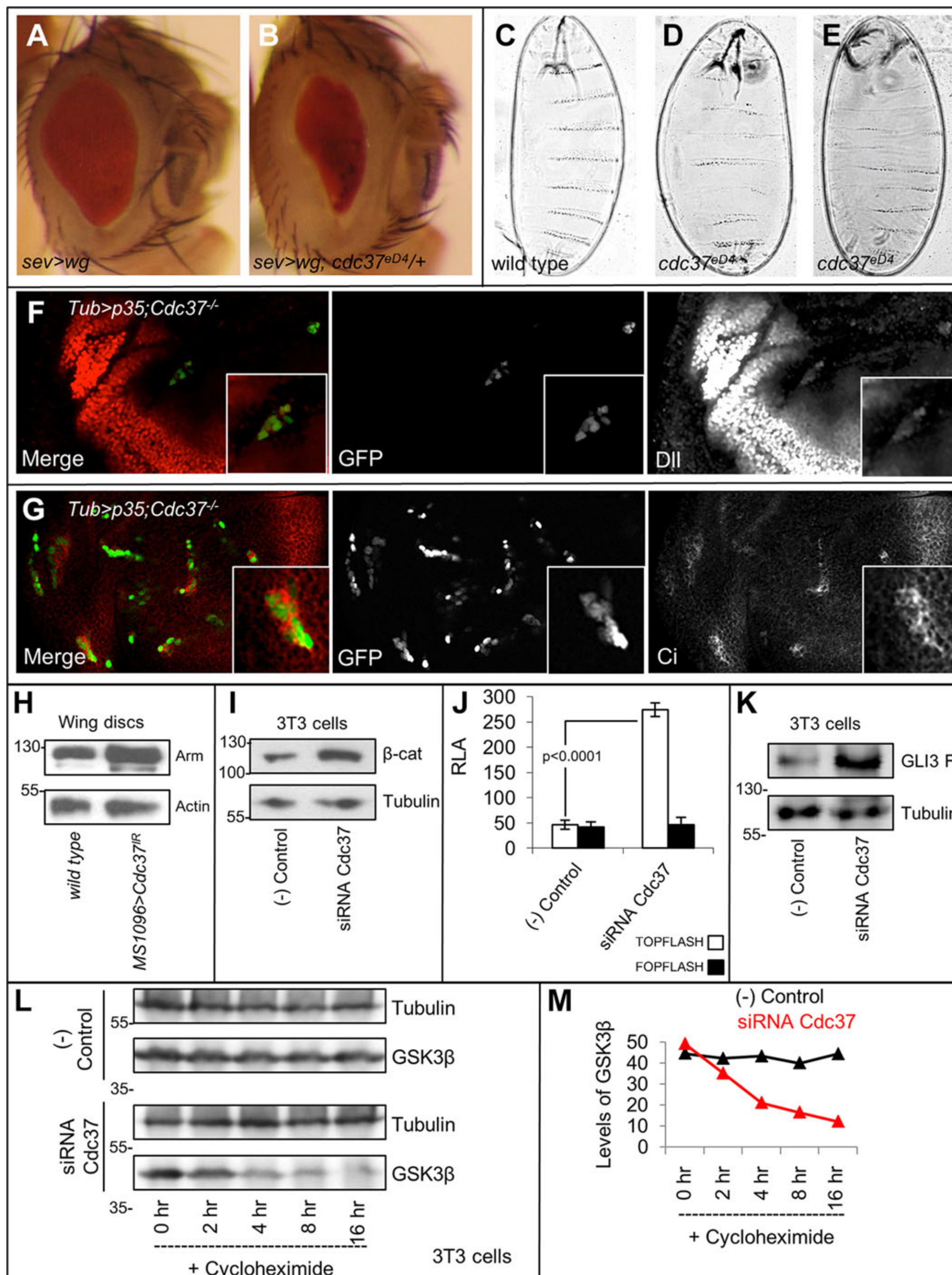

Fig. 7. Cdc37 destabilizes the effectors of the Wnt and Hh pathways to inhibit signaling. (A,B) Expression of *wg* from the *sev* promoter (*sev>wg*) results in a small rough eye, which is enhanced upon heterozygosity for the $cdc37^{eD4}$ allele. (C-E) Homozygous mutant $cdc37^{eD4}$ embryos display cuticle defects reminiscent of *wg* gain-of-function defects, including loss of denticle belts. (F,G) MARCM clones (with *p35* transgene) of $cdc37^{eD4}$ (marked by nuclear GFP) in the wing disc resulted in an increase in Dll (F) and Ci (G) levels. (H) Protein lysate from wing discs reduced for *Cdc37* function had elevated levels of stabilized Arm compared with wild-type discs. (I) Compared with the mock-transfected control, siRNA-mediated knockdown of *Cdc37* in 3T3 cells enhanced the levels of stabilized β-catenin. (J) Increased Wnt pathway activity is observed following siRNA-mediated knockdown of *Cdc37*, as measured from the TOPFLASH reporter but not FOPFLASH (negative control) (RLA=Relative Luciferase Activity). Student's *t*-test was performed and s.d. was calculated. (K) siRNA-mediated knockdown of *Cdc37* in 3T3 cells enhanced the levels of full-length GLI3 compared with the mock-transfected control. (L,M) siRNA-mediated knockdown of *Cdc37* in cycloheximide-treated 3T3 cells displayed progressively lower levels of GSK3β over the course of 16 h, compared with the mock-transfected control, as seen in western blot (L) and following densitometry (M). The levels of GSK3β were normalized to the loading control. Molecular weights are indicated in kDa next to each blot.

is an additional step that regulates Ci import into nucleus. We propose that only a small amount of Ci enters the nucleus in *cdc37* clones, which may explain why there are no apparent changes in *ptc* (which requires high-level Hh for expression), although we do observe a change in *dpp*, which is a low level target. Knockdown of *cdc37* caused increased cell proliferation in discs, as indicated by increased levels of phospho-Histone H3 (PH3; supplementary material Fig. S9B), but this proliferation cannot solely explain the expression of targets in regions outside the normal domain of signaling. Thus, these results indicate that loss of *cdc37* enhances both Wg and Hh signaling outputs.

To examine whether the function of Cdc37 is evolutionarily conserved, we carried out biochemical studies in mammalian cells. Knockdown of *Cdc37* with siRNA in 3T3 cells strongly enhanced levels of stabilized β-catenin (Fig. 7J). Furthermore, knockdown of *Cdc37* also induced signaling in unstimulated 3T3 cells, as measured using the Wnt pathway-specific TOPFLASH reporter, compared with mock-transfected 3T3 cells (Fig. 7K). Knockdown of *Cdc37* also enhanced the levels of full-length GLI3 (Fig. 7L). We were unable to detect the truncated form of GLI3 in our assay. These effects of Cdc37 in mammalian cells mimic those of negative regulators of the Wnt and Hh pathways, such as GSK3β and CK1α. As Cdc37 is a kinase-associated chaperone, we propose that it functions to promote the stability of GSK3β and/or CK1α, which both constitutively destabilize the effectors of the Wnt and Hh pathways. Accordingly, cycloheximide-treated 3T3 cells reduced for *Cdc37* displayed progressively lower levels of GSK3β due to its shorter half-life, compared with mock-transfected cells (Fig. 7M,N). We did not

evaluate whether Cdc37 regulates the stability of CK1α in our assay. Thus, our analyses suggest that Cdc37 has a novel evolutionarily conserved function from *Drosophila* to mammalian cells to promote the stability of GSK3β and inhibit both the Wnt and Hh pathways.

Gish/CK1γ promotes the Hh pathway by phosphorylating Smo

Gish (*Drosophila* CK1γ) is a plasma membrane-associated kinase that has been described to promote the Wnt pathway by phosphorylating the co-receptor LRP (Davidson et al., 2005; Zhang et al., 2006). Consistently, it was recovered as a high-confidence Wnt regulator (Fig. 2L,O). Thus far, no role has been ascribed to this kinase in the regulation of the Hh pathway. Although knockdown of *gish* decreased the expression of targets of the Wnt, Notch and Hh pathways in the wing disc (Fig. 6A), the levels of Delta (Dl) and cleaved-Caspase 3 (Casp 3) were unaffected (supplementary material Fig. S9C). Thus, *gish* does not have a non-specific effect on gene transcription or cell death. Compared with wild type, a *gish* mutant wing disc displayed lower levels of Ci (Fig. 8A). Hh signaling stabilizes Ci to regulate expression of *dpp-lacZ* within the morphogenetic furrow (MF) of the eye disc (Fig. 8B). A somatic clone of *gish* in the eye disc had decreased levels of Ci and correspondingly *dpp-lacZ* expression (Fig. 8C), thereby confirming its role as a positive regulator of Hh signaling across multiple tissues.

We propose that Gish/CK1γ regulates the phosphorylation of the transmembrane protein Smo to promote the Hh pathway, analogous to its role in regulating the phosphorylation of LRP in the Wnt pathway. We tested this hypothesis using protein lysates from wing discs in a gel mobility shift assay (Fig. 8D). Knockdown of *gish* throughout the wing blade using *71B-Gal4* reduced the phosphorylation of Smo in disc protein lysates (as detected by a faster migrating band). As Smo phosphorylation is a prerequisite to its accumulation, knockdown of *gish* also decreased Smo levels. Conversely, overexpression of the Hh ligand, *hh-N*, expectedly enhanced the phosphorylation (as detected by a slower migrating band in lane 3) and levels of Smo (Fig. 8D). The simultaneous knockdown of *gish* in the presence of ectopic *hh-N* reduced the phosphorylation (migration) and levels of Smo, compared with ectopic *hh-N* alone (determined by densitometry), thus indicating that Gish acts downstream of the Hh ligand.

To determine whether the effect of Gish on the Hh pathway is evolutionarily conserved, the effect of CK1γ on signaling was assessed in mammalian cells. 3T3 cells transfected with CK1γ displayed increased levels of full-length GLI3, compared with mock-transfected cells (Fig. 8E). When exogenous CK1γ and Smo were co-transfected into 3T3 cells, they were detected in a complex in a co-immunoprecipitation assay (Fig. 8F). Furthermore, CK1γ robustly phosphorylated Smo at one or more serine and/or threonine residues (as detected by phospho-specific antibodies) in the presence of ATP in an *in vitro* kinase assay (Fig. 8G). Thus, Gish/CK1γ regulates the phosphorylation of Smo to promote the Hh pathway in *Drosophila* and mammalian cells.

DISCUSSION

Divergent disease states have been attributed to be a cause or consequence of aberrant protein phosphorylation (Reiter et al.,

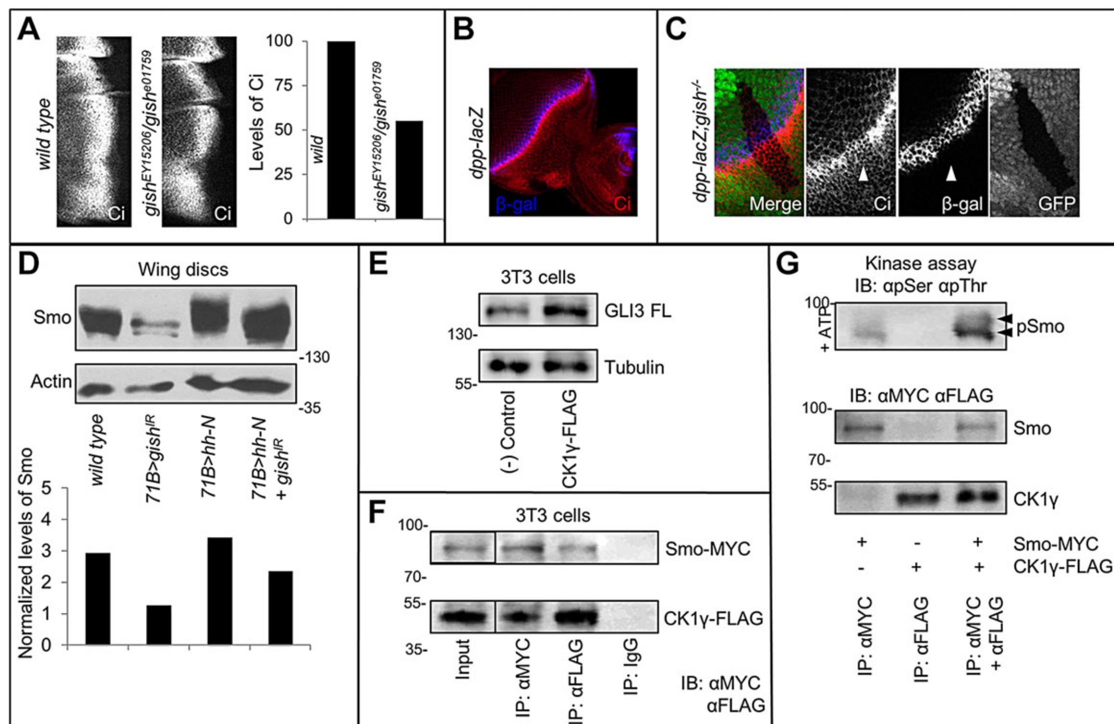

Fig. 8. Gish/CK1γ promotes the Hh pathway by phosphorylating Smo. (A) Ci protein levels are reduced in a *gish* mutant wing disc compared with wild type. (B) The Hh pathway stabilizes Ci to promote *dpp-lacZ* expression within the MF of the eye disc. (C) A *gish* somatic clone (GFP negative) in the eye disc had decreased levels of Ci and *dpp-lacZ* (detected by anti-β-gal; arrowheads). (D) Knockdown of *gish* using *71B-Gal4* in wing discs reduced the phosphorylation (detected by mobility shift) and levels of Smo, compared with wild type. Expression of *hh-N* with *71B-Gal4* enhanced the phosphorylation (as detected by mobility shift) and levels of Smo. The simultaneous knockdown of *gish* in the presence of ectopic *hh-N* reduced the phosphorylation (as detected by mobility shift) and levels of Smo. (E) Overexpression of CK1γ in 3T3 cells resulted in increased levels of full-length GLI3 compared with the mock-transfected control. (F) CK1γ co-precipitated with Smo when transfected into 3T3 cells. Epitope-tagged exogenous proteins were precipitated with corresponding antibodies or with IgG (negative control). (G) Smo was phosphorylated by CK1γ in an *in vitro* kinase assay, as detected by antibodies against phosphorylated serine and threonine residues. The loading control had equal amounts of immunoprecipitated proteins. Molecular weights are indicated in kDa next to each blot.

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2001). Wnt signaling is phosphor-regulated both in its silent and active states, but thus far our understanding of kinases, phosphatases and associated factors of the pathway has been limited. In this study, we performed the first genome-wide *in vivo* screen under physiological conditions in the *Drosophila* wing disc for phospho-regulators of the Wnt pathway. We identified 54 high-confidence regulators, 22 of which are novel. The results of our analyses do not indicate whether a high-confidence regulator has a direct or indirect effect on signaling. However, as ∼60% of the high-confidence regulators identified have been previously validated to have a direct effect on Wnt signaling, we predict that at least some of the novel high-confidence regulators identified would also have a direct effect on the pathway. Indeed, subsequent analyses of Myopic revealed a novel role in regulating Wg secretion (Pradhan-Sundd and Verheyen, 2014). Although the mechanism and components of the Wnt pathway are for the most part conserved between *Drosophila* and humans, there are possibly vertebrate-specific phospho-regulators of signaling that would not have been identified in our analyses. Our dataset represents the largest list of putative phospho-regulators of the Wnt pathway identified to date, almost all of which have identified human orthologs (supplementary material Figs S1, S2) and are therefore likely to be functionally conserved.

As part of this study, we also established previously unknown relationships between the Wnt and Hh pathways *in vivo* by identifying 12 novel dual regulators that we propose function at analogous levels of signaling (Fig. 5B). As proof of concept, we biochemically characterized the roles of Cdc37 and Gish/CK1γ to demonstrate that their functions are conserved from *Drosophila* to mammalian cells. We also describe an initial analysis of candidate regulators of Notch signaling during wing disc development. Although these findings are preliminary, they highlight an emerging theme of phospho-regulation of Notch that likely hold parallels in vertebrate biology. The comparison of signaling pathways *in vivo* and the identification of specific versus shared phospho-regulators facilitate our understanding of human development and disease states.

MATERIALS AND METHODS

Drosophila genetics

The following *Drosophila* strains were used: w^{1118} (wild type), *dpp-Gal4/TM6B*, *C5-Gal4*, *MS1096-Gal4*, *omb-Gal4*, *71B-Gal4*, *UAS-flp*, *UAS-dicer-2*, *UAS-p35*, *dpp-lacZ/CyO*, *Cdc37*^{eD4} *FRT79/TM6B*, *FRT82,GFP/TM6B*, *MARCM79*, *hs-flp;;FRT82,GFP/TM6B* and *gish*^{EY15206} (Bloomington *Drosophila* Stock Center); *hh-Gal4/TM6B* and *eyFlp;ey-Gal4,GMR-Gal4; sev>y+>wg* (Port et al., 2011); *UAS-hhN* (Su et al., 2011); and *FRT82 gish*^{e01759}*/TM6B* (Gault et al., 2012). The transgenic RNAi strains used for the screens were obtained from the Vienna Drosophila RNAi Center (Dietzl et al., 2007), National Institute of Genetics and Harvard Transgenic RNAi Project (supplementary material Fig. S4, Fig. 5). The percentage of inverted repeats with predicted sequence-dependent off-target effects are as follows: 65% (0), 27% (1-2) and 8% (>2). All genes (at least two independent RNAi lines per gene) were first tested with the *dpp-Gal4* driver. If a phenotype was only observed with one RNAi line for *dpp-Gal4* or with two lines targeting the same region of the mRNA, the gene was re-tested with *hh-Gal4*. The one or more RNAi lines that were re-tested with *hh-Gal4* were either the same lines that were used with *dpp-Gal4* or additional lines (if new ones were available). If a RNAi line displayed a phenotype with both the *dpp-Gal4* and *hh-Gal4* drivers in the primary screen, the *hh-Gal4 driver* was used in the secondary screen. Wing discs from 20 larvae of each genotype were immunostained (30 larvae for genotypes that were non-homozygous with a balancer chromosome). Knockdown of no gene precluded analysis due to lethality before the third instar stage. Twenty flies of each genotype were scored for adult phenotypes. The penetrance of all phenotypes indicated is between 80 and 100%. False-positive results due to cell death, cell proliferation or non-specific gene transcription were evaluated through testing the ability of candidate regulators to affect different signaling pathways in the wing disc. *cdc37* MARCM clones were generated by crossing the *UAS-p35; Cdc37*^{eD4} *FRT79/TM6B* and *MARCM79* strains, and progeny were heat-shocked 48 h after egg laying (AEL) for 2 h at 38°C. *gish* somatic clones in the wing disc were generated by crossing the *UAS-flp; FRT82 gish*^{e01759}*/TM6B* and *omb-Gal4;;FRT82,GFP/TM6B* strains. *gish* somatic clones in the eye disc were generated by crossing the *hs-flp;;FRT82, GFP/TM6B* and *FRT82 gish*^{e01759}*/TM6B* strains, and progeny were heat-shocked 48 h AEL for 2 h at 38°C.

Immunostaining of wing discs

Drosophila wing and eye discs from third instar larvae were dissected, immunostained and mounted according to standard procedures (Swarup and Verheyen, 2011). The following primary antibodies were used: anti-Ci 2A1 (1:50), anti-Ptc (1:50), anti-Wg 4D4 (1:100), anti-Ct 2B10 (1:75), anti-Delta C594.9B (1:50) (Developmental Studies Hybridoma Bank), anti-Phospho Histone H3 (1:100), anti-β-galactosidase (1:1500), anti-Cleaved Caspase 3 (1:100) (Cell Signaling Technology), anti-Sens (1:1000) (Nolo et al., 2000) and anti-Dll (1:400) (Duncan et al., 1998; Panganiban et al., 1995). Fluorescent secondary antibodies (1:400) were from Jackson Immunolabs. Wing disc images were obtained on a Nikon A1R laser scanning confocal microscope and all images are derived from stacked *z*-series. A fixed-size box (width=width of stabilized Ci domain in wild type, height=bottom of disc to top of wing pouch) was used to compare the integrated density of wild type and *gish* mutant. For quantification of immunostained discs, integrated density (pixel area multiplied by baseline subtracted intensity) was calculated.

Cell culture

NIH-3T3 cells (American Type Culture Collection) were cultured at 37°C in DMEM supplemented with 10% FBS (Invitrogen). 3T3 cells were transfected with CK1γ-FLAG (Davidson et al., 2005), Smo-MYC (Chen et al., 2011), TOPFLASH, FOPFLASH, or Renilla luciferase (Ishitani et al., 2003) using Polyfect Reagent (Qiagen) and harvested 36-48 h post-transfection. Knockdown of Cdc37 was performed with siRNA and Lipofectamine RNAiMAX Reagent (Invitrogen). As a negative control, cells were mock-transfected with empty vector. Cells were treated with cycloheximide (25 µg/ml) (Sigma) 24 h after transfection with Cdc37 siRNA [a mixture of two distinct siRNAs targeted to different parts of the genes from Ambion (136227 and 136228)] and harvested at the indicated time points.

Biochemical assays

Protein lysates were prepared from 3T3 cells and wing discs. Proteins of interest were immunoprecipitated from lysates using antibodies and Protein-G Sepharose beads (Sigma). The kinase assay with non-radiolabeled ATP/kinase assay buffer (Cell Signaling Technology) was performed with immunoprecipitated proteins at 30°C for 30 min. The de-phosphorylation assay was performed on protein lysate using lambda protein phosphatase at 30°C for 1 h (New England Biolabs).

Western blot analyses

Western blot analyses were performed using the following primary antibodies: anti-Tubulin (1:1000), anti-Myc (1:1000), anti-FLAG (1:1000) (Sigma), anti-GSK3β (1:500) (Abcam), anti-GLI3 N19 (1:200) (Santa Cruz Biotechnology), anti-Phosphoserine (1:500), anti-Phosphothreonine (1:500) (Sigma), anti-Smo 20C6 (1:10) and anti-Armadillo N27A1 (1:200) (Developmental Studies Hybridoma Bank). For quantification of western blot bands, integrated density (pixel area multiplied by baseline subtracted intensity) of each band was calculated within an appropriate exposure range and then normalized to the loading control.

Transcriptional assays

Transcriptional assays were performed using TOPFLASH, FOPFLASH and Renilla (control) luciferase reporter plasmids. The experiment was performed using the Dual Luciferase Reporter Assay System (Promega).

DEVELOPMENT

The value for each data point is the average of three individual experiments. The s.d. was calculated and Student's *t*-test was performed for statistical significance.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
S.S., E.M.V. designed the experiments. S.S., T.P.-S. performed the experiments. S.S., T.P.-S., E.M.V. analyzed the data. S.S., E.M.V. wrote the manuscript.

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Supplementary material
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References
Aberle, H., Bauer, A., Stappert, J., Kispert, A. and Kemler, R. (1997). beta-catenin is a target for the ubiquitin-proteasome pathway. *EMBO J.* **16**, 3797-3804.
Amit, S., Hatzubai, A., Birman, Y., Andersen, J. S., Ben-Shushan, E., Mann, M., Ben-Neriah, Y. and Alkalay, I. (2002). Axin-mediated CKI phosphorylation of beta-catenin at Ser 45: a molecular switch for the Wnt pathway. *Genes Dev.* **16**, 1066-1076.
Anton, R., Chatterjee, S. S., Simundza, J., Cowin, P. and DasGupta, R. (2011). A systematic screen for micro-RNAs regulating the canonical Wnt pathway. *PLoS ONE* **6**, e26257.
Apionishev, S., Katanayeva, N. M., Marks, S. A., Kalderon, D. and Tomlinson, A. (2005). Drosophila Smoothened phosphorylation sites essential for Hedgehog signal transduction. *Nat. Cell Biol.* **7**, 86-92.
Aza-Blanc, P., Ramírez-Weber, F.-A., Laget, M.-P., Schwartz, C. and Kornberg, T. B. (1997). Proteolysis that is inhibited by hedgehog targets Cubitus interruptus protein to the nucleus and converts it to a repressor. *Cell* **89**, 1043-1053.
Barolo, S. (2006). Transgenic Wnt/TCF pathway reporters: all you need is Lef? *Oncogene* **25**, 7505-7511.
Behrens, J., von Kries, J. P., Kühl, M., Bruhn, L., Wedlich, D., Grosschedl, R. and Birchmeier, W. (1996). Functional interaction of beta-catenin with the transcription factor LEF-1. *Nature* **382**, 638-642.
Bejsovec, A. (2006). Flying at the head of the pack: Wnt biology in Drosophila. *Oncogene* **25**, 7442-7449.
Bodenmiller, B., Malmstrom, J., Gerrits, B., Campbell, D., Lam, H., Schmidt, A., Rinner, O., Mueller, L. N., Shannon, P. T., Pedrioli, P. G. et al. (2007). PhosphoPep—a phosphoproteome resource for systems biology research in Drosophila Kc167 cells. *Mol. Syst. Biol.* **3**, 139.
Buechling, T. and Boutros, M. (2011). Wnt signaling: signaling at and above the receptor level. *Curr. Top. Dev. Biol.* **97**, 21-53.
Buechling, T., Chaudhary, V., Spirohn, K., Weiss, M. and Boutros, M. (2011). p24 proteins are required for secretion of Wnt ligands. *EMBO Rep.* **12**, 1265-1272.
Caplan, A. J., Mandal, A. K. and Theodoraki, M. A. (2007). Molecular chaperones and protein kinase quality control. *Trends Cell Biol.* **17**, 87-92.
Caspi, E. and Rosin-Arbesfeld, R. (2008). A novel functional screen in human cells identifies MOCA as a negative regulator of Wnt signaling. *Mol. Biol. Cell* **19**, 4660-4674.
Cavallo, R. A., Cox, R. T., Moline, M. M., Roose, J., Polevoy, G. A., Clevers, H., Peifer, M. and Bejsovec, A. (1998). Drosophila Tcf and Groucho interact to repress Wingless signalling activity. *Nature* **395**, 604-608.
Chen, Y., Gallaher, N., Goodman, R. H. and Smolik, S. M. (1998). Protein kinase A directly regulates the activity and proteolysis of cubitus interruptus. *Proc. Natl. Acad. Sci. USA* **95**, 2349-2354.
Chen, Y., Sasai, N., Ma, G., Yue, T., Jia, J., Briscoe, J. and Jiang, J. (2011). Sonic Hedgehog dependent phosphorylation by CK1α and GRK2 is required for ciliary accumulation and activation of smoothened. *PLoS Biol.* **9**, e1001083.
Cliffe, A., Hamada, F. and Bienz, M. (2003). A role of dishevelled in relocating axin to the plasma membrane during wingless signaling. *Curr. Biol.* **13**, 960-966.
Cohen, P. (1992). Signal integration at the level of protein kinases, protein phosphatases and their substrates. *Trends Biochem. Sci.* **17**, 408-413.
Couso, J., Bishop, S. and Martinez-Arias, A. (1994). The wingless signalling pathway and the patterning of the wing margin in Drosophila. *Development* **120**, 621-636.
Cselenyi, C. S., Jernigan, K. K., Tahinci, E., Thorne, C. A., Lee, L. A. and Lee, E. (2008). LRP6 transduces a canonical Wnt signal independently of Axin degradation by inhibiting GSK3's phosphorylation of beta-catenin. *Proc. Natl. Acad. Sci. USA* **105**, 8032-8037.
DasGupta, R., Kaykas, A., Moon, R. T. and Perrimon, N. (2005). Functional genomic analysis of the Wnt-wingless signaling pathway. *Science* **308**, 826-833.
Davidson, G., Wu, W., Shen, J., Bilic, J., Fenger, U., Stannek, P., Glinka, A. and Niehrs, C. (2005). Casein kinase 1 gamma couples Wnt receptor activation to cytoplasmic signal transduction. *Nature* **438**, 867-872.
Davidson, G., Shen, J., Huang, Y.-L., Su, Y., Karaulanov, E., Bartscherer, K., Hassler, C., Stannek, P., Boutros, M. and Niehrs, C. (2009). Cell cycle control of wnt receptor activation. *Dev. Cell* **17**, 788-799.
Denef, N., Neubüser, D., Perez, L. and Cohen, S. M. (2000). Hedgehog induces opposite changes in turnover and subcellular localization of patched and smoothened. *Cell* **102**, 521-531.
Diaz-Benjumea, F. and Cohen, S. (1995). Serrate signals through Notch to establish a Wingless-dependent organizer at the dorsal/ventral compartment boundary of the Drosophila wing. *Development* **121**, 4215-4225.
Dietzl, G., Chen, D., Schnorrer, F., Su, K.-C., Barinova, Y., Fellner, M., Gasser, B., Kinsey, K., Oppel, S., Scheiblauer, S. et al. (2007). A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. *Nature* **448**, 151-156.
Duncan, D. M., Burgess, E. A. and Duncan, I. (1998). Control of distal antennal identity and tarsal development in Drosophila by spineless-aristapedia, a homolog of the mammalian dioxin receptor. *Genes Dev.* **12**, 1290-1303.
Firestein, R., Bass, A. J., Kim, S. Y., Dunn, I. F., Silver, S. J., Guney, I., Freed, E., Ligon, A. H., Vena, N., Ogino, S. et al. (2008). CDK8 is a colorectal cancer oncogene that regulates beta-catenin activity. *Nature* **455**, 547-551.
Fortini, M. E., Skupski, M. P., Boguski, M. S. and Hariharan, I. K. (2000). A survey of human disease gene counterparts in the Drosophila genome. *J. Cell Biol.* **150**, F23-F30.
Gao, C., Xiao, G. and Hu, J. (2014). Regulation of Wnt/β-catenin signaling by posttranslational modifications. *Cell Biosci.* **4**, 13.
Gault, W. J., Olguin, P., Weber, U. and Mlodzik, M. (2012). Drosophila CK1-γ, gilgamesh, controls PCP-mediated morphogenesis through regulation of vesicle trafficking. *J. Cell Biol.* **196**, 605-621.
Greaves, S., Sanson, B., White, P. and Vincent, J. P. (1999). A screen for identifying genes interacting with armadillo, the drosophila homolog of {beta}-catenin. *Genetics* **153**, 1753-1766.
Groenendyk, J. and Michalak, M. (2011). A genome-wide siRNA screen identifies novel phospho-enzymes affecting Wnt/β-catenin signaling in mouse embryonic stem cells. *Stem Cell Rev. Rep.* **7**, 910-926.
Herrera, S. C., Martín, R. and Morata, G. (2013). Tissue homeostasis in the wing disc of drosophila melanogaster: immediate response to massive damage during development. *PLoS Genet.* **9**, e1003446.
Hunter, T. (1995). Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. *Cell* **80**, 225-236.
Ishitani, T., Ninomiya-Tsuji, J. and Matsumoto, K. (2003). Regulation of lymphoid enhancer factor 1/T-cell factor by mitogen-activated protein kinase-related Nemo-like kinase-dependent phosphorylation in Wnt/beta-catenin signaling. *Mol. Cell. Biol.* **23**, 1379-1389.
Jacob, L. S., Wu, X., Dodge, M. E., Fan, C.-W., Kulak, O., Chen, B., Tang, W., Wang, B., Amatruda, J. F. and Lum, L. (2011). Genome-wide RNAi screen reveals disease-associated genes that are common to Hedgehog and Wnt signaling. *Sci. Signal.* **4**, ra4.
James, R. G., Biechele, T. L., Conrad, W. H., Camp, N. D., Fass, D. M., Major, M. B., Sommer, K., Yi, X., Roberts, B. S., Cleary, M. A. et al. (2009). Bruton's tyrosine kinase revealed as a negative regulator of Wnt-beta-catenin signaling. *Sci. Signal.* **2**, ra25.
Jia, J., Amanai, K., Wang, G., Tang, J., Wang, B. and Jiang, J. (2002). Shaggy/GSK3 antagonizes Hedgehog signalling by regulating Cubitus interruptus. *Nature* **416**, 548-552.
Jia, J., Tong, C. and Jiang, J. (2003). Smoothened transduces Hedgehog signal by physically interacting with Costal2/Fused complex through its C-terminal tail. *Genes Dev.* **17**, 2709-2720.
Jia, J., Tong, C., Wang, B., Luo, L. and Jiang, J. (2004). Hedgehog signalling activity of Smoothened requires phosphorylation by protein kinase A and casein kinase I. *Nature* **432**, 1045-1050.
Jia, J., Zhang, L., Zhang, Q., Tong, C., Wang, B., Hou, F., Amanai, K. and Jiang, J. (2005). Phosphorylation by double-time/CKIepsilon and CKIalpha targets cubitus interruptus for Slimb/beta-TRCP-mediated proteolytic processing. *Dev. Cell* **9**, 819-830.
Jiang, J. and Struhl, G. (1998). Regulation of the Hedgehog and Wingless signalling pathways by the F-box/WD40-repeat protein Slimb. *Nature* **391**, 493-496.

Kalderon, D. (2002). Similarities between the Hedgehog and Wnt signaling pathways. *Trends Cell Biol.* **12**, 523-531.

Kategaya, L. S., Changkakoty, B., Biechele, T., Conrad, W. H., Kaykas, A., Dasgupta, R. and Moon, R. T. (2009). Bili inhibits Wnt/beta-catenin signaling by regulating the recruitment of axin to LRP6. *PLoS ONE* **4**, e6129.

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

Kitagawa, M., Hatakeyama, S., Shirane, M., Matsumoto, M., Ishida, N., Hattori, K., Nakamichi, I., Kikuchi, A., Nakayama, K.-i. and Nakayama, K. (1999). An F-box protein, FWD1, mediates ubiquitin-dependent proteolysis of beta-catenin. *EMBO J.* **18**, 2401-2410.

Lange, B. M. H., Rebollo, E., Herold, A. and González, C. (2002). Cdc37 is essential for chromosome segregation and cytokinesis in higher eukaryotes. *EMBO J.* **21**, 5364-5374.

Liu, C., Kato, Y., Zhang, Z., Do, V. M., Yankner, B. A. and He, X. (1999). Beta-TrCP couples beta-catenin phosphorylation-degradation and regulates Xenopus axis formation. *Proc. Natl. Acad. Sci. USA* **96**, 6273-6278.

Liu, C., Li, Y., Semenov, M., Han, C., Baeg, G.-H., Tan, Y., Zhang, Z., Lin, X. and He, X. (2002). Control of beta-catenin phosphorylation/degradation by a dual-kinase mechanism. *Cell* **108**, 837-847.

Liu, Y., Cao, X., Jiang, J. and Jia, J. (2007). Fused Costal2 protein complex regulates Hedgehog-induced Smo phosphorylation and cell-surface accumulation. *Genes Dev.* **21**, 1949-1963.

Lum, L., Zhang, C., Oh, S., Mann, R. K., von Kessler, D. P., Taipale, J., Weis-Garcia, F., Gong, R., Wang, B. and Beachy, P. A. (2003). Hedgehog signal transduction via smoothened association with a cytoplasmic complex scaffolded by the atypical kinesin, costal-2. *Mol. Cell* **12**, 1261-1274.

MacDonald, B. T., Tamai, K. and He, X. (2009). Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Dev. Cell* **17**, 9-26.

Major, M. B., Roberts, B. S., Berndt, J. D., Marine, S., Anastas, J., Chung, N., Ferrer, M., Yi, X., Stoick-Cooper, C. L., von Haller, P. D. et al. (2008). New regulators of Wnt/beta-catenin signaling revealed by integrative molecular screening. *Sci. Signal.* **1**, ra12.

Méthot, N. and Basler, K. (1999). Hedgehog controls limb development by regulating the activities of distinct transcriptional activator and repressor forms of Cubitus interruptus. *Cell* **96**, 819-831.

Mi, K., Dolan, P. J. and Johnson, G. V. W. (2006). The low density lipoprotein receptor-related protein 6 interacts with glycogen synthase kinase 3 and attenuates activity. *J. Biol. Chem.* **281**, 4787-4794.

Micchelli, C. A., Rulifson, E. J. and Blair, S. S. (1997). The function and regulation of cut expression on the wing margin of Drosophila: Notch, Wingless and a dominant negative role for Delta and Serrate. *Development* **124**, 1485-1495.

Miller, B. W., Lau, G., Grouios, C., Mollica, E., Barrios-Rodiles, M., Liu, Y., Datti, A., Morris, Q., Wrana, J. L. and Attisano, L. (2009). Application of an integrated physical and functional screening approach to identify inhibitors of the Wnt pathway. *Mol. Syst. Biol.* **5**, 315.

Miura, G. I., Roignant, J.-Y., Wassef, M. and Treisman, J. E. (2008). Myopic acts in the endocytic pathway to enhance signaling by the Drosophila EGF receptor. *Development* **135**, 1913-1922.

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

Neumann, C. J. and Cohen, S. M. (1996). A hierarchy of cross-regulation involving Notch, wingless, vestigial and cut organizes the dorsal/ventral axis of the Drosophila wing. *Development* **122**, 3477-3485.

Neumann, C. and Cohen, S. (1997). Long-range action of Wingless organizes the dorsal-ventral axis of the Drosophila wing. *Development* **124**, 871-880.

Nolo, R., Abbott, L. A. and Bellen, H. J. (2000). Senseless, a Zn finger transcription factor, is necessary and sufficient for sensory organ development in Drosophila. *Cell* **102**, 349-362.

Ogden, S. K., Ascano, M., Stegman, M. A., Suber, L. M., Hooper, J. E. and Robbins, D. J. (2003). Identification of a functional interaction between the transmembrane protein smoothened and the kinesin-related protein costal2. *Curr. Biol.* **13**, 1998-2003.

Ohlmeyer, J. T. and Kalderon, D. (1998). Hedgehog stimulates maturation of Cubitus interruptus into a labile transcriptional activator. *Nature* **396**, 749-753.

Panganiban, G., Sebring, A., Nagy, L. and Carroll, S. (1995). The development of crustacean limbs and the evolution of arthropods. *Science* **270**, 1363-1366.

Parker, D. S., White, M. A., Ramos, A. I., Cohen, B. A. and Barolo, S. (2011). The cis-regulatory logic of Hedgehog gradient responses: key roles for gli binding affinity, competition, and cooperativity. *Sci. Signal.* **4**, ra38.

Port, F., Hausmann, G. and Basler, K. (2011). A genome-wide RNA interference screen uncovers two p24 proteins as regulators of Wingless secretion. *EMBO Rep.* **12**, 1144-1152.

Pradhan-Sundd, T. and Verheyen, E. M. (2014). The role of Bro1 domain-containing protein Myopic in endosomal trafficking of Wnt/Wingless. *Dev. Biol.* **392**, 93-107.

Price, M. A. and Kalderon, D. (1999). Proteolysis of cubitus interruptus in Drosophila requires phosphorylation by protein kinase A. *Development* **126**, 4331-4339.

Price, M. A. and Kalderon, D. (2002). Proteolysis of the Hedgehog signaling effector Cubitus interruptus requires phosphorylation by Glycogen Synthase Kinase 3 and Casein Kinase 1. *Cell* **108**, 823-835.

Reiter, L. T., Potocki, L., Chien, S., Gribskov, M. and Bier, E. (2001). A systematic analysis of human disease-associated gene sequences in Drosophila melanogaster. *Genome Res.* **11**, 1114-1125.

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

Ruel, L., Rodriguez, R., Gallet, A., Lavenant-Staccini, L. and Thérond, P. P. (2003). Stability and association of Smoothened, Costal2 and Fused with Cubitus interruptus are regulated by Hedgehog. *Nat. Cell Biol.* **5**, 907-913.

Rulifson, E. and Blair, S. (1995). Notch regulates wingless expression and is not required for reception of the paracrine wingless signal during wing margin neurogenesis in Drosophila. *Development* **121**, 2813-2824.

Rulifson, E. J., Micchelli, C. A., Axelrod, J. D., Perrimon, N. and Blair, S. S. (1996). wingless refines its own expression domain on the Drosophila wing margin. *Nature* **384**, 72-74.

Ryoo, H. D., Gorenc, T. and Steller, H. (2004). Apoptotic cells can induce compensatory cell proliferation through the JNK and the Wingless signaling pathways. *Dev. Cell* **7**, 491-501.

Salazar, C. and Höfer, T. (2009). Multisite protein phosphorylation - from molecular mechanisms to kinetic models. *FEBS J.* **276**, 3177-3198.

Serysheva, E., Berhane, H., Grumolato, L., Demir, K., Balmer, S., Bodak, M., Boutros, M., Aaronson, S., Mlodzik, M. and Jenny, A. (2013). Wnk kinases are positive regulators of canonical Wnt/β-catenin signalling. *EMBO Rep.* **14**, 718-725.

Silhankova, M., Port, F., Harterink, M., Basler, K. and Korswagen, H. C. (2010). Wnt signalling requires MTM-6 and MTM-9 myotubularin lipid-phosphatase function in Wnt-producing cells. *EMBO J.* **29**, 4094-4105.

Smelkinson, M. G. and Kalderon, D. (2006). Processing of the Drosophila hedgehog signaling effector Ci-155 to the repressor Ci-75 is mediated by direct binding to the SCF component Slimb. *Curr. Biol.* **16**, 110-116.

Smelkinson, M. G., Zhou, Q. and Kalderon, D. (2007). Regulation of Ci-SCFSlimb binding, Ci proteolysis, and hedgehog pathway activity by Ci phosphorylation. *Dev. Cell* **13**, 481-495.

Strigini, M. and Cohen, S. (1997). A Hedgehog activity gradient contributes to AP axial patterning of the Drosophila wing. *Development* **124**, 4697-4705.

Su, Y., Ospina, J. K., Zhang, J., Michelson, A. P., Schoen, A. M. and Zhu, A. J. (2011). Sequential phosphorylation of smoothened transduces graded hedgehog signaling. *Sci. Signal.* **4**, ra43.

Swarup, S. and Verheyen, E. M. (2011). Drosophila homeodomain-interacting protein kinase inhibits the Skp1-Cul1-F-box E3 ligase complex to dually promote Wingless and Hedgehog signaling. *Proc. Natl. Acad. Sci. USA* **108**, 9887-9892.

Tabata, T. and Takei, Y. (2004). Morphogens, their identification and regulation. *Development* **131**, 703-712.

Tamai, K., Zeng, X., Liu, C., Zhang, X., Harada, Y., Chang, Z. and He, X. (2004). A mechanism for Wnt coreceptor activation. *Mol. Cell* **13**, 149-156.

Tang, L.-Y., Deng, N., Wang, L.-S., Dai, J., Wang, Z.-L., Jiang, X.-S., Li, S.-J., Li, L., Sheng, Q.-H., Wu, D.-Q. et al. (2007). Quantitative phosphoproteome profiling of Wnt3a-mediated signaling network: indicating the involvement of ribonucleoside-diphosphate reductase M2 subunit phosphorylation at residue serine 20 in canonical Wnt signal transduction. *Mol. Cell. Proteomics* **6**, 1952-1967.

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

Tempé, D., Casas, M., Karaz, S., Blanchet-Tournier, M.-F. and Concordet, J.-P. (2006). Multisite protein kinase A and glycogen synthase kinase 3beta phosphorylation leads to Gli3 ubiquitination by SCFbetaTrCP. *Mol. Cell. Biol.* **26**, 4316-4326.

Umbhauer, M., Djiane, A., Goisset, C., Penzo-Méndez, A., Riou, J.-F., Boucaut, J.-C. and Shi, D.-L. (2000). The C-terminal cytoplasmic Lys-thr-X-X-X-Trp motif in frizzled receptors mediates Wnt/beta-catenin signalling. *EMBO J.* **19**, 4944-4954.

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

Varjosalo, M. and Taipale, J. (2008). Hedgehog: functions and mechanisms. *Genes Dev.* **22**, 2454-2472.

Development (2015) 142, 1502-1515 doi:10.1242/dev.116715

Verheyen, E. M. and Gottardi, C. J. (2010). Regulation of Wnt/beta-catenin signaling by protein kinases. *Dev. Dyn.* **239**, 34-44.

Wang, G., Wang, B. and Jiang, J. (1999). Protein kinase A antagonizes Hedgehog signaling by regulating both the activator and repressor forms of Cubitus interruptus. *Genes Dev.* **13**, 2828-2837.

Wells, B. S., Yoshida, E. and Johnston, L. A. (2006). Compensatory proliferation in Drosophila imaginal discs requires Dronc-dependent p53 activity. *Curr. Biol.* **16**, 1606-1615.

Willert, K., Shibamoto, S. and Nusse, R. (1999). Wnt-induced dephosphorylation of Axin releases beta-catenin from the Axin complex. *Genes Dev.* **13**, 1768-1773.

Wodarz, A. and Nusse, R. (1998). Mechanisms of Wnt signaling in development. *Annu. Rev. Cell Dev. Biol.* **14**, 59-88.

Wong, H.-C., Bourdelas, A., Krauss, A., Lee, H.-J., Shao, Y., Wu, D., Mlodzik, M., Shi, D.-L. and Zheng, J. (2003). Direct binding of the PDZ domain of Dishevelled to a conserved internal sequence in the C-terminal region of Frizzled. *Mol. Cell* **12**, 1251-1260.

Yang-Snyder, J., Miller, J. R., Brown, J. D., Lai, C.-J. and Moon, R. T. (1996). A frizzled homolog functions in a vertebrate Wnt signaling pathway. *Curr. Biol.* **6**, 1302-1306.

Zecca, M., Basler, K. and Struhl, G. (1996). Direct and long-range action of a wingless morphogen gradient. *Cell* **87**, 833-844.

Zeng, X., Tamai, K., Doble, B., Li, S., Huang, H., Habas, R., Okamura, H., Woodgett, J. and He, X. (2005). A dual-kinase mechanism for Wnt co-receptor phosphorylation and activation. *Nature* **438**, 873-877.

Zhang, C., Williams, E. H., Guo, Y., Lum, L. and Beachy, P. A. (2004). Extensive phosphorylation of Smoothened in Hedgehog pathway activation. *Proc. Natl. Acad. Sci. USA* **101**, 17900-17907.

Zhang, W., Zhao, Y., Tong, C., Wang, G., Wang, B., Jia, J. and Jiang, J. (2005). Hedgehog-regulated Costal2-kinase complexes control phosphorylation and proteolytic processing of Cubitus interruptus. *Dev. Cell* **8**, 267-278.

Zhang, L., Jia, J., Wang, B., Amanai, K., Wharton, K. A. and Jiang, J. (2006). Regulation of wingless signaling by the CKI family in Drosophila limb development. *Dev. Biol.* **299**, 221-237.

Zheng, X., Mann, R. K., Sever, N. and Beachy, P. A. (2010). Genetic and biochemical definition of the Hedgehog receptor. *Genes Dev.* **24**, 57-71.

Zhu, A. J., Zheng, I., Suyama, K. and Scott, M. P. (2003). Altered localization of Drosophila Smoothened protein activates Hedgehog signal transduction. *Genes Dev.* **17**, 1240-1252.