

# 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, prove to be a rapid means of identifying regulators of signaling pathways implicated in development and disease.

Wnt proteins are a family of conserved signaling molecules involved in a plethora of fundamental developmental and cell biological processes such as cell proliferation, differentiation, and 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 that activate receptor-mediated pathways (6), which lead 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 ( $\beta$ -cat) [called Armadillo (Arm) in *Drosophila*], which is otherwise degraded by the proteasome pathway. Initially identified as an important player in stabilizing cell-cell adherens junctions,  $\beta$ -cat is now known to participate in transcriptional regulation by forming a complex with the T cell-specific transcription factor (TCF) and lymphoid enhancer-binding factor (LEF) families of high-mobility-group (HMG)-box transcription factors (7, 8). In cells stimulated by Wnts, stabilized  $\beta$ -cat translocates to the

nucleus, where, together with TCF/LEF transcription factors, it activates downstream target genes (7, 8). The Wnt pathway can also be activated through inhibition of its negative regulators such as glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ), adenomatous polyposis coli (APC), and Axin, which promote degradation of  $\beta$ -cat, or by the introduction of activating mutations in  $\beta$ -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 (9, 10).

**Whole-genome RNA interference screens.** Genetic and biochemical approaches have identified many of the genes that regulate the Wnt-Wg pathway in *Drosophila* (11) and other model organisms. However, many components may remain unidentified if mutants do not display a distinguishable “Wnt phenotype.” Indeed, it is estimated that only 25% of all known *Drosophila* genes are associated with a readily obvious phenotype (12–15). The availability of the *Drosophila* genome sequence, a well-established RNA interference (RNAi)-based screening technology, and the fact that ~75% of the fly genome remains uncharacterized, provided us with an opportunity to rapidly and systematically characterize gene function at a genomewide scale to find new components in the Wnt signaling pathway (16–19).

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, as well as 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 (20, 21). The screen was performed in *Drosophila* imaginal disc-derived clone 8 cells, which are epithelial in origin (22, 23). The Wg pathway is active in the imaginal discs, and thus clone 8 cells are likely to contain the majority of the components required to respond to Wg (24). The assay involved transfection of the TOP-Flash reporter, along with a *Renilla* luciferase vector (PoIII-RL) as a control for transfection efficiency, and an expression vector encoding *wg* (pMK33-*wg*) to stimulate the pathway (24–27) (see fig. S1). The activity of the Wg signaling pathway was quantified by measurement of normalized (N) luciferase expression or relative luciferase activity units (RLUs), 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-to-noise ratio in the high-density screen format (fig. S2) (28). STF16 comprises 16 TCF-binding sites and a minimal TATA box from the thymidine kinase 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 the reporters exhibited different basal activities, both allowed use of small volumes of cells and transfection reagents and displayed strong signal-to-noise ratios in multiple *Drosophila* cell lines including clone 8 and S2 receptor-positive (S2R<sup>+</sup>) cells (24) (fig. S2B). Both reporters were expressed in a robust fashion after pathway stimulation by Wg, as well as by downstream activators in the pathway such as a  $\Delta$ NLrp6, a constitutively active form of the Wg coreceptor low-density lipoprotein (LDL)

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receptor-related protein-6 (LRP6) (29) (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 *Drosophila* TCF (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 when induced by Wg or LRP6 (Fig. 1A; 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) (28), we used a library of ~22,000 double-stranded RNAs (dsRNAs) (30). The library represents >95% of genes in the *Drosophila* genome and has been used successfully in several screens (24, 26, 27, 31). 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, we analyzed the data from each individual plate with four distinct protocols, and we assigned candidate genes on the basis of their deviation from the plate average for each given criterion [see Methods (28)]. Genes that satisfied two or more statistical criteria were considered strong candidates; 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 were identified, including Wnt-wingless (*wg*) (32), *arrow* (*arr*)/LRP-6 (33), *frizzled* (*fz*) (34), *frizzled-4* (*fz4*), *dallylike protein* (*dlp*), *naked cuticle* (*nkd*) (35), *axin* (*axn*) (36), *supernumerary-limbs* (*slmb*) (37), *casein kinase 1 alpha* (*ck1a*), *disheveled* (*dsh*) (38),  *$\beta$ -catenin-armadillo* ( *$\beta$ -cat-arm*) (39), *dTCF/pangolin* (*dTCF/pan*) (40), the gene for *Drosophila* cAMP-responsive element-binding protein (CREB)-binding protein (*dCBP/nejire* (*nej*) (41), *pygopus* (*pygo*) (42), and *legless* (*lgs*) (43), thus underscoring the robustness and validity of the Wnt screen in this HTS format (Fig. 2B). Comparison of the z scores (which measure the number of standard deviations 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). Note that ~90% (213 out of 238) of the candidate genes that were selected for further analyses were verified in secondary screens (table S1A). About 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) HMG- and homeodomain-box transcription factors, (ii) kinases and phosphatases, (iii) proteosomal components and ubiquitin ligases, (iv) small GTPases (guanosine triphosphatases: monomeric guanine nucleotide-binding proteins) family, (v) membrane-associated proteins, and (vi) cellular enzymes (Fig. 2C).

Among the 52 potential transcription factors identified in the screen, several contain HMG-box protein domains. In fact, the proteins of the TCF/LEF family that interact with  $\beta$ -cat in the nucleus to activate Wnt target genes themselves encode HMG transcription factors. Additionally, recent studies in *Xenopus* embryos have

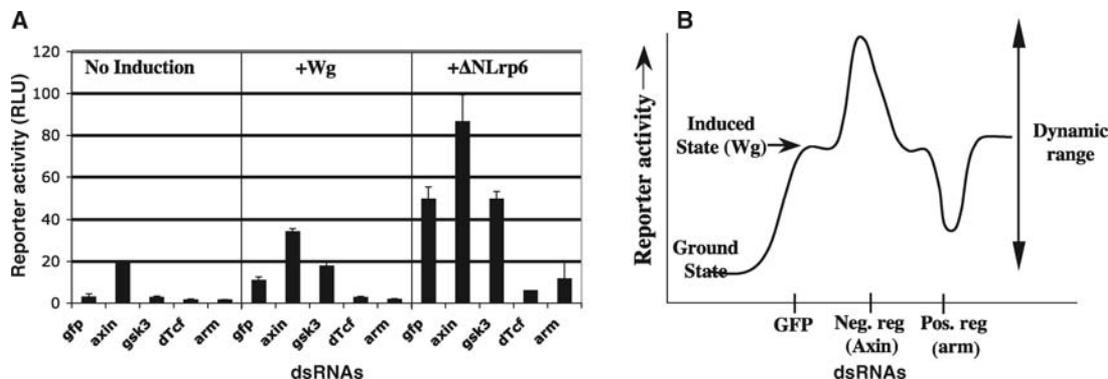
suggested that  $\beta$ -cat can physically interact with other HMG-box transcription factors, such as Sox family members, to regulate transcription of endodermal genes (44). Even though the specificity of these interactions in the Wnt pathway will have to be further tested, our results indicate that there may be other HMG transcription factors that cooperate with  $\beta$ -cat in the regulation of downstream Wnt target genes.

Several members of the TATA-binding protein (TBP)-associated factors (Taf) family of transcription factors were identified. There is evidence from both in vitro studies in mammalian cells and in vivo studies in *Drosophila* that  $\beta$ -cat physically interacts with TBP and that there are other cofactors such as Pontin and Reptin (Repressing Pontin) that interact with both TBP and  $\beta$ -cat to regulate Wnt target gene activity antagonistically (45, 46). Intriguingly, dsRNA knockdown of most Tafs led to an increase in Wnt reporter activity, which suggests 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 (47). 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 (48).

Protein phosphorylation and dephosphorylation by protein kinases and phosphatases have been especially implicated in the regulation of  $\beta$ -cat protein stability and degradation (7–9). Recent studies have also suggested that Wnt signaling stimulates and requires the phosphorylation of Lrp5 and 6-Arrow intracellular domain (PPPSP motif) to create an inducible docking site for Axin, a scaffolding protein controlling  $\beta$ -cat stability (49). We identified

**Fig. 1. Wg reporter assay. (A)**

Optimization of reporter assay in 384-well plate format with both Wg and  $\Delta$ NLrp6 as activators. dsRNA knockdown of the 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  $\Delta$ NLrp6-mediated induction of reporter. Knockdown of Gsk3 $\beta$  did not affect reporter activity in clone 8 cells(1d) even though its knockdown resulted in activation of STF16 or dTF12 reporters in the absence of Wg



induction, in S2R<sup>+</sup> cells (fig. S2). (B) Schematic representation of 1A demonstrating the use of the reporter to screen for both positive and negative regulators in a single assay.

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 *ck1a*; genes whose function in the Wnt pathway has not been previously recognized—such as *warts* and *PDGF- and VEGF-receptor related (pvr)* [platelet-derived growth factor and vascular endothelial growth factor, respectively]; and genes encoding kinases that have no annotated function (see table S1A).

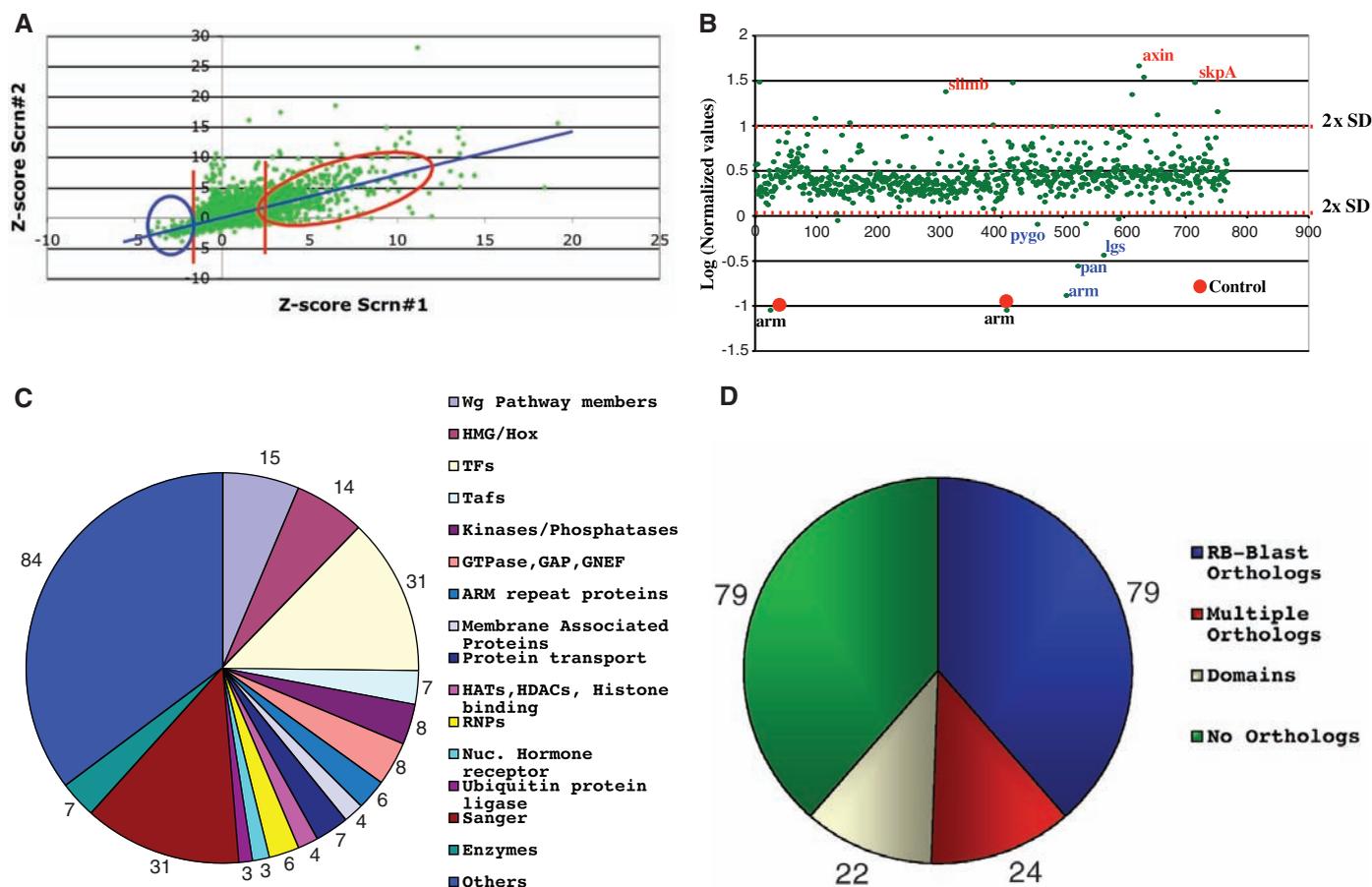
We also identified a class of proteins containing one or more Armadillo repeats (Arm repeats). The Arm-repeat protein motif was first identified in the *Drosophila arm* gene and is a tandemly repeated sequence motif about 40 amino acids long. Arm-repeat proteins function in various processes, such as intracellular signaling and cytoskeletal regulation, and include such proteins as  $\beta$ -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  $\beta$ -cat and other important regulators of the Wnt pathway (50). 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 >50% of the genes identified in the RNAi screen have vertebrate orthologs, which suggests their potential conserved role in the Wnt signaling pathway across evolution (see Fig. 2C and below 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 se-

lected candidate genes in multiple *Drosophila* cell lines including S2R<sup>+</sup> 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 is not specific to clone 8 cells but is 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 be able 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 on the basis of 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 relation according to their roles at various steps in the pathway in relation to



**Fig. 2.** Data analysis for the Wg screen. (A) Scatter plot comparison of z scores obtained from duplicate whole-genome screens, screen 1 versus screen 2. “Edge effect” outliers were removed. The comparison reveals a high correlation between the duplicate screens, with most data points mapping to a diagonal line (blue) in the 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) Candidate genes obtained from the primary screen as potential regulators of the Wg pathway based on their “Gene Ontology” and molecular function or protein domains. (D) The percentage of the 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).

known negative and positive regulators of the pathway (Fig. 3).

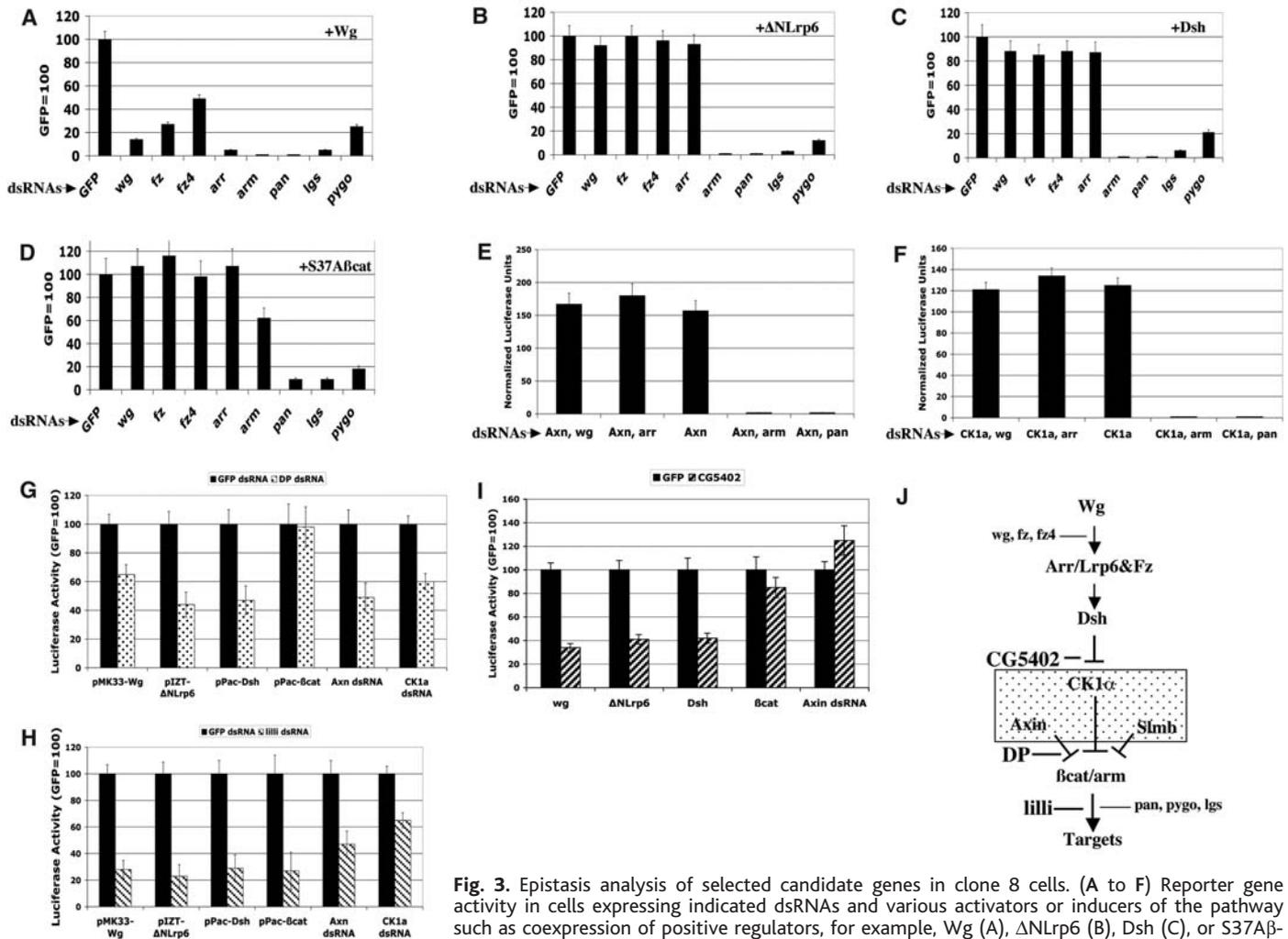
To conduct epistasis experiments, we activated the signaling pathway either by transfecting individual DNA constructs encoding activators of the pathway [Wg,  $\Delta$ NLrp6, Dsh, or  $\beta$ -cat (Fig. 3, A to D)] along with the reporter gene or by dsRNA-mediated inhibition of known negative regulators [Axin, CK1 $\alpha$  (Fig. 3, E and F), or Slimb]. We used RNAi to knock down 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 *ck1a*) could be efficiently inhibited by RNAi of downstream positive regulators (such as *arm* or *pan*) but not by dsRNAs directed toward components (such as *wg*, *arr*, or *fz*) that act upstream of *axin* and *ck1a* (Fig. 3, E and F).

These results allowed us tentatively to place selected candidate genes in a hierarchy either upstream or downstream of known positive and negative regulators. Specific examples of three potential regulators that we identified in the screen (Fig. 3, G to I) include 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 clone 8 cells placed each of the three candidate genes at three distinct steps in the pathway (Fig. 3, G

to I). *CG5402* acts upstream of Axin but downstream of Wg, Fz, or Arr (Fig. 3I); DP functions downstream of Axin and CK1 $\alpha$  but upstream of  $\beta$ -cat (Fig. 3G); and Lilli functions downstream of  $\beta$ -cat (Fig. 3H). It is interesting that *lilli* encodes an HMG-box transcription factor. *lilli* has also been shown to interact genetically with *arm*, which further corroborates its role in the Wnt pathway (51). It is important to note that *lilli* interacts genetically with members of several signaling pathways, including the receptor tyrosine kinase (RTK)/Ras and the Decapentaplegic (Dpp) pathway, which underscores 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 (52, 53).

Overall, our epistasis analysis of the potential positive regulators in the clone 8 cells failed to place any new gene between Wg-Fz-Arr ligand-receptor complex and Dsh (54), even though known intermediates such as Arr and



**Fig. 3.** Epistasis analysis of selected candidate genes in clone 8 cells. (A to F) Reporter gene activity in cells expressing indicated dsRNAs and various activators or inducers of the pathway such as coexpression of positive regulators, for example, Wg (A),  $\Delta$ NLrp6 (B), Dsh (C), or S37A $\beta$ -cat cDNA (D); or dsRNA-mediated knockdown of negative regulators such as Axin (E) or CK1 $\alpha$  (F). Relative luciferase activity units (RLU) with reporter activity of cells containing GFP dsRNA and the inducer scaled to 100 units. (G to I) Effect of dsRNA-mediated knockdown of three selected candidate genes on TOP-Flash reporter activity in clone 8 cells, including DP transcription factor, Lilli, and *CG5402*, after induction of the pathway. (J) Epistatic ordering of the selected candidate genes in G to I and positive controls in A to F.

Relative luciferase activity units (RLU) with reporter activity of cells containing GFP dsRNA and the inducer scaled to 100 units. (G to I) Effect of dsRNA-mediated knockdown of three selected candidate genes on TOP-Flash reporter activity in clone 8 cells, including DP transcription factor, Lilli, and *CG5402*, after induction of the pathway. (J) Epistatic ordering of the selected candidate genes in G to I and positive controls in A to F.

Fz were placed between Wg and Dsh by this method (Fig. 3, A to 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  $\beta$ -cat (*axin*, *ck1a*, and *slmb*) and those that acted downstream of  $\beta$ -cat (54). 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.

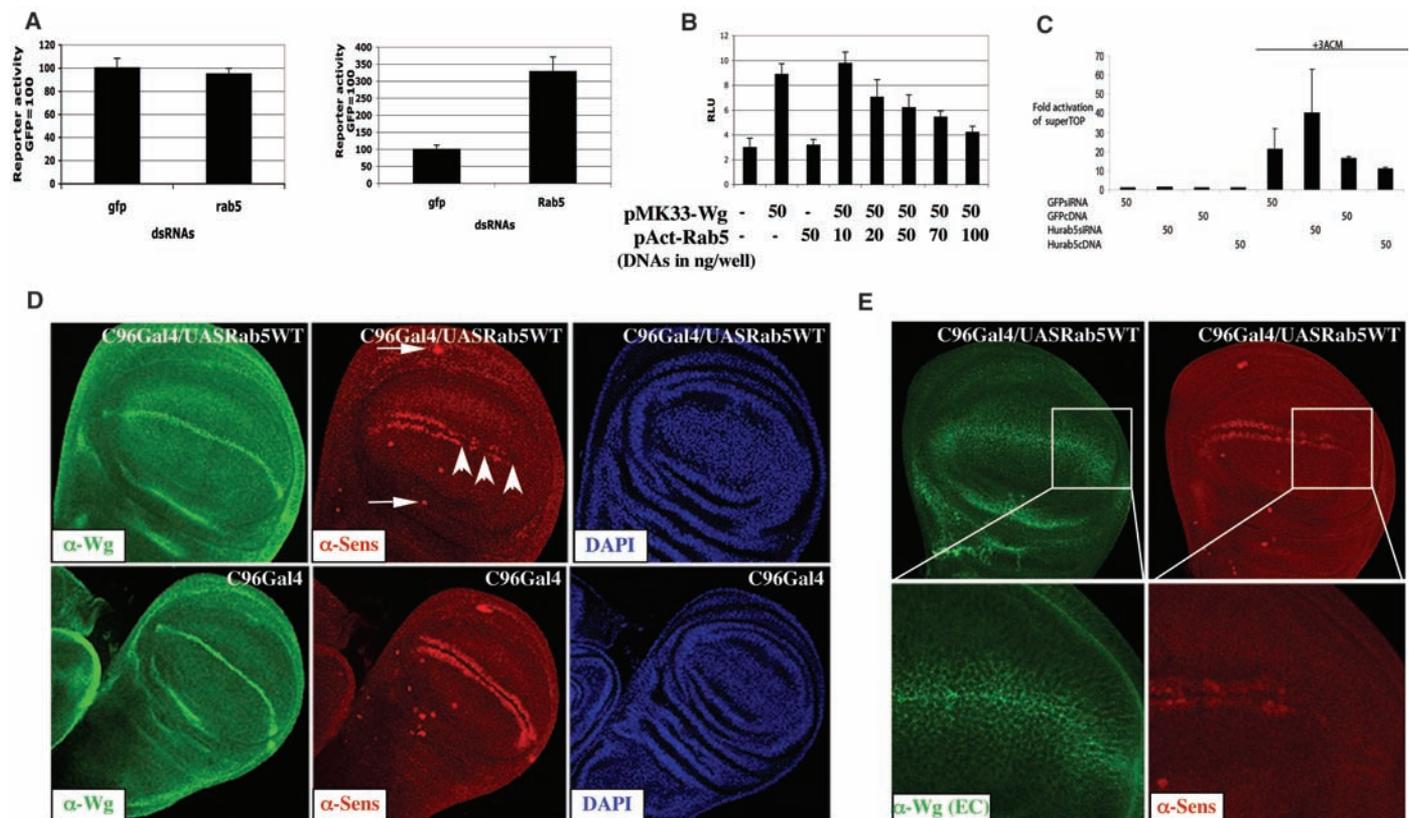
The candidate genes that increased reporter activity when their expression was inhibited were further tested in order to categorize them into specific functional groups. First, we determined whether RNAi of potential negative regulators could ectopically activate the TOP-Flash reporter in the absence of Wg stimulus. Of the 129 negative regulators tested, 63% (83 out of 129) activated reporter activity after dsRNA-mediated knockdown, which suggests 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/ $\beta$ -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, which suggests a role specifically in Wg-stimulated cells. This second class of genes could be functioning either at the level of ligand-receptor regulation or receptor-mediated endocytosis, or they may be involved in the regulation of the stable pool Arm/ $\beta$ -cat that is present only in a stimulated cell. This class includes regulators, such as *nkd* and *Dlp*, that have been shown to regulate the intracellular and extracellular trafficking of Wg, respectively (55–57).

We tested whether decreased expression of “candidate” negative regulators required downstream effectors such as Arm and Pan to activate the Wnt- $\beta$ -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), which placed 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 (58, 59). 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, Dpp, in the *Drosophila* wing by regulating the endocytosis of ligand-receptor complex (60). Rab5-interacting proteins, such as APPL1 and APPL2, as well as other proteins involved in the formation of clathrin-coated vesicles (CCVs) (such as Eps15, epsin, and  $\beta$ -arrestin 2), can undergo nucleocytoplasmic shuttling and can interact with nuclear transcription factors to regulate expression of target genes (61, 62). These studies indicate that the endocytic machinery may be directly involved in nuclear signaling functions as well (62).

In our screen, RNAi-mediated depletion of Rab5 only promoted reporter activity if cells



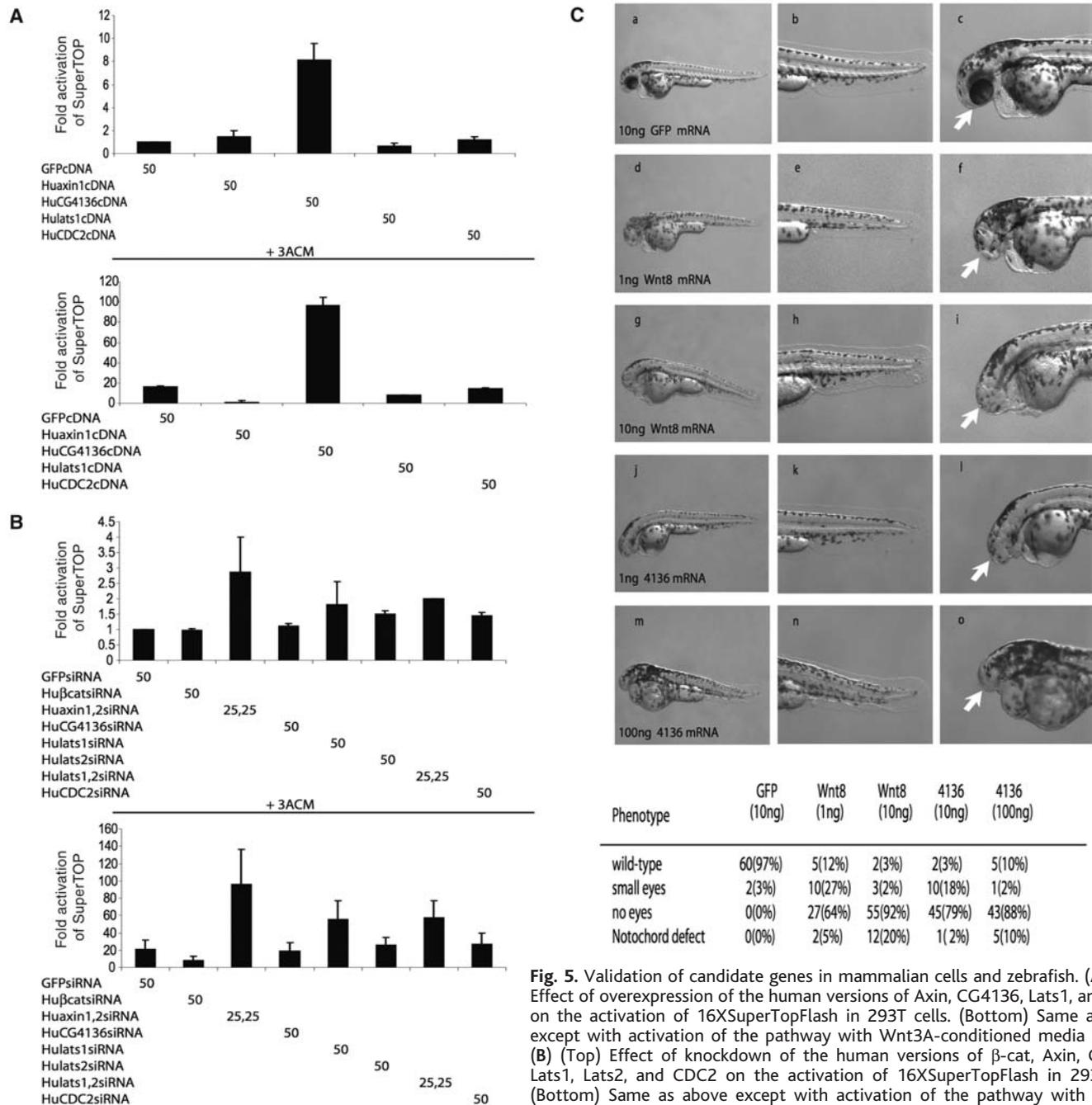
**Fig. 4.** Effect of Rab5 RNAi and overexpression in vitro and in vivo on the Wg signaling pathway. (A) Effect of Rab5 knockdown on reporter activity in control cells (left) or cells expressing Wg (right). (B) Rab5 overexpression in S2 cells results in a dose-dependent repression of Wg-induced reporter activity. (C) Effect of Rab5 overexpression and siRNA knockdown in mammalian 293T cells. (D) Effect of overexpression of wild-type (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 forms fluorescent complexes with natural dsDNA, was used to mark the nuclei of cells in the imaginal discs.

were also stimulated with Wg (table S4 and Fig. 4A). Conversely, cotransfection 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 small interfering RNA (siRNA)-mediated knockdown was similar to the effects obtained in fly cells in culture (Fig. 4C).

To assess whether Rab5 could similarly affect Wg signaling in vivo, we used the GAL4-UAS (upstream activation sequence) system to drive the expression of wild-type *rab5* in the *Drosophila* wing imaginal disc with 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 with 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). Because Rab5 has been implicated in receptor-mediated endocytosis and degradation of morphogenetic signals, we thought overexpression



**Fig. 5.** Validation of candidate genes in mammalian cells and zebrafish. (A) (Top) Effect of overexpression of the human versions of Axin, CG4136, Lats1, and CDC2 on the activation of 16XSuperTopFlash in 293T cells. (Bottom) Same as above except with activation of the pathway with Wnt3A-conditioned media (3ACM). (B) (Top) Effect of knockdown of the human versions of  $\beta$ -cat, Axin, CG4136, Lats1, Lats2, and CDC2 on the activation of 16XSuperTopFlash in 293T cells. (Bottom) Same as above except with activation of the pathway with Wnt3A-conditioned media (3ACM). (C) Effect of overexpression of zebrafish Wnt8 ORF1

(panels d to i) or human CG4136 (panels j to 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, from embryos injected with both Wnt8 and CG4136 [arrowheads in panels i and l] as compared with wild-type eyes in the GFP mRNA-injected embryos [arrow in panel c]. (Below) Table of the various phenotypes of zebrafish injected with Wnt8 ORF1 or CG4136 mRNA.

of Rab5 might influence endocytosis of the endogenous Wg protein and thus might 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, insets). 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 coreceptors Fzd2 and Arrow (Lrp6). However, any significant change in the distribution of receptors is unlikely based on our analysis of extracellular 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 (63). Nonetheless, we cannot rule out subtle changes at the level of receptors and/or coreceptors. Alternatively, Rab5 could be regulating trafficking of the stabilized pool of Arm/ $\beta$ -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 whether 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- $\beta$ -cat-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- $\beta$ -cat-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 three to four short-interfering RNAs (siRNAs) against human Lats1, Lats2, CDC2, and CG4136. Transfection of plasmids encoding pools of siRNA for  $\beta$ -cat inhibited Wnt-3A activation of STF16 as expected (fig. S2). Transfection of pools of siRNAs for Axin1 and 2, Lats1, Lats2, both Lats1 and 2, and CDC2 all increased basal Wnt- $\beta$ -cat-responsive reporter

activity and synergized with Wnt-3A activation of the reporter (Fig. 5B). Pools of siRNA for human CG4136 (HuCG4136) had no effect on activation of the Wnt- $\beta$ -cat-responsive reporter (Fig. 5B), although one caveat is that 293T cells may not express HuCG4136. Because the gain of function of HuCG4136 gave a strong activation of the Wnt- $\beta$ -cat-responsive reporter, it is clear that it can regulate Wnt- $\beta$ -cat signaling and strongly implicates it as a new positive regulator of Wnt signaling in vertebrates. Because all of the vertebrate orthologs tested thus far affect Wnt- $\beta$ -cat signaling, we are in the process of generating expression constructs and siRNAs for multiple additional human orthologs to test their roles in Wnt signaling (64).

To determine whether 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, one cell-stage zebrafish embryos were injected with RNAs encoding HuCG4136, *wnt-8* (as a positive control), and green fluorescent protein (GFP) (as a negative control). Embryos injected with GFP (10 ng) developed normally (Fig. 5C, panels a to 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 of injected embryos (Fig. 5C, panels d to i) (65). Some of the embryos injected with 10 ng also had a defect in notochord formation (see table of Fig. 5C). Injection of RNA encoding HuCG4136 phenocopied injection with *wnt-8*, albeit at higher doses of RNA (Fig. 5C, panels j to o). This coupled with the reporter data in 293T cells indicates that HuCG4136 activates Wnt- $\beta$ -cat signaling. Injection of RNA encoding Huls1 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 (64). A more detailed analysis of this phenotype will be required to determine whether 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- $\beta$ -cat 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 this Research Article, 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 (66). 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; (67) and table S5] underscores the potential broad applicability and importance of such screens in 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 and to identify potential novel regulators of the Wnt pathway.

Although elucidating specific molecular mechanisms for selected candidate genes is beyond the scope of this study, our data strongly suggest 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, as well as 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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**Supporting Online Material**  
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 Materials and Methods  
 Figs. S1 to S4  
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## MicroRNAs Regulate Brain Morphogenesis in Zebrafish

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MicroRNAs (miRNAs) are small RNAs that regulate gene expression posttranscriptionally. To block all miRNA formation in zebrafish, we generated maternal-zygotic *dicer* (*MZdicer*) mutants that disrupt the Dicer ribonuclease III and double-stranded RNA-binding domains. Mutant embryos do not process precursor miRNAs into mature miRNAs, but injection of preprocessed miRNAs restores gene silencing, indicating that the disrupted domains are dispensable for later steps in silencing. *MZdicer* mutants undergo axis formation and differentiate multiple cell types but display abnormal morphogenesis during gastrulation, brain formation, somitogenesis, and heart development. Injection of miR-430 miRNAs rescues the brain defects in *MZdicer* mutants, revealing essential roles for miRNAs during morphogenesis.

MicroRNAs are evolutionarily conserved small non-protein-coding RNA gene products that regulate gene expression at the posttranscriptional level (1–3). In animals, mature miRNAs are ~22 nucleotides (nt) long and are generated from a primary transcript (termed pri-miRNA) through sequential processing by nucleases belonging to the ribonuclease III (RNaseIII) family. Initially, Droscha cleaves the pri-miRNA and excises a stem-loop precursor of ~70 nt (termed pre-miRNA), which is then cleaved by Dicer (4–7). One strand of the processed duplex is incorporated into a silencing complex and guides it to target sequences (1, 3). This re-

sults in the cleavage of target mRNAs and/or the inhibition of their productive translation (1–3).

Several hundred vertebrate miRNAs and several thousand miRNA targets have been predicted or identified, but little is known about miRNA function during development (1, 2, 8, 9). Clues to vertebrate miRNA function have come from several approaches, including expression analyses (1–3, 10–12), computational prediction of miRNA targets (8, 13–15), experimental support of predicted targets (13, 14, 16, 17), cell culture studies (16), and gain-of-function approaches (18). These studies have led to the suggestions that

vertebrate miRNAs might be involved in processes such as stem cell maintenance (12, 19) or cell fate determination (17, 18, 20); however, no loss-of-function analysis has assigned a role for a particular miRNA or miRNA family in vivo, and it has been unclear how widespread the role of miRNAs is during vertebrate embryogenesis.

One approach to reveal the global role of vertebrate miRNAs is to abolish the generation of mature miRNAs with the use of *dicer* mutants. For example, *dicer* mutant embryonic stem cells fail to differentiate in vivo and in vitro (20), and *dicer* mutant mice die before axis formation (19), suggesting that mature miRNAs (or other Dicer products) are essential for early mammalian development. In zebrafish, maternal *dicer* activity has hampered the analysis of the single *dicer* gene. Mutants for the zygotic function of *dicer* (*Zdicer*) retain pre-miRNA processing activity up to 10 days postfertilization,

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**Functional Genomic Analysis of the Wnt-Wingless Signaling Pathway**

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