RESEARCH ARTICLE

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

Sharan Swarup, Tirthadipa Pradhan-Sundd and Esther M. Verheyen*

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/CK1α 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; Rosenstein 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 (Cseleyny 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 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 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
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 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).

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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
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 \textit{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 \textit{string} (\textit{stg}) (Davidson et al., 2009) and \textit{gilgamesh} (\textit{gish}) (Davidson et al., 2005; Zhang et al., 2006), and a novel regulator, \textit{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 \textit{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 \textit{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 \textit{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 \textit{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).

\textbf{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
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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### Fig. 4. Summary of phospho-regulators of the Wnt pathway identified in the Drosophila wing disc.

<table>
<thead>
<tr>
<th>Kinase (associated factor)</th>
<th>Phosphatase (associated factor)</th>
<th>Wnt pathway</th>
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<td>Cad37</td>
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**Group I:** Positive regulator

**Group II:** Negative regulator

**Group III:** Previously identified

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### B. Genome: Kinases

- **Wnt pathway:**
  - Non-protein kinase: 15.1% (31)
  - Phosphatase-associated: 8.3% (17)
  - Class II DSP: 11.7% (24)
  - Class III: 2.4% (5)

- **Phosphatase:**
  - Non-protein phosphatase: 14.3% (3)
  - Phosphatase-associated: 4.6% (1)
  - FCP/SCP: 9.5% (2)
  - Classical: 9.5% (2)

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### C. Genome: Phosphatases

- **PPP:** 17.6% (38)
- **PPM:** 7.3% (15)
- **Classical:** 8.3% (17)
- **Unclassified:** 8.3% (17)
- **HAD:** 9.9% (8)
- **Class III:** 9.9% (2)
- **Class II DSP:** 9.9% (2)
- **PPP:** 52.4% (11)

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(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...
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<sup>227</sup> allele. Wg signaling is required for specification of regions of naked cuticle
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.
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. 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.
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 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.

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 cdc37eD4 allele. (C-E) Homozygous mutant cdc37eD4 embryos display cuticle defects reminiscent of wg gain-of-function defects, including loss of denticle belts. (F,G) MARCM clones (with p35 transgene) of cdc37eD4 (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.

(l) 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.
evaluate whether Cdc37 regulates the stability of CK1γ 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 its role in regulating the phosphorylation of LRP in the Wnt pathway. 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 moving 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.,
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: w1118 (wild type), dpp-Gal4/TM6B, CS-Gal4, MS1099-Gal4, emb-Gal4, 71B-Gal4, UAS-flp, UAS-FLP2, hs-flp, dpp:ZC:O, Cdc37- and FRT79/TM6B, FRT82, GFP/TM6B, MARCM79, hs-flp; FRT82, GFP/TM6B, and gishEY15206 (Bloomington Drosophila Stock Center); hh-Gal4/TM6B and eyFlp; ey-Gal4, GMR-Gal4; sevΔy+; wog (Port et al., 2011); UAS-hhN (Su et al., 2011); and FRT82 gishEY15206/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 Figs S4, S5). 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 with 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-PhosphoERK (1:500), anti-PhosphoH3 (1:500) (Sigma), anti-Smo 20C6 (1:10) and anti-Armadillo N27A (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.

**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-radioabeled 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-Phosphohistone H3 (1:1000), anti-β-galactosidase (1:1500), anti-Cleaved Caspase 3 (1:1000) (Cell Signaling Technology), anti-Sens (1:1000) (Nolo et al., 2000) and anti-DI (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.

**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).
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.

Acknowledgements

We are grateful to the numerous people who provided fly strains, plasmids and antibodies, especially Hugo Bellen, Ian Duncan, and Sean Carroll. We are also grateful to the RNAi stock centers (Vienna Drosophila RNAi Center, National Institute of Genetics and Harvard Transgenic RNAi Project) for generating the strains used in this study. Thanks to past and present members of the Verheyen lab for discussions, as well as to Hans Clevers, Roel Nusse and Nick Harden for comments on the study.

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.

Funding

This work was supported by an operating grant from the Canadian Institutes of Health Research.

Supplementary material

Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.116715/-/DC1

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