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Functional Genomic Analysis of the Wnt-Wingless Signaling Pathway

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The Wnt-Wingless (Wg) pathway is one of a core set of evolutionarily conserved signaling pathways that regulates many aspects of metazoan development. Aberrant Wnt signaling has been linked to human disease. In the present study we used a genomewide RNA interference (RNAi) screen in *Drosophila* cells to screen for regulators of the Wnt pathway. We identified 238 potential regulators, which include known pathway components, genes with functions not previously linked to this pathway, and genes with no previously assigned functions. Reciprocal-Best-Blast analyses reveal that 50% of the genes identified in the screen have human orthologs of which ~18% are associated with human disease. Functional assays of selected genes from the cell-based screen in *Drosophila*, mammalian cells, and zebrafish embryos demonstrated that these genes have evolutionarily conserved functions in Wnt signaling. High throughput RNAi screens in cultured cells, followed by functional analyses in model organisms, proves to be a rapid means of identifying regulators of signaling pathways implicated in development and disease.

Wnt proteins (called Wingless in *Drosophila*) are a family of conserved signaling molecules involved in a plethora of fundamental developmental and cell biological processes such as cell proliferation, differentiation and cell polarity (*1*–*3*). Several components of the pathway are tumorigenic when mutated in hepatic, colorectal, breast and skin cancers (*1*, *4*, *5*). Wnts encode secreted glycoproteins (http://stke.sciencemag.org/cgi/cm/stkecm;CMN_5537) that activate receptor-mediated pathways (http://stke.sciencemag.org/cgi/cm/stkecm;CMP_5533) leading to numerous transcriptional and cellular responses. The main function of the Wnt–beta-catenin pathway is to stabilize the cytoplasmic pool of a key mediator, beta-catenin (β-cat) [called Armadillo (Arm) in *Drosophila*] (http://stke.sciencemag.org/cgi/cm/stkecm;CMN_5554), which is otherwise degraded by the proteosome pathway. Initially identified as an important player in stabilizing cell-cell adherens junctions, β-cat is now known to participate in transcriptional regulation by forming a complex with the T cell–specific transcription factor (TCF) (http://stke.sciencemag.org/cgi/cm/stkecm;CMN_5566) and lymphoid enhancer factor (LEF) (http://stke.sciencemag.org/cgi/cm/stkecm;CMN_5890) families of high mobility group (HMG)-box transcription factors (*6*, *7*). In cells stimulated by Wnts, stabilized β-cat translocates to the nucleus, where, together with TCF/LEF transcription factors, it activates downstream target genes (*6*, *7*) (http://stke.sciencemag.org/cgi/cm/stkecm;CMN_6480). The Wnt pathway can also be activated through inhibition of its negative regulators such as glycogen synthase kinase-3beta (GSK-3β; http://stke.sciencemag.org/cgi/cm/stkecm;CMN_5562), adenomatous polyposis coli (APC; http://stke.sciencemag.org/cgi/cm/stkecm;CMN_5841) and Axin (http://stke.sciencemag.org/cgi/cm/stkecm;CMN_5847), which promote degradation of β-cat, or by the introduction of activating mutations in β-cat that render it incapable of interacting with the degradation complex. Wnt signaling can also activate an alternative signaling pathway involved in planar cell polarity (PCP), that may lead to protein kinase C (PKC) and Jun kinase (JNK) activation, resulting in calcium release and cytoskeletal rearrangements (*8*, *9*).

Whole-genome RNAi screens. Genetic and biochemical approaches have identified many of the genes that regulate the Wnt pathway in *Drosophila* (http://stke.sciencemag.org/cgi/cm/stkecm;CMP_6459) and other model organisms. However, many components may remain unidentified if mutants do not display a distinguishable "Wnt phenotype". In fact it is estimated that only 25% of all known *Drosophila* genes are associated with a readily obvious phenotype (*10*–*13*). The availability of the *Drosophila* genome sequence, a well-established RNAi-based screening technology, and the fact that ~75% of the fly genome remains uncharacterized, thus provided us with an

opportunity to rapidly and systematically characterize gene function at a genome-wide scale to find new components in the Wnt signaling pathway (*14–17*).

Here we present the results from a genome-wide RNAi screen in *Drosophila* cells that identified 238 potential regulators of the Wnt pathway. These include many known genes that have not been implicated previously in the Wnt pathway, and others that have not yet been assigned any gene function. We further demonstrate the conserved involvement of selected candidate genes in the Wnt-Wg pathway by conducting functional assays in *Drosophila* and mammalian cells. Finally, these cell-based assays were complemented by analysis of the functions of selected genes at the organismic level, specifically in *Drosophila* and in the zebrafish embryo.

Wnt-reporter genes and screen design. The assay for the RNAi screen was based on the Wnt reporter TOP-Flash (Tcf optimal promoter), which consists of multimerized Tcf binding sites driving the expression of a cDNA encoding the firefly luciferase gene (*18*, *19*). The screen was performed in *Drosophila* imaginal-disc-derived clone8 (cl8) cells which are epithelial in origin (*20*, *21*). The Wg pathway is active in the imaginal discs, and thus cl8 cells are likely to contain the majority of the components required to respond to Wg (*23*). The assay involved transfection of the TOP-Flash reporter along with a *Renilla* luciferase vector (PolIII-RL) as a control for transfection efficiency and an expression vector encoding *wg* (pMK33-wg) to stimulate the pathway (*22–25*) (see fig. S1). The activity of the Wg signaling pathway was quantified by measurement of normalized (N) luciferase expression or RLU (relative luciferase units), which equated to the ratio of the absolute activity of firefly luciferase to that of renilla luciferase.

To optimize the Wg-assay for a high-throughput screen (HTS) in a 384-well plate format, we designed two new TOP-Flash-like reporters, STF16 and dTF12, because existing reporters did not display robust signal/noise ratio in the high-density screen format (fig. S2) (*49*). STF16 comprises 16 Tcf binding sites and a minimal TATA box from the thymidine kinase (TK) promoter, whereas dTF12 contains 12 Tcf binding sites upstream of the *Drosophila* heat shock minimal promoter (fig. S2A). We first optimized the reporter assays in 96-well plate format (fig. S2, B and C). Although exhibiting different basal activities, both reporters allowed use of small volumes of cells and transfection reagents and displayed strong signal/noise ratios in multiple *Drosophila* cell lines including clone8 and S2R+ (*23*) cells (fig. S2B). Both reporters were expressed in a robust fashion upon pathway stimulation by Wg as well as downstream activators in the pathway such as a ΔNlrp6, a constitutively active form of the Wg co-receptor Lrp6 (*26*) (http://stke.sciencemag.org/cgi/cm/stkecm;CMN_6750) (fig. S2B). The use of two independent reporters interchangeably in primary and secondary screens ensured robustness of the assay by minimizing any reporter-specific differences and/or artifacts. The specificity of the reporters was confirmed by the use of FOP-Flash (in which the 12 Tcf binding sites are mutated), which did not display any significant activity above background (fig. S2C).

RNAi-mediated knockdown of positive regulators, such as Arm and dTcf, suppressed Wg-enhanced reporter activity, whereas RNAi-knockdown of negative regulators, such as Axin, ectopically activated the reporter in the absence of stimulus, or further synergistically activated the reporter upon Wg or Lrp6 induction (Fig. 1A and fig. S2). Thus we could use this reporter to identify both positive and negative modulators (Fig. 1B).

Data analysis and validity of primary screen. For the whole genome RNAi screen for the Wnt pathway (fig. S1) (*49*), we used a library of ~22,000 dsRNAs (www.flyrnai.org). The library represents >95% of genes in the *Drosophila* genome and has been used successfully in several screens (*14*, *16*, *17*, *27*). The screen was performed in duplicate to reduce the rate of false positives and to ensure the reproducibility of and hence confidence in individual candidate genes. To ascertain potential candidate genes involved in the Wnt pathway the data from each individual plate was analyzed by four distinct protocols, and candidate genes were assigned on the basis of their deviation from the plate average for each given criterion (see Methods) (*49*). Genes that satisfied two or more statistical criteria were considered strong candidates, whereas those that scored positive only by one imposed condition were considered weak candidates.

We identified 238 candidates that showed consistent response in both screens) that either reduced or increased Wnt pathway activity as measured by the TOP-Flash reporter activity (table S1, A and B). A majority of the known core Wnt pathway members, including Wnt-*wingless* (*wg*) (http://stke.sciencemag.org/cgi/cm/stkecm;CMN_6466) (http://www.sdbonline.org/fly/segment/wingles1.htm), *arrow* (*arr*)/*LDL-receptor-related protein-6* (*Lrp-6*) (http://stke.sciencemag.org/cgi/cm/stkecm;CMN_6926) (http://www.sdbonline.org/fly/segment/arrow1.htm), *frizzled* (*fz*) (http://stke.sciencemag.org/cgi/cm/stkecm;CMN_6496) (http://www.sdbonline.org/fly/neural/frizzled.htm), *frizzled-4* (*fz4*), *dally-like protein* (*dlp*) (http://stke.sciencemag.org/cgi/cm/stkecm;CMN_11382), *naked cuticle* (*nkd*) (http://stke.sciencemag.org/cgi/cm/stkecm;CMN_6509) (http://www.sdbonline.org/fly/segment/naked1.htm), *axin* (*axn*) (http://stke.sciencemag.org/cgi/cm/stkecm;CMN_6499) (http://www.sdbonline.org/fly/segment/axin1.htm), *supernumerary-limbs* (*slmb*) (http://stke.sciencemag.org/cgi/cm/stkecm;CMN_16386)

(http://www.sdbonline.org/fly/dbzhnsky/slimb1.htm), *casein kinase-1 alpha* (*ck1α*) (http://stke.sciencemag.org/cgi/cm/stkecm;CMN_16368), *disheveled* (*dsh*) (http://stke.sciencemag.org/cgi/cm/stkecm;CMN_6495) (http://www.sdbonline.org/fly/segment/dishevel.htm), *beta-catenin-armadillo* (*β-cat-arm*) (http://stke.sciencemag.org/cgi/cm/stkecm;CMN_6500) (http://www.sdbonline.org/fly/segment/armadilo.htm), *dTCF/pangolin* (*dTCF/pan*) (http://stke.sciencemag.org/cgi/cm/stkecm;CMN_6501) (http://www.sdbonline.org/fly/segment/pangoln1.htm), *dCBP/nejire* (*nej*) (http://stke.sciencemag.org/cgi/cm/stkecm;CMN_16384) (http://www.sdbonline.org/fly/hjmuller/crebbp1.htm), *pygopus* (*pygo*) (http://stke.sciencemag.org/cgi/cm/stkecm;CMN_11346) (http://www.sdbonline.org/fly/segment/pygopus1.htm), and *legless* (*lgs*) (http://stke.sciencemag.org/cgi/cm/stkecm;CMN_11347) (http://www.sdbonline.org/fly/segment/legless1.htm), thus underscoring the robustness and validity of the Wnt-screen in this HTS format (Fig. 2B). Comparison of the Z-scores (which is a measure of the number of standard deviation away from the mean for any particular normalized luciferase value) between the duplicate screens revealed high reproducibility both qualitatively and quantitatively with a correlation coefficient of 0.63 (Fig. 2A). Importantly, ~90% (213/238) of the candidate genes that were selected for further analyses, were verified in secondary screens (table S1A). Approximately 50% of the genes identified in the screen had an associated Gene Ontology annotation or had an identifiable InterPro protein domain. Many of these genes corresponded to certain molecular complexes or biological functions, including: (i) high-mobility-group (HMG) and Homeodomain transcription factors, (ii) kinases and phosphatases, (iii) proteosomal components and ubiquitin ligases, (iv) G-protein family, (v) membrane-associated proteins, and (vi) cellular enzymes (Fig. 2C).

Interestingly, among the 52 potential transcription factors identified in the screen, several contain HMG-box protein domains. In fact, the TCF/LEF-family of proteins that interact with β-cat in the nucleus to activate Wnt target genes, themselves encode HMG-transcription factors. Additionally, recent studies in *Xenopus* embryos have suggested that β-cat can physically interact with other HMG-box transcription factors, such as Sox family members (http://stke.sciencemag.org/cgi/cm/stkecm;CMN_6091), to regulate transcription of endodermal genes (*28*). Even though the specificity of these interactions in the Wnt pathway will have to be further tested, our results indicate that there may be other HMG-transcription factors that cooperate with β-cat in the regulation of downstream Wnt target genes.

Several members of the TBP-associated factors (Taf) family of transcription factors were identified. There is evidence from both *in vitro* studies in mammalian cells as well as *in vivo* studies in *Drosophila* that β-cat physically interacts with TATA-Binding Protein (TBP; http://stke.sciencemag.org/cgi/cm/stkecm;CMN_6040) and that there are other co-factors such as Reptins (http://stke.sciencemag.org/cgi/cm/stkecm;CMN_6798) and Pontins (http://stke.sciencemag.org/cgi/cm/stkecm;CMN_6037) that interact with both TBP and β-cat to antagonistically regulate Wnt target gene activity (*29*, *30*). Intriguingly, dsRNA knockdown of most Tafs led to an increase in Wnt-reporter activity suggesting that Tafs might contribute to repression of Wnt target genes. Other classes of transcription factors identified in the screen include several Homeodomain-containing and HOX genes. There is precedence for cross-talk between Wnts and Homeodomain or Hox transcription factors. HOXB13 inhibits TCF-4-mediated Wnt signaling activity in prostate cells by decreasing expression of Tcf-4 and its target genes (*31*). On the other hand, zebrafish *wnt8* transcriptionally regulates *vent* and *vox* genes encoding two homeodomain transcription factors in the establishment of the ventral pattern in the early embryo (*32*).

Protein phosphorylation and dephosphorylation by protein kinases and phosphatases have been especially implicated in the regulation of β-cat protein stability and degradation (*6–8*). Recent studies have also suggested that Wnt signaling stimulates and requires the phosphorylation of Lrp5/6/Arrow intracellular domain (PPPSP motif) to create an inducible docking site for Axin, a scaffolding protein controlling β-cat stability (*33*). We identified several protein kinases that negatively or positively affected the activity of the Wnt reporter gene. These include genes that encode known members of the pathway such as *ck1α* (http://stke.sciencemag.org/cgi/cm/stkecm;CMN_5895) genes whose function in the Wnt pathway has not been previously recognized- such as *warts* and *platelet-derived growth factor* (*PDGF*)*-and vascular endothelial growth* factor (VEGF)*-receptor related* (*pvr*); and genes encoding kinases that have no annotated function (see table S1A).

We also identified a class of "ARM (Armadillo)-repeat" containing proteins. The ARM-repeat protein motif was first identified in the *Drosophila arm* gene and is an approximately 40 amino acid-long tandemly repeated sequence motif. ARM-repeat proteins function in various processes, including intracellular signaling and cytoskeletal regulation, and include such proteins as β-cat, the junctional plaque protein plakoglobin, the APC tumor suppressor protein, and the nuclear transport factor importin-alpha.

These repeats have a key role in mediating protein-protein interactions between β-cat and other important regulators of the Wnt pathway (*34*). Importantly, a subset of these proteins is conserved across eukaryotic kingdoms. Taken together, our results indicate that there are likely to be additional ARM-repeat proteins that participate in the regulation of the Wnt pathway.

Additionally, we used "Reciprocal-Best-BLAST" (RBB) and other BLAST protocols to identify potential human homologs of the genes identified in the screen (for details, see table S2). These analyses indicated that more than 50% of the genes identified in the RNAi screen have vertebrate orthologs, suggesting their potential conserved role in the Wnt signaling pathway across evolution (see Fig. 2C and Figs. 4 and 5 for functional validation in mammalian cells). To test whether the genes identified in the screen were involved in the regulation of the Wnt pathway in multiple cell types, we performed the reporter assay for the selected candidate genes in multiple *Drosophila* cell lines including S2R+ and Kc167 cells (table S3). Of the 200+ genes, we found ~140 genes that appear to regulate Wnt signaling activity in two or more cell types. Our analysis suggests that a majority of the candidate genes are not clone8 cell-type specific but are more generally required for the modulation of the Wnt signaling pathway in multiple cell types.

Secondary screens. A challenge presented by any high-throughput primary screen is to extract meaningful information from the list of candidate genes. One useful approach is to categorize groups of genes according to their putative function in specific secondary assays that can be designed based on previous knowledge of the signaling pathway. To accomplish that for the Wnt screen, we ordered the candidate genes in the Wnt pathway in an epistatic relationship according to their roles at various steps in the pathway in relation to known negative and positive regulators of the pathway (Fig. 3).

To conduct epistasis experiments we activated the signaling pathway by either transfecting individual DNA constructs encoding activators of the pathway (Wg, ΔNlrp6, Dsh (http://stke.sciencemag.org/cgi/cm/stkecm;CMN_5545) or β-cat; see Fig. 3, A–D) along with the reporter gene or by dsRNA-mediated inhibition of known negative regulators (Axin, CK1alpha; see Fig. 3, E and F, or Slmb http://stke.sciencemag.org/cgi/cm/stkecm;CMN_5854).We used RNAi to reduce expression of individual candidate genes during simultaneous activation of the pathway by different inducers. We used both Wg-reporters (fig. S1A) in our secondary screens for independent confirmation of our assays. Simultaneous expression of dsRNA for known downstream positive regulators together with genes encoding activators of the pathway inhibited reporter activation. For example, activation of the pathway by overexpression of Dsh was blocked by RNAi knockdown of genes encoding downstream effectors (*arm, pan, pygo* or *lgs*) but not that of upstream pathway members encoding the ligand-receptor complex (*wg, arr, fz* or *fz4*) (Fig. 3C). Alternatively, ectopic activation of the reporters that occurred after dsRNA-mediated knockdown of negative regulators (such as *axin* or *ck1α*), could be efficiently inhibited by RNAi of downstream positive regulators (such as *arm* or *pan*) but not by dsRNAs directed towards components (such as *wg, arr* or *fz*), that act upstream of *axin* and *ck1α* (Fig. 3, E and F).

These results allowed us to tentatively place selected candidate genes in a hierarchy either upstream or downstream of known positive and negative regulators. Figure 3, G–I, represents specific examples of three potential regulators that we identified in the screen, including two known transcription factors, DP (Dimerization Partner) and Lilli (Lilliputian), and a novel gene CG5402, as activators in the Wnt pathway in the primary screen. *In vitro* epistasis experiments in cl8 cells placed each of the three candidate genes at three distinct steps in the pathway (Fig. 3, G-I). CG5402 acts upstream of Axin but downstream of Wg, Fz or Arr (Fig. 3I); DP functions downstream of Axin and Ck1α but upstream of β-cat (Fig. 3G); and Lilli functions downstream of β-cat (Fig. 3H). It is interesting to note that *lilli* encodes an HMG-box transcription factor. *lilli* has also been shown to genetically interact with *arm* thus further corroborating its role in the Wnt pathway (*35*). It is important to note that *lilli* interacts genetically with members of several signaling pathways including the receptor tyrosine kinase (RTK)/Ras and the Decapantaplegic (Dpp) pathway, thus underscoring the power of the RNAi approach in assigning functions to genes with pleiotropic functions that may be critical factors involved in cross-talk between multiple signaling pathways (*36*, *37*).

Overall our epistasis analysis of the potential positive regulators in the cl8 cells failed to place any new gene between Wg-Fz-Arr ligand-receptor complex and Dsh (R. DasGupta and N. Perrimon, unpublished data), even though known intermediates such as Arr and Fz were placed between Wg and Dsh by this method (Fig. 3, A-D and J). Preliminary epistasis analysis of most genes encoding potential positive regulators revealed that they affect the pathway downstream of Dsh. These genes were further categorized into those that acted upstream or downstream of genes involved in phosphorylation or degradation of β-cat (Axin, Ck1α and Slimb) and those that acted downstream of β-cat (R. DasGupta and N. Perrimon, unpublished data). Altogether, the *in vitro* epistasis studies provide a starting point from which to investigate the mechanism of action of candidate genes identified in the screen.

The candidate genes that increased reporter activity when their expression was inhibited were further tested in order to categorize them into specific functional groups. First we

determined if RNAi of potential negative regulators could ectopically activate the TOP-Flash reporter in the absence of Wg stimulus. Of the 129 negative regulators tested, 63% (83/129) activated reporter activity upon dsRNA-mediated knockdown, suggesting a potential role in the regulation of basal Wg-activity in a cell (table S4). Genes in this category could be either directly or indirectly acting at the level of regulation of Arm/β-cat stability and/or phosphorylation or at the level of target gene regulation. RNAi knockdown of the remaining 47 genes promoted expression of the TOP-Flash reporter only in the presence of Wg, suggesting a role specifically in Wg-stimulated cells. This second class of genes could be functioning either at the level of ligand-receptor regulation, receptor-mediated endocytosis or they may be involved in the regulation of the stable pool Arm/β-cat that is present only in a stimulated cell. In fact this class includes known regulators such as *nkd* and *Dlp*, which have been shown to regulate the intracellular and extracellular trafficking of Wg, respectively (*38–40*).

We tested whether decreased expression of "candidate" negative regulators required downstream effectors such as Arm and Pan to activate the Wnt–β-cat responsive-reporter gene (fig. S3). We transfected cells with *arm* or *pan* dsRNA together with individual dsRNAs specific for selected negative regulators. With the exception of two genes, *CR31616* and *CG4699*, Arm and Pan were indeed required for activation of the TOP-Flash reporter (in the absence of Wg-stimulus), thus placing them epistatically downstream of most negative regulators (fig. S3).

***In vivo* validation of hits in *Drosophila*.** To further test the relevance of the genes identified as potential regulators of the Wnt pathway, we overexpressed selected candidates in cells in culture and in *Drosophila* wing imaginal discs *in vivo* (Fig. 4). One of the candidate genes encoded the small GTPase Rab5 (*41*, *42*). Rab5 has a central role in early endocytic trafficking by directing the budding of endocytic vesicles from the plasma membrane, their movement along microtubules, and their fusion with sorting endosomes. Rab5 has been implicated in controlling the shape of the long-range gradient of the transforming growth factor superfamily member, *Decapentaplegic* (*Dpp*), in the *Drosophila* wing by regulating the endocytosis of ligand-receptor complex (*43*). Interestingly, Rab5-interacting proteins, such as APPL1 and APPL2, and other proteins involved in the formation of Clathrin-Coated Vesicles (CCVs) (such as Eps15, Epsin and β-arrestin2), can undergo nucleo-cytoplasmic shuttling and can interact with nuclear transcription factors to regulate expression of target genes (*44*, *45*). These studies indicate that the endocytic machinery may be directly involved in nuclear signaling functions as well (*45*).

In our screen, RNAi-mediated depletion of Rab5 only promoted reporter activity if cells were also stimulated with Wg (table S4 and Fig. 4A). Conversely, co-transfection of increasing amounts of Rab5 cDNA together with the Wg cDNA in *Drosophila* cells displayed a dose-dependent repression of Wg-mediated TOP-flash reporter activity (Fig. 4B). The effect on STF-reporter activity in mammalian 293T cells upon Rab5 overexpression and siRNA-mediated knockdown yielded results that were similar to those obtained in fly cells in culture (Fig. 4C).

In order to assess whether Rab5 could similarly affect Wg-signaling *in vivo* we used the GAL4-UAS system to drive the expression of wild type *rab5* in the *Drosophila* wing imaginal disc using a specific wing-margin driver, C96-GAL4 (Fig. 4, D and E). We monitored the expression of *senseless*, a proneural gene that is a target of the Wg signaling pathway at the wing margin (straddling the dorsal-ventral boundary) as a readout for pathway activity. Overexpression of Rab5 (C96GAL4-UASRab5WT) resulted in a partial to complete loss of *senseless* expression at the wing margin (Fig. 4D, arrowheads) compared to that in control discs (C96GAL4). Expression of *wg* itself was not affected (Fig. 4D). Nor was expression of *senseless* in the proneural clusters at the distal regions of the wing pouch (Fig. 4D, arrows). Since Rab5 has been implicated in receptor-mediated endocytosis and degradation of morphogenetic signals, we thought over-expression of Rab5 might influence endocytosis of the endogenous Wg protein and thus alter signaling activity at the plasma membrane but antibody-staining against extracellular-Wg revealed no difference in the levels of secreted Wg protein between regions that displayed high and low levels of *senseless* expression (Fig. 4E, inset). Thus Rab5 appears to have a role in the control of Wg-signaling activity in which it acts to inhibit Wg-dependent activation of target genes.

Our observations suggest that overexpression of Rab5 does not affect the extracellular distribution of Wg protein per se. It is possible that Rab5 could be perturbing the distribution of the receptors and co-receptors Fzd2 and Arrow (Lrp6). However any significant change in the distribution of receptors is unlikely based on our analysis of extra-cellular Wg and previous studies that have demonstrated the role of Frizzled-2 receptor in regulating extracellular distribution of Wingless and shaping the Wg-gradient in the wing imaginal disc (*46*). Nonetheless, we cannot rule out subtle changes at the level of receptors/co-receptors. Alternatively, Rab5 could be regulating trafficking of the stabilized pool of Arm/β-cat, which is present only in a Wg-induced cell and thus affecting the downstream Wingless-readout as judged by antibody staining for Senseless.

Validation of mammalian orthologs in 293T cells and the zebrafish embryo. All major components of the Wnt pathway are conserved in metazoans. To determine if the *Drosophila* genes newly identified in the RNAi screen are *bona fide* components of the conserved Wnt-Wg pathway we

tested their signaling activity and functions in vertebrates. We used gain- and loss-of-function of selected vertebrate orthologs of *Drosophila* genes to assess effects on Wnt signaling in human cells and in developing zebrafish embryos.

We cloned multiple human orthologs and performed Wnt–β-catenin-responsive reporter assays in human embryonic kidney (HEK) 293T cells. Transfection of plasmids encoding human Lats (also called Warts in *Drosophila*), a serine-threonine kinase, cyclin dependent kinase 2 (CDC2), or Axin1 (as a control) inhibited the ability of Wnt-3a to activate the Wnt–β-catenin responsive-reporter STF16 in 293T cells (Fig. 5A). Conversely, expression of the human ortholog of CG4136, a pair-like homeobox gene, activated the Wnt-pathway in the presence or absence of Wnt-3A (Fig. 5A). We also generated 3 to 4 short-interfering RNAs (siRNAs) against human Lats1, Lats 2, CDC2, and CG4136. Transfection of plasmids encoding pools of siRNA for β-catenin inhibited Wnt-3A activation of STF16 as expected (Fig. 5B). Transfection of pools of siRNAs for Axin1 and 2, Lats1, Lats2, both Lats1 and 2, and CDC2 all increased basal Wnt–β-catenin-responsive reporter activity and synergized with Wnt-3A activation of the reporter (Fig. 5B). Pools of siRNA for human CG4136 had no effect on activation of the Wnt–β-catenin-responsive reporter (Fig. 5B), though one caveat is that 293T cells may not express human CG4136 (HuCG4136). Since the gain of function of HuCG4136 gave a strong activation of the Wnt–β-catenin responsive-reporter it is clear that it can regulate Wnt–β-catenin signaling and strongly implicates it as a new positive regulator of Wnt-signaling in vertebrates. Since all of the vertebrate orthologs tested thus far affect Wnt–β-catenin signaling, we are in the process of generating expression constructs and siRNAs for multiple additional human orthologs to test their roles in Wnt signaling (R. T. Moon and A. Kaykas, data not shown).

To determine if any of the vertebrate orthologs function in the Wnt pathway *in vivo* at the organismic level we performed both gain- and loss-of-function assays for some of the genes that we had validated in 293T cells. For gain-of-function, 1-cell stage zebrafish embryos were injected with RNAs encoding HuCG4136, *wnt-8* (as a positive control), and GFP (as a negative control). Embryos injected with GFP (10 ng) developed normally (Fig. 5C, panels a-c). However, embryos injected with *wnt-8* RNA (1 or 10 ng) developed anterior truncations and had either small eyes or no eyes in the majority on injected embryos (Fig. 5C, panels d-i) (*47*). Some of the 10 ng-injected embryos also had a defect in notochord formation (see table in Fig. 5C). Injection of RNA encoding HuCG4136 phenocopied injection with *wnt-8* albeit at higher doses of RNA (Fig. 5C, panels j-o). This coupled with the reporter data in 293T cells indicates that HuCG4136 activates Wnt–β-catenin signaling. Injection of RNA encoding Hulats1 or CDC2 had no obvious phenotype in zebrafish. However, depletion of zebrafish Lats1 by injection of antisense morpholino oligonucleotides gave a severe phenotype and the embryos arrested before epiboly (A. Kaykas and RT. Moon, unpublished data). A more detailed analysis of this phenotype will be required to determine if it is a consequence of altered Wnt signaling. . Taken together, the data from human cells and zebrafish strongly suggest that some of the hits from the *Drosophila* RNAi screen have a conserved role in Wnt–β-catenin signaling in vertebrates.

Conclusions. In the future, global understanding of the complexities of and interplay between multiple signaling pathways will rely upon the systematic identification and functional characterization of unexpected regulators of signal transduction cascades. This combined with powerful genetic and biochemical analyses of molecular mechanisms might lead to breakthroughs in the fields of development and disease biology. In the current study we present a whole-genome RNAi screen for the *Drosophila* Wnt-Wg signaling pathway, which in humans is implicated in hepatic, colorectal, breast and skin cancers, bone density syndromes, Alzheimer's Disease, and the retinal disease Familial Exudative Vitreoretinopathy (*48*). Even though the primary screen was done in *Drosophila* cells, the majority of identified pathway modulators appear to share a conserved role in the regulation of the Wnt-Wg pathway in multiple *Drosophila* cell types and in mammalian cells, as judged by the functional validation of their vertebrate orthologs in 293T cells. This combined with the fact that 18% of the candidate genes identified in the screen have disease-related human orthologs (Blast E value < 10^{-20}, fig. S4; http://superfly.ucsd.edu/homophila/ and table S5), underscores the potential broad applicability and importance of such screens in the future understanding and treatment of human disease. Finally, we demonstrated that selected hits from the RNAi screen function in Wnt-Wg signaling *in vivo* in both invertebrates (*Drosophila*) and vertebrates (zebrafish embryo). This approach has enabled us to assign new functions to previously known genes as well as to identify potential novel regulators of the Wnt pathway.

Although elucidating specific molecular mechanisms for selected candidate genes are beyond the scope of this study, our data strongly suggests that the RNAi-based screening in the *Drosophila* cell-based assay system is efficient in the identification of genes, and will have far-reaching consequences in the expansion of our understanding of the Wnt-Wg pathway. Future studies elucidating the molecular mechanism of individual candidate genes in multiple cell types and model organisms will shed light on the complexities and nuances of this important signaling pathway. Finally, the cross-comparison of whole-genome RNAi screens for multiple signaling pathways, and the identification of specific versus common regulators, will help us better understand the multifactorial processes that regulate the intricate steps of animal development and disease states.

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49. Materials and methods are available as supporting material on Science Online.
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Supporting Online Material

www.sciencemag.org/cgi/content/full/1109374/DC1
Materials and Methods
Fig. S1 to S4
Tables S1 to S5
References and Notes

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Fig 1. Wg-reporter assay: (**A**) Optimization of reporter assay in 384-well-plate format with both Wg and ΔNlrp6 as activators. dsRNA knockdown of known negative regulator, Axin, activates the reporter in uninduced cells whereas knockdown of control positive regulators such as Arm and dTCF represses Wg-induced activation of the TOP-Flash reporter. Note the further activation of the Wg-reporter upon dsRNA-mediated knockdown of Axin over and above Wg or ΔNlrp6-mediated induction of reporter. Knockdown of Gsk3β did not affect reporter activity in cl8 cells (1d) even though its knockdown resulted in activation of STF16 or dTF12 reporters in the absence of Wg-induction, in S2R+ cells (as above). (**B**) Schematic representation of 1A demonstrating the use of the reporter to screen for both positive and negative-regulators in a single assay.

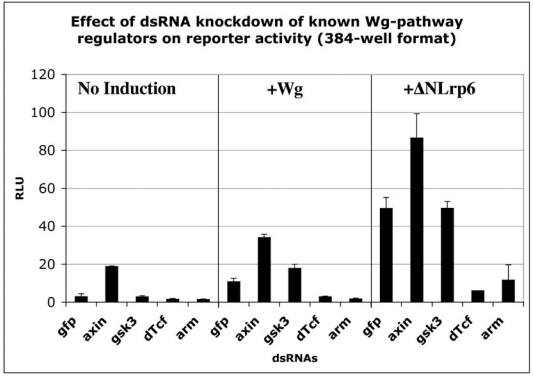
Fig 2. Data analysis for the Wg-screen: (**A**) XY-scatter plot comparison of Z-scores obtained from duplicate whole-genome screens, Screen #1 versus Screen #2. The comparison reveals a high correlation between the duplicate screens with most data points mapping to a diagonal line (blue) in the XY-scatter plot. The correlation coefficient between the two screens was 0.63. Data points within the blue oval were considered to be candidate genes that act as potential positive regulators of the Wg-pathway in Clone 8 cells whereas the ones within the red oval were considered potential negative regulators. (**B**) Scatter plot of two representative plates that contained several of the known positive (blue) and negative (red) regulators of the pathway with respect to other data points and the controls from cells expressing arm dsRNA (red dots). (**C**) Pie-chart representation of candidate genes obtained from the screen as potential regulators of the Wg-pathway based on their "Gene Ontology" and molecular function or protein domains. (**D**) Pie chart representing the percentage of total number of candidate genes obtained from the Wg-screen that have potential vertebrate orthologs, as judged by Reciprocal Best Blast (details in table S2).

Fig 3. Epistasis analysis of selected candidate genes in Clone8 cells: (**A-F**) Histograms showing reporter gene activity in cells expressing indicated dsRNAs and various activators or inducers of the pathway such as coexpression of positive regulators such as Wg (A), ΔNLrp6 (B), Dsh (C) or S37Aβ-cat cDNA (D) or dsRNA-mediated knockdown of negative regulators such as Axin (E) or Ck1α (F). Y-axis represents relative luciferase activity with reporter activity of cells containing GFP dsRNA and the inducer scaled to 100 units. (**G-I**) Effect of dsRNA-mediated knockdown of 3 selected candidate genes on TOPFlash reporter activity in clone8 cells including DP transcription factor, Lilli and CG5402 upon induction of the pathway. (**J**) Schematic representation of epistatic ordering of the selected candidate genes in G-I and positive controls in A-F.

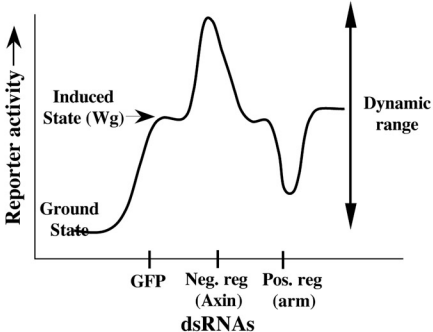
Fig 4. Effect of Rab5 RNAi and overexpression *in vitro* and *in vivo* on the Wg-signaling pathway: (**A**) Effect of Rab5 knockdown on reporter activity in control cells (left) or cells expressing Wg (right). (**B**) Rab5 overexpression in S2 cells results in a dose-dependent repression of Wg-induced reporter activity. (**C**) Effect of Rab5 overexpression and siRNA knockdown in mammalian 293T cells. (**D**) Effect of overexpression of WT Rab5 (upper panels) in the wing margin of the larval imaginal disc on expression of Senseless (Sens) or Wg. Control discs (lower panel). (**E**) Failure of Rab5 overexpression to change amounts of extracellular Wg protein in regions of diminished Senseless expression. DAPI, (4'-6-Diamidino-2-phenylindole) which form fluorescent complexes with natural double-stranded DNA, was used to mark the nuclei of cells in the imaginal discs.

Fig 5. Validation of candidate genes in mammalian cells and zebrafish: (**A**) (Upper panel) Effect of overexpression of the human versions of Axin, CG4136, Lats1, and CDC2 on the activation of 16XSuperTopFlash in 293T cells. (Lower panel) Same as above except with activation of the pathway with Wnt3A conditioned media (3ACM). (**B**) (Upper panel) Effect of knock down of the human versions of β-catenin, Axin, GC4136, Lats1, Lats2, and CDC2 on the activation of 16XSuperTopFlash in 293T cells. (Lower panel) Same as above except with activation of the pathway with Wnt3A conditioned media (3ACM). (**C**) Effect of overexpresssion of zebrafish Wnt8 ORF1 (panels d-i) or Human CG4136 (panels j-o) mRNA on the development of zebrafish at 48 hours post fertilization. A couple of lateral views are shown to highlight the loss of anterior structures, such as the eye, in both Wnt8 and CG4136 injected embryos (arrowheads in 5C, panels i and l) as compared to wild type eyes in the gfp mRNA injected embryos (arrow in 5C, panel c). (Right) Table of the various phenotypes of zebrafish injected with Wnt8 ORF1 or CG4136 mRNA.

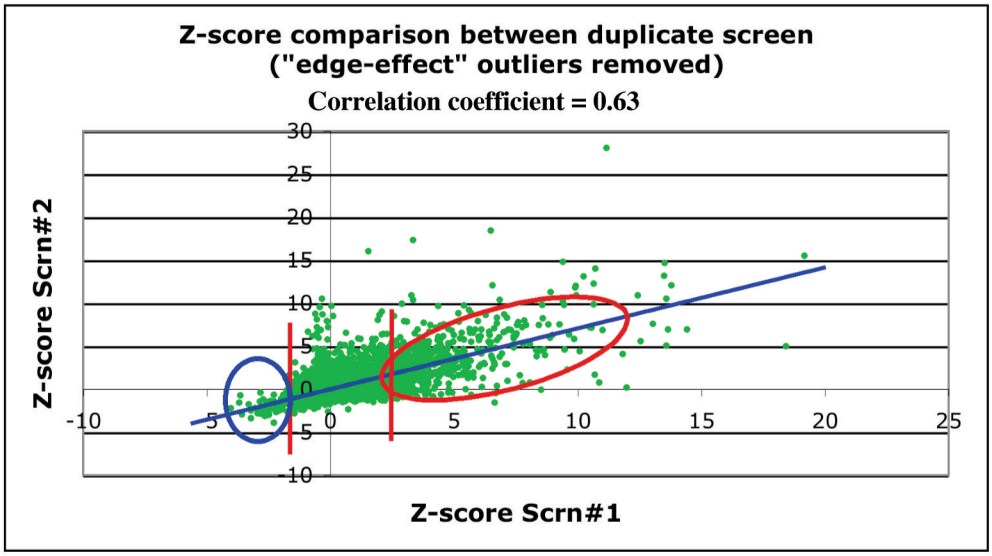
A


Effect of dsRNA knockdown of known Wg-pathway regulators on reporter activity (384-well format)
No Induction
+Wg
+ΔNLrp6
RLU
120
100
80
60
40
20
0
gfp
axin
gsk3
dTcf
arm
dsRNAs

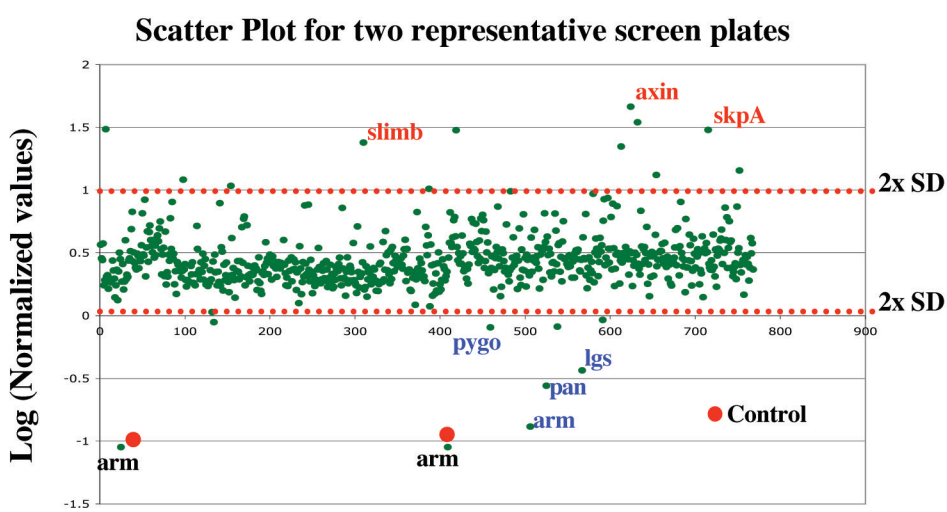
B


Reporter activity
Induced State (Wg)
Ground State
Dynamic range
GFP
Neg. reg (Axin)
Pos. reg (arm)
dsRNAs

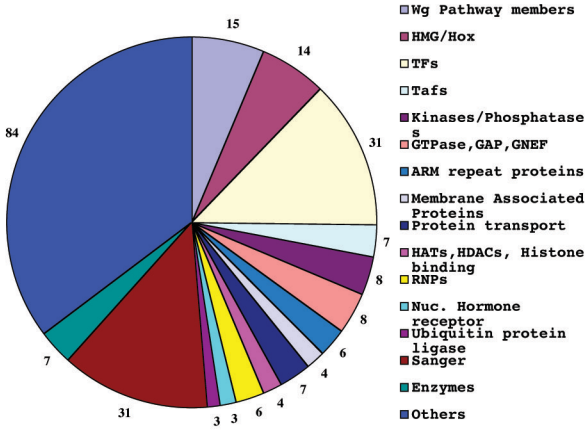
A


Z-score comparison between duplicate screen
("edge-effect" outliers removed)
Correlation coefficient = 0.63
Z-score Scrn#2
Z-score Scrn#1
30
25
20
15
10
5
0
-5
-10
-10
-5
0
5
10
15
20
25

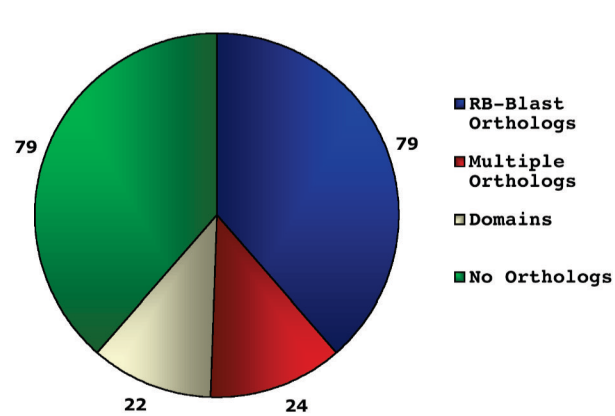
B

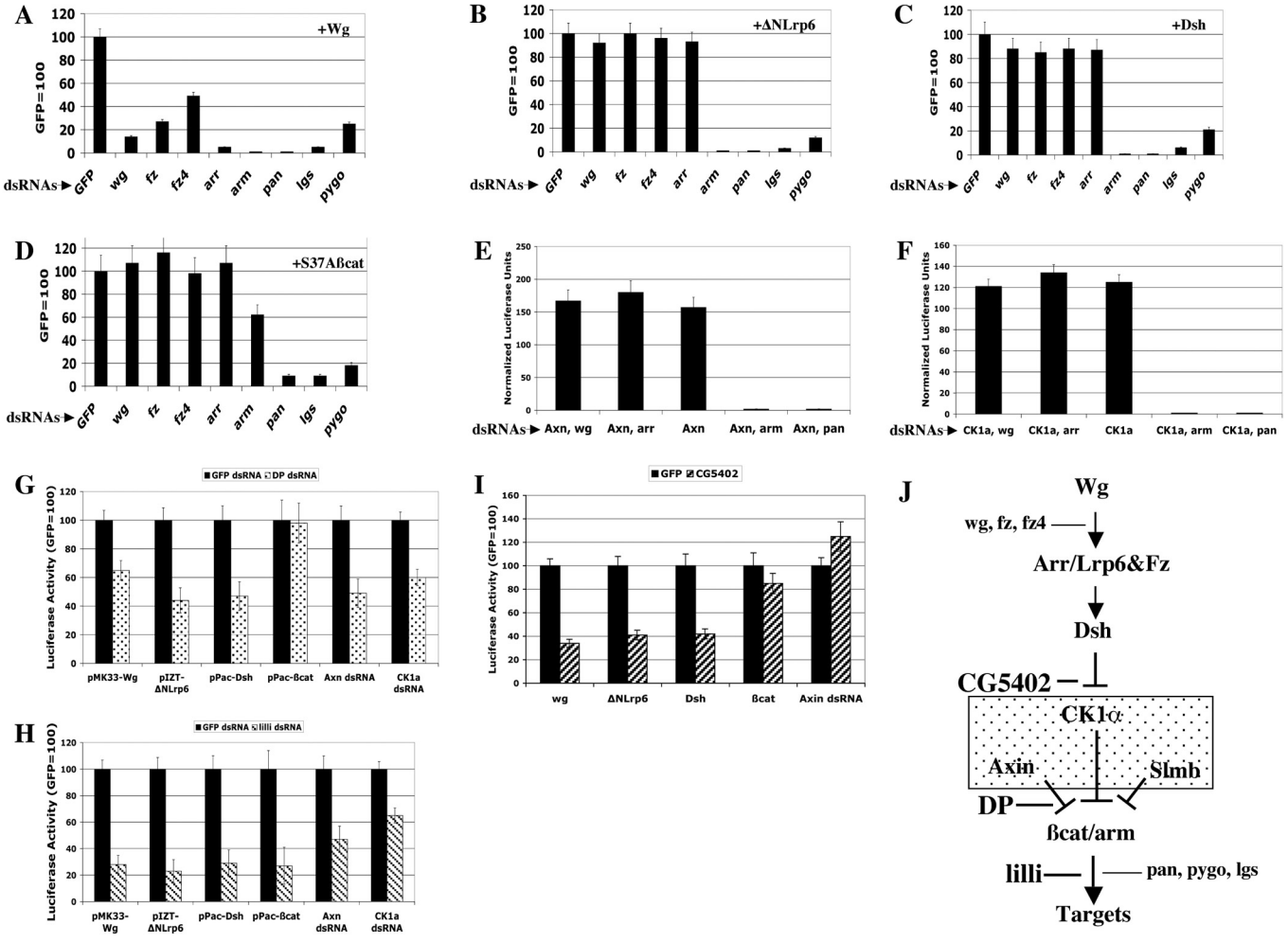

Scatter Plot for two representative screen plates
Log (Normalized values)
axin
skpA
slimb
2x SD
2x SD
pygo
lgs
pan
arm
Control
arm
arm
2
1.5
1
0.5
0
-0.5
-1
-1.5
0
100
200
300
400
500
600
700
800
900

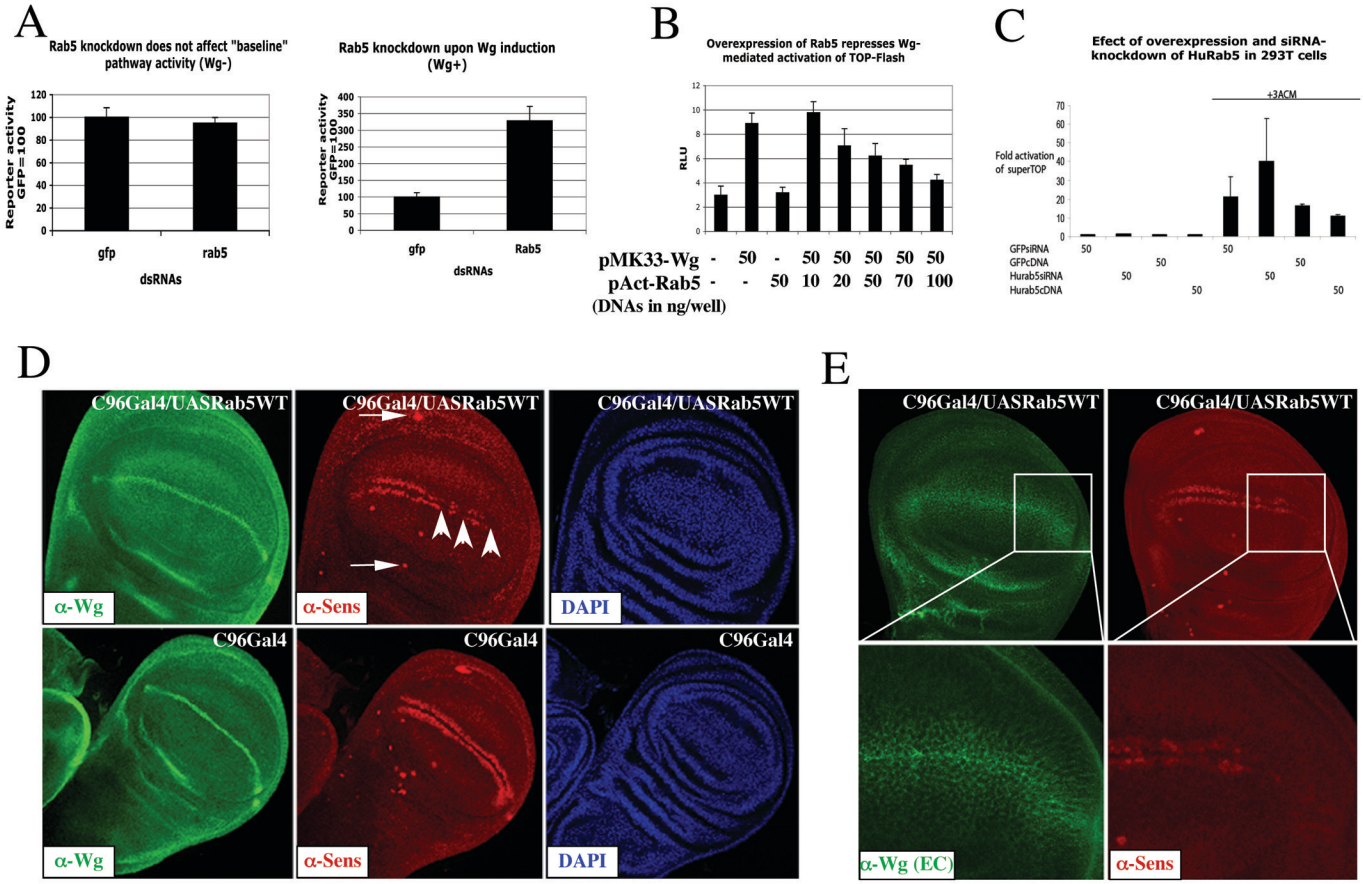
C

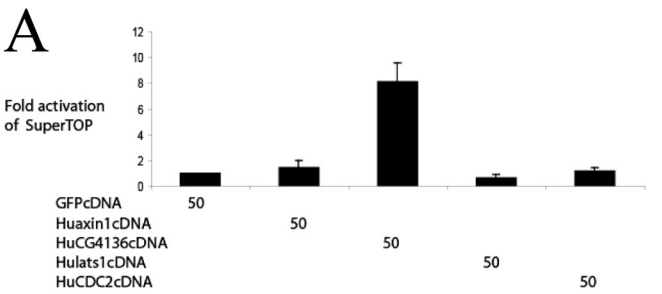

Primary Screen (duplicates)
15
14
31
7
8
8
6
4
7
4
6
3
3
31
7
84
Wg Pathway members
HMG/Hox
TFs
Tafs
Kinases/Phosphatases
GTPase,GAP,GNEF
ARM repeat proteins
Membrane Associated Proteins
Protein transport
HATs,HDACs, Histone binding
RNPs
Nuc. Hormone receptor
Ubiquitin protein ligase
Sanger
Enzymes
Others

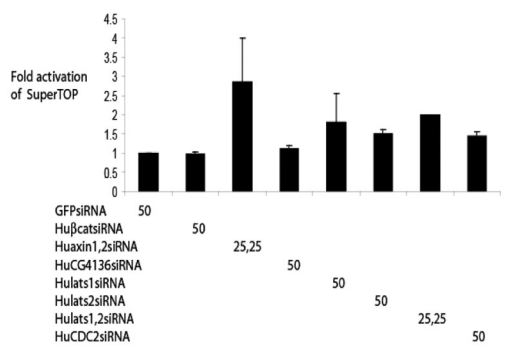
D

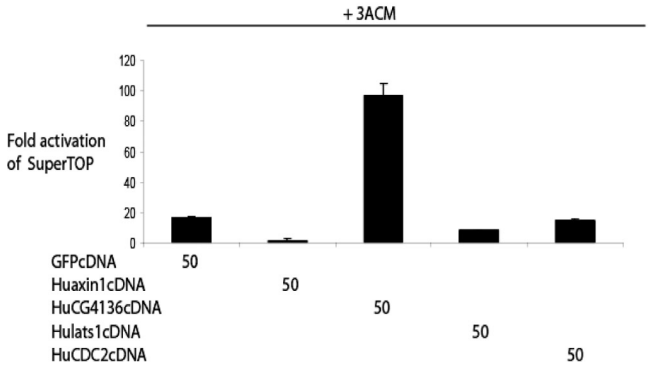

Vertebrate orthologs
79
79
22
24
RB-Blast Orthologs
Multiple Orthologs
Domains
No Orthologs

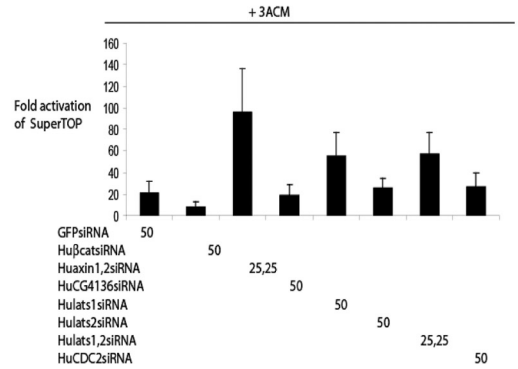

A
+Wg
GFP=100
dsRNAs ► GFP wg fz fz4 arr arm pan lgs pygo
B
+ΔNLrp6
GFP=100
dsRNAs ► GFP wg fz fz4 arr arm pan lgs pygo
C
+Dsh
GFP=100
dsRNAs ► GFP wg fz fz4 arr arm pan lgs pygo
D
+S37Aßcat
GFP=100
dsRNAs ► GFP wg fz fz4 arr arm pan lgs pygo
E
Normalized Luciferase Units
dsRNAs ► Axn, wg Axn, arr Axn Axn, arm Axn, pan
F
Normalized Luciferase Units
dsRNAs ► CK1a, wg CK1a, arr CK1a CK1a, arm CK1a, pan
G
GFP dsRNA DP dsRNA
Luciferase Activity (GFP=100)
pMK33-Wg pIZT-ΔNLrp6 pPac-Dsh pPac-ßcat Axn dsRNA CK1a dsRNA
H
GFP dsRNA lilli dsRNA
Luciferase Activity (GFP=100)
pMK33-Wg pIZT-ΔNLrp6 pPac-Dsh pPac-ßcat Axn dsRNA CK1a dsRNA
I
GFP CG5402
Luciferase Activity (GFP=100)
wg ΔNLrp6 Dsh ßcat Axin dsRNA
J
Wg
wg, fz, fz4
Arr/Lrp6&Fz
Dsh
CG5402
CK1α
Axin
Slmb
DP
ßcat/arm
lilli
pan, pygo, lgs
Targets

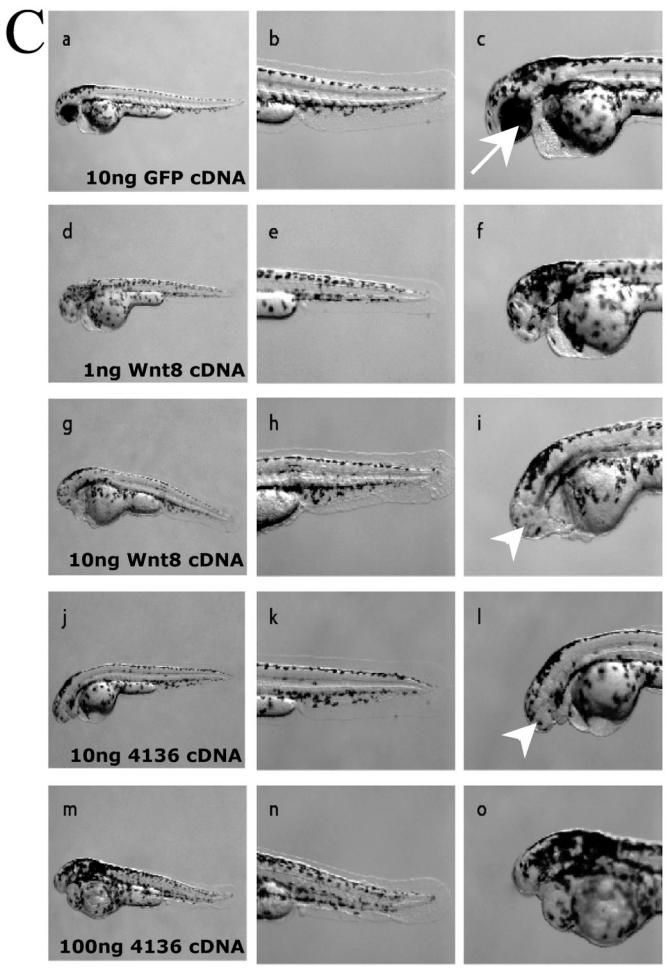

A
Rab5 knockdown does not affect "baseline" pathway activity (Wg-)
Reporter activity GFP=100
120
100
80
60
40
20
0
gfp
rab5
dsRNAs
Rab5 knockdown upon Wg induction (Wg+)
Reporter activity GFP=100
400
350
300
250
200
150
100
50
0
gfp
Rab5
dsRNAs
B
Overexpression of Rab5 represses Wg-mediated activation of TOP-Flash
RLU
12
10
8
6
4
2
0
pMK33-Wg - 50 - 50 50 50 50 50
pAct-Rab5 - - 50 10 20 50 70 100
(DNAs in ng/well)
C
Efect of overexpression and siRNA-knockdown of HuRab5 in 293T cells
+3ACM
Fold activation of superTOP
70
60
50
40
30
20
10
0
GFPsiRNA 50 50 50
GFPcDNA 50 50
Hurab5siRNA 50 50
Hurab5cDNA 50 50
D
C96Gal4/UASRab5WT
α-Wg
C96Gal4/UASRab5WT
α-Sens
C96Gal4/UASRab5WT
DAPI
C96Gal4
α-Wg
C96Gal4
α-Sens
C96Gal4
DAPI
E
C96Gal4/UASRab5WT
C96Gal4/UASRab5WT
α-Wg (EC)
α-Sens











Phenotype	GFP (10ng)	Wnt8 (1ng)	Wnt8 (10ng)	4136 (10ng)	4136 (100ng)
wild-type	60(97%)	5(12%)	2(3%)	2(3%)	5(10%)
small eyes	2(3%)	10(27%)	3(2%)	10(18%)	1(2%)
no eyes	0(0%)	27(64%)	55(92%)	45(79%)	43(88%)
Notochord defect	0(0%)	2(5%)	12(20%)	1(2%)	5(10%)