Minireview

Canonical Wnt signaling: high-throughput RNAi widens the path

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Abstract

The canonical Wnt signaling pathway is highly conserved in evolution, widely used throughout animal development, and frequently hyperactive in cancer. Although Wnt signaling has been the subject of extensive genetic analysis in the past, some 200 genes have now been identified as candidate modulators of this pathway by a recent study using high-throughput RNAi screening.

The discovery of small interfering RNAs (siRNAs), in conjunction with whole-genome sequence data, has spawned a 21st-century strategy for carrying out surrogate genetics on a grand scale - high-throughput RNA interference (RNAi) analysis [1,2]. Using libraries of siRNAs that target potentially all transcripts, one can screen for the phenotypic effects of knocking down individual genes on a genome-wide scale. Perhaps more than any other technique, high-throughput RNAi encapsulates the full meaning of functional genomics. DasGupta and colleagues [3] have recently described the use of such screening for identifying genes that modulate one of the major signal transduction mechanisms in animal cells, the Wnt/β-catenin pathway.

Wnt proteins activate one of the most frequently encountered intracellular signaling pathways in all of developmental biology: one that has innumerable roles in the development of multicellular animals, from sea anemones to humans [4]. The Wnt family of secreted signaling factors is also one of the most ancient, pre-dating the divergence of cnidarians and bilateral metazoans 650 million years ago [5]. In mammals, besides its importance in embryogenesis and postnatal development, Wnt signaling is also of major medical significance. Aberrant activation of Wnt signaling is an initiating or contributing factor in a wide range of human cancers, including most colorectal cancers, and mutations in components of the pathway have been associated with specific hereditary diseases such as bone-density defects, failure of tooth development, and vascular defects in the eye [4,6,7]. At the cellular level, Wnt signaling is perhaps best known for its effects on cell-fate decisions, although in different settings it can regulate cell proliferation, apoptosis, differentiation, adhesion, and migration [4,6]. Recent evidence also implicates Wnt signaling in regulating the self-renewal of pluripotent stem cells in various tissues, suggesting that it will have a major role to play in stem-cell therapeutics [8].

The 19 different Wnt proteins in mammals share extensive sequence similarity and many are functionally redundant. Their principal signaling pathway involves β-catenin as a key signaling intermediate [4,6,7]. This pathway is variously known as the Wnt/β-catenin or canonical Wnt signaling pathway, to distinguish it from non-canonical Wnt signaling mechanisms that do not involve β-catenin and which are much less well characterized at present [9]. With intentional simplification, our current understanding of the canonical pathway can be reduced to the behavior of three multiprotein complexes: the Wnt receptor complex at the cell surface, the β-catenin destruction complex in the cytoplasm, and the β-catenin/TCF (T-cell factor) transcription factor complex in the nucleus (Figure 1). Wnt ligands interact with receptor complexes composed of a seven-transmembrane-domain Frizzled protein and one of the low-density lipoprotein (LDL) receptor-related proteins LRP5 or LRP6. Formation of a Wnt-Frizzled-LRP complex initiates a signal, via the cytoplasmic proteins Dishevelled and Axin, which inhibits the function of the β-catenin destruction complex. The latter normally serves to phosphorylate β-catenin and so target it for destruction by proteolysis. Wnt-mediated inhibition of the destruction complex therefore results in stabilization of

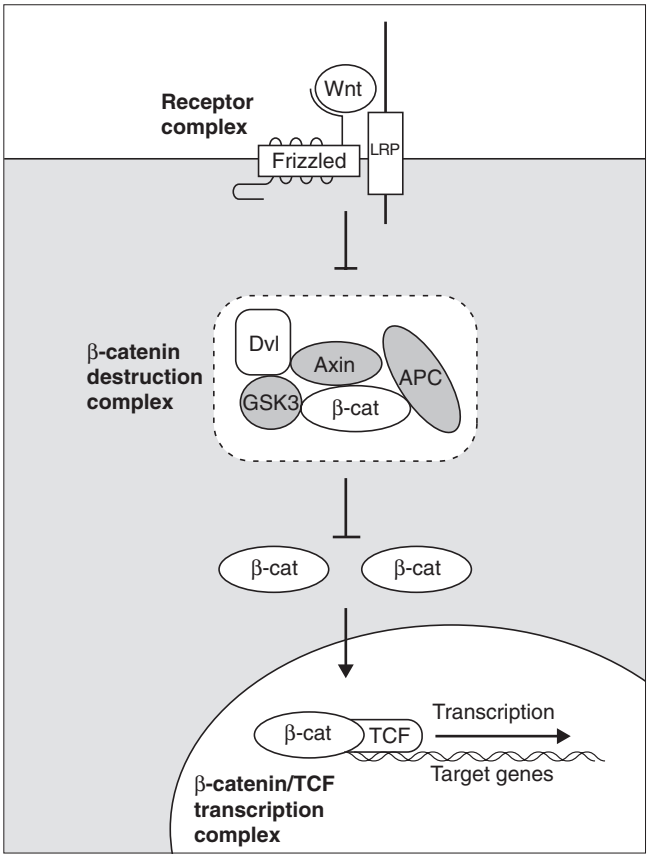

Figure 1
Key components of the canonical Wnt/β-catenin signaling pathway. Complexes formed by a Wnt ligand, Frizzled receptor protein and LRP5 or LRP6 initiate signaling within the cytoplasm. The signal acts via Dishevelled (Dvl) and Axin to inhibit the β-catenin destruction complex and thus increase the stabilization of β-catenin (β-cat) , which then accumulates in the cytosol and nucleus. In the nucleus, β-catenin forms a complex with TCF proteins that activates the transcription of specific target genes. For simplicity, Dvl and Axin are shown as part of the β-catenin destruction complex, but each protein can also be found associated with the receptor complex. Components shown shaded in gray have an inhibitory effect on downstream signaling in the nucleus. The nomenclature used is as for mammalian cells (see Table 1 for the nomenclature of equivalent components in *Drosophila*). APC, the adenomatous polyposis coli protein; GSK3, glycogen synthase kinase 3.

β-catenin, which then accumulates in both cytoplasm and nucleus. The nuclear fraction forms complexes with TCF proteins and other factors, and directly activates the transcription of diverse target genes whose promoters contain TCF-binding sites.

Many of the central elements of the canonical Wnt pathway were originally identified in *Drosophila* through classical genetic analysis of patterning in the fly embryo [10-12]. Here, the *Drosophila* Wnt1 ortholog (Wingless) signals via the β-catenin ortholog (Armadillo) to regulate the specific fate of epidermal cells within each segment (see Table 1 for details of the nomenclature differences between fly and human). It seems appropriate that a signaling pathway whose current framework is derived so much from conventional genetics should be one of the first to be taken to a new level of complexity by functional genomics and high-throughput RNAi. The choice of *Drosophila* as the biological system here has practical advantages. The lower complexity of the genome predicts fewer redundancies among key components and so improves the chances of detecting signaling changes from the knockdown of individual genes.

DasGupta *et al.* [3] developed a high-throughput assay based on the known ability of canonical Wnt signaling to activate transcription of luciferase reporter constructs in transfected cells. Improving on the widely used construct TOP-Flash [13], they generated two new reporters each containing multiple TCF-binding sites upstream of a different minimal promoter. Because only the TCF sites were common between the reporters, off-target effects unrelated to β-catenin/TCF signaling were minimized. Reporters with mutated TCF-binding sites also served as specificity controls. The authors first validated the behavior of these reporters in transfection assays of *Drosophila* cell lines. Then they scaled up the transfections to incorporate approximately 22,000 double-stranded RNAs (dsRNAs), so as to induce RNAi [3], and tested the individual effects on Wingless-induced signaling. The library of dsRNA sequences, previously used in other high-throughput RNAi screens, is directed at all known open reading frames in the *Drosophila* genome and is thought to be more than 95% complete [14,15]. High-throughput screening was achieved in a 384-well plate format, with an individual RNA in each well, together with transfection mixture, reporter plasmids, a *wingless*-expressing plasmid, and the *Drosophila* cells. After 5 days, luciferase activity was measured in the wells and a variety of statistical parameters were applied to highlight those RNAs that had the most credible effects on reporter activity. Of these, 238 were identified and re-tested in secondary screens. Their ability to modulate reporter activity was verified in 213 cases, an impressive proportion (90%) of the primary screen's harvest.

Reassuringly, this protocol rounded up most of the 'usual suspects' - that is, key components of the pathway already known to regulate Wingless signaling. These included *wingless* itself, *frizzled*, *arrow*, *dishevelled*, *axin*, *armadillo* (β-catenin), *pangolin* (dTCF), and eight others, although absent from the list were *Dfz2* (Frizzled2), *APC*, and *zw3/shaggy* (GSK3). Attention could then be confidently turned to the nearly 200 new sequences identified, one of the challenges being to find ways of prioritizing the genes and to separate more effectively the wheat from the chaff. The authors approached this in several ways. One was to re-test the dsRNAs in additional *Drosophila* cells and to focus on those that modulated reporter activity in two or more different cell lines. Another was to test the ability of the

Table 1

Principal components of the Wingless signaling pathway in *Drosophila* and their human orthologs

Drosophila	Human
Wingless	Wnts 1-16
Frizzled2	Frizzleds 1-10
Arrow	LRP5, and LRP6
Dishevelled	Dvl-1, Dvl-2, and Dvl-3
Axin	Axin-1 and Axin-2
APC, APC2	APC and APC2
Zw3/Shaggy	GSK3
Armadillo	β-catenin
Pangolin/dTCF	TCFs 1-4

There is greater redundancy of Wnt pathway components in humans than in *Drosophila*. In addition to Wingless and Frizzled2, there are six other Wnt proteins in *Drosophila* and three other Frizzled family members. In contrast, humans have 16 Wnts and 10 Frizzleds. There are two APC paralogs in both species, but other components downstream of Frizzled in *Drosophila* are unique. APC, the adenomatous polyposis coli protein; GSK3, glycogen synthase kinase 3; Zw3, zeste-white 3.

dsRNA to affect the TCF-luciferase reporter in the absence of Wingless ligand. As the positive sequences here affect baseline activity of the pathway, or perhaps the expression of key components, some may be of less interest than the 37% that specifically modulate Wingless-induced signaling.

Clues to possible functions of the approximately 200 new candidate modulators were obtained from functional annotations assigned by the Gene Ontology Consortium and from the presence of protein domains identified by the InterPro database. This allowed tentative grouping into functional categories. The most numerous of these were transcription factors, including several HMG-box proteins (the group to which TCF proteins belong), homeodomain-containing proteins, Taf family proteins (TATA-binding protein-associated factors), and the basic helix-loop-helix (bHLH) protein Twist. The latter has previously been identified as a target gene of Wnt/β-catenin signaling [16,17] and its identification here as a negative regulator therefore suggests the possibility of a negative feedback mechanism. Other prominent functional categories included kinases and phosphatases, small GTPases, RNA-binding proteins, and proteins containing Armadillo repeats. The latter contain sequences related to the protein-interaction motifs found in Armadillo/β-catenin, but which can be associated with a variety of cellular functions.

In several cases it was possible to obtain preliminary evidence of where in the pathway the new players act, relative to known landmarks. This was achieved by the equivalent of epistasis tests in transfection assays. These experiments asked whether dsRNA targeting a candidate positive regulator could block signaling in the presence of positive stimuli downstream of Wingless, such as expression of the activated co-receptor LRP6, Dishevelled, or stabilized mutant β-catenin. The results showed, for example, that the gene product Dimerization Partner (DP) functions upstream of β-catenin. As DP is a transcription factor, a result such as this could potentially imply that DP plays a role in transcription of β-catenin, rather than being a direct component of the signaling pathway. In contrast, Lilli, a transcription factor known to interact genetically with *armadillo* [18], was placed downstream of β-catenin and is a stronger candidate for direct involvement in Wnt-induced transcriptional regulation.

Another computational approach for analyzing the dataset was to use the Reciprocal-Best-BLAST method to identify human orthologs of the 213 candidate modulators. This was successful in approximately 50% of the cases. Besides the obvious mammalian relevance of those that 'made the cut', these are also more likely to be ubiquitous components or modulators of the pathway. This is because virtually all the key elements of the canonical Wnt pathway known so far are well conserved between flies and humans. In some cases, the authors validated the functional significance of the human orthologs in vertebrate systems by testing their ability to modulate TCF-luciferase reporter activity in human 293T cells. The most impressive of these was the human homolog of the gene named CG4136, which encodes a paired-like homeobox protein. This same sequence, when expressed in zebrafish embryos by injection of RNA at the single-cell stage, gave a developmental phenotype similar to that of Wnt8, indicating that it has a positive effect on Wnt signaling in a variety of systems, both invertebrate and vertebrate.

One of the negative regulators identified in the screen was Rab5, a small GTPase known to have a major role in the formation of endocytic vesicles and their subcellular distribution [19]. This is of particular interest in view of extensive current research into the local modulation of Wnt signaling within cells and tissues by the surface availability of Wnt ligands and receptors, and the subcellular localization of other components [20]. DasGupta and colleagues [3] confirmed the antagonistic effect of Rab5 on Wingless-mediated signaling in cells, and found that it had similar effects in *Drosophila* when ectopically expressed in the wing imaginal disk. How it exerts these effects remains to be seen, as it caused no obvious change in the extracellular distribution of Wingless protein as revealed by immunofluorescence. It remains possible, however, that Rab5 affects the surface distribution of Wingless receptor components, or perhaps a critical subset of Wingless molecules that is distinct from the bulk fraction detected by antibody staining.

In highlighting the importance of the high-throughput RNAi screen by DasGupta *et al*. [3], and the promise that it offers for finding new signaling components, it is also worth considering the genes that were not found by this approach. A number of other signaling pathways have been reported to

exhibit cross-talk with Wnt/β-catenin signaling or to interact with specific components so as to modulate signaling. These include the Notch pathway, the phosphatidylinositol (PI) 3-kinase pathway, and pathways activated by certain tyrosine kinase receptors [21-24]. Non-canonical modes of Wnt signaling may also regulate the canonical Wnt/β-catenin pathway, possibly by acting through Nemo or Nemo-like kinase [25,26]. No high-throughput screen of the sort discussed here is flawless but, given its success in finding most of the known components of Wingless signaling, it is perhaps surprising that there is little evidence of these other interacting pathways in the dataset. In some cases it is possible that the modulation of Wnt/β-catenin signaling by these pathways is only seen in specific cellular contexts, that functional redundancy precludes their detection, or that they have a relatively minor role. Alternatively, their effects may have been masked by the presence of Wingless in the primary screen. This could be an explanation in the case of Notch, as dsRNA targeted against Notch can substantially induce TCF-reporter activity in transfection assays very similar to those used here, but performed in the absence of Wingless [23]. Absence of these predicted modulators may also reflect the reduced sensitivity or reliability of a high-throughput screen relative to individual low-throughput experiments.

From the long list of new potential regulators of Wnt signaling revealed by this study, many readers will have their own most interesting candidates and some may wish to explore the dataset more directly. To facilitate this, there is a searchable database of this and other high-throughput RNAi screens at the *Drosophila* RNAi screening center's website [27]. The site also includes other useful resources and links relating to RNAi screening in general. Public availability of RNAi datasets such as these will be a crucial aspect of their future value. While some individual sequences from the screens will undoubtedly rise to prominence through further experimental analysis of their individual roles, the significance of others will be likely to emerge through computational integration of functional genomics data from related screens.

As a result of high-throughput RNAi screening, the canonical Wnt signaling pathway has suddenly become wider, with a large new batch of contenders for inclusion. The bad news is that the signaling diagrams will be getting more crowded and complex. The good news is that computational biology will take us closer to a more realistic view of signal transduction and modulation *in vivo*. Given the immense biomedical implications of manipulating Wnt signaling in the treatment of cancer and other diseases, as well as in stem-cell therapies, functional genomics will help us to do this more intelligently.

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