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Towards an integrated view of Wnt signaling in development

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Wnt signaling is crucial for embryonic development in all animal species studied to date. The interaction between Wnt proteins and cell surface receptors can result in a variety of intracellular responses. A key remaining question is how these specific responses take shape in the context of a complex, multicellular organism. Recent studies suggest that we have to revise some of our most basic ideas about Wnt signal transduction. Rather than thinking about Wnt signaling in terms of distinct, linear, cellular signaling pathways, we propose a novel view that considers the integration of multiple, often simultaneous, inputs at the level of both Wnt-receptor binding and the downstream, intracellular response.

Introduction

Whether the outcome is a roundworm of the species *C. elegans*, consisting of exactly 959 somatic cells, or a newborn baby, in which a fertilized oocyte eventually gives rise to trillions of cells, the proper execution of any developmental program remains awe-inspiring. At the cellular level, all developmental processes are ultimately controlled by the cooperative actions of different signal transduction pathways. Among them, Wnt signaling is indispensable for orchestrating the complex cell behaviors that occur throughout development (reviewed by Croce and McClay, 2008). Wnt signaling controls cell proliferation, stem cell maintenance and cell fate decisions, as well as organized cell movements and the establishment of tissue polarity. It is also frequently deregulated in human cancers and has been implicated in degenerative diseases. As a potential target for therapeutic intervention, it thus holds new promises in the fields of stem cell biology and regenerative medicine.

As might be inferred from its involvement in such a variety of biological processes, Wnt signaling is itself inherently complex. Roughly speaking, this complexity can be attributed to two main aspects. First, both the ligands and receptors involved in Wnt signal transduction belong to large multi-gene families, allowing for a dazzling number of possible ligand-receptor interactions (reviewed by Kikuchi et al., 2009). Second, Wnt-receptor interactions can elicit a variety of intracellular responses, the best-known of which results in the activation of β-catenin/TCF transcriptional complexes. However, Wnt proteins are also implicated in the activation of other intracellular messengers, including calcium fluxes, Jnk and Src kinases, to name but a few. Over the years, we have come to view the different responses downstream of Wnt-receptor binding as distinct, linear pathways (for reviews, see James et al., 2008; Komiya and Habas, 2008) (Box 1). However, in light of emerging experimental evidence, we believe that we should move away from this concept, as the current models are insufficient to explain the complex responses that are often observed in developing organisms.

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Notably, components at virtually every level of the Wnt signal transduction cascade have been shown to affect both β-catenin-dependent and -independent responses, depending on the cellular context. As we discuss below, this holds true for the Wnt proteins themselves, as well as for their receptors and some intracellular messengers. Rather than concluding that these proteins are shared between pathways, we instead propose that it is the total net balance of signals that ultimately determines the response of the receiving cell. In the context of an intact and developing organism, cells receive multiple, dynamic, often simultaneous and sometimes even conflicting inputs, all of which are integrated to elicit the appropriate cell behavior in response. As such, the different signaling pathways might thus be more intimately intertwined than previously envisioned. In fact, from a developmental biologist's perspective, it would be wrong to look at them as separate pathways at all. Below, we consider this novel concept in more detail, focusing on the integration of signals at the level of Wnt-receptor interactions.

A historical perspective of Wnt signaling in development

The groundwork for Wnt signal transduction research was performed in the late 1980s and early 1990s, when the gene products of the *Drosophila wingless* (*wg*) and mouse *Int1* (subsequently *Wnt1*) genes were found to belong to a large, evolutionarily conserved family of extracellular signaling molecules (Rijsewijk et al., 1987). The name 'Wnt' is derived from a combination of *wingless* and *Int1*. The *Int1* gene was originally identified as an oncogene that, upon insertional activation by the mouse mammary tumor virus (MMTV), contributed to the formation of mammary carcinomas (Nusse and Varmus, 1982). When the *Drosophila* developmental gene *wg* was isolated subsequently, it was shown to be homologous to *Int1* (Baker, 1987; Cabrera et al., 1987; Rijsewijk et al., 1987).

Other Wnt signal transduction components, identified in forward genetic screens and functionally mapped by epistasis experiments (which test the ability of one gene to suppress the effects of another gene, thereby allowing them to be placed upstream or downstream of each other) in *Drosophila*, were also shown to be conserved across species. These include the Frizzled (Fz) transmembrane receptor and the downstream effectors Dishevelled (Dsh; Dvl), β-catenin/Armadillo and T cell factor (TCF)/Pangolin (Brunner et al., 1997; Dominguez et al., 1995; Noordermeer et al., 1994; Peifer et al., 1991; Siegfried et al., 1994; Sokol et al., 1995; Sussman et al., 1994; van de Wetering et al., 1997). As such, these studies built the framework for what has become known as the Wnt/β-catenin pathway (Box 1).

For a number of years, the Wnt field focused on elucidating the biochemical mechanisms that control the activity of β-catenin/TCF (reviewed by Barker, 2008; Huang and He, 2008; Moon et al., 2004). This was largely driven by the fact that β-catenin was frequently found to be mutated in human cancers, causing hyperactivation of Wnt/β-catenin signaling in virtually all intestinal cancers and in a variety of other malignancies. Only recently has

attention shifted back to the cell membrane, where signal transduction is initiated by the binding of Wnt proteins to membrane receptors.

We now know that the genomes of *Drosophila*, *C. elegans*, *Xenopus* and higher vertebrates harbor multiple Wnt genes (a total of 19 in mammals). Notably, a remarkably diverse Wnt repertoire is already present in Cnidarians, an ancient metazoan phylum that includes fresh water polyps and sea anemones (Guder et al., 2006). This not only indicates that Wnt gene diversity arose early in evolution, but also that it has remained essential for the proper development of multicellular animals. Such genetic complexity is also found at the level of the prototypical Wnt receptor, Frizzled, a cell surface receptor with seven transmembrane-spanning segments and a Wnt-binding site in the form of a cysteine-rich domain (CRD) (Bhanot et al., 1996). A total of four Frizzled receptors have been identified in *Drosophila* and *C. elegans*, and ten Frizzled receptors are found in mice and humans [see Table 1 for an overview of the Wnt and receptor proteins that have been identified in commonly utilized model organisms; a table providing a detailed overview of different Wnt-receptor interactions was recently published elsewhere (Kikuchi et al., 2009)].

From the outset, researchers have attempted to group individual Wnt proteins into classes to which specific activities could be assigned. This has resulted in the subdivision of Wnts into 'canonical' or 'non-canonical' based on the ability of the former, but not the latter, to induce an ectopic axis in *Xenopus* embryos (McMahon and Moon, 1989) and to cause the morphological transformation of mouse C57MG mammary cells (Wong et al., 1994). Both of these activities correlate with an increase in the levels of β-catenin and a concomitant increase in β-catenin/TCF signaling (Shimizu et al., 1997). Studies in *Drosophila* had shown previously that *frizzled* and *dishevelled* were required to establish tissue polarity independently of β-catenin/TCF (Strutt et al., 1997; Theisen et al., 1994; Vinson and Adler, 1987). When certain non-canonical Wnts were shown to be involved in related processes that control convergent extension movements during vertebrate development (Heisenberg et al., 2000; Tada and Smith, 2000;

Box 1. Intracellular responses downstream of Wnt-receptor binding

Wnt-receptor binding can elicit a variety of intracellular responses. (**A**) The interaction of Wnt with Frizzled and low-density lipoprotein receptor-related protein (LRP) results in the activation of β-catenin/T cell factor (TCF) signaling. This requires inactivation of a 'destruction complex', comprising Adenomatosis polyposis coli (APC), Axin, glycogen synthase kinase 3b (Gsk3b) and Casein kinase I (CKI) that normally sequesters and phosphorylates newly synthesized β-catenin, targeting it for degradation by the proteasome. Phosphorylation of the cytoplasmic tail of LRP has also been shown to be crucial for Wnt/β-catenin signaling. Although direct evidence demonstrating an endogenous Wnt-Frizzled-LRP complex is lacking, there are sufficient data to conclude that such a trimeric complex exists in vivo (e.g. Bilic et al., 2007; Holmen et al., 2005; Liu et al., 2005). (**B**) Binding of Wnts to the receptor tyrosine kinase (RTK) Ror2 can inhibit β-catenin/TCF signaling and activate Jnk. The mechanisms that underlie these activities are still incompletely understood. (**C**) Frizzled receptors are implicated in the establishment of planar cell polarity (PCP) and in the control of polarized cell migration (reviewed by Simons and Mlodzik, 2008). In flies, but not vertebrates, these responses appear to occur independently of a Wnt-Frizzled interaction (as indicated by a question mark in the figure). Rather than β-catenin/TCF complexes, the establishment of PCP is thought to involve a set of distinct downstream messengers that include Dishevelled (Dvl), small Rho GTPases and Jnk. In some cases, Frizzled receptors can induce Ca^{2+} fluxes. (**D**) Wnt proteins have also been shown to bind to Ryk RTKs, mostly in the context of neuronal development, resulting in the activation of Src proteins.

Although this article focuses on the integration of signals at the level of Wnt-receptor binding, it is important to note that integration also occurs further downstream, as it is a general feature of any signaling network. Many of the depicted players have been implicated in more than one cellular response. For instance, Dvl is generally thought to function at the crossroad of Wnt/β-catenin and Frizzled/PCP signaling (Boutros and Mlodzik, 1999), whereas Rac and Jnk, which have been implicated in a β-catenin-independent response downstream of Dvl, were recently shown to activate β-catenin/TCF (Wu et al., 2008). Other proteins, such as Nkd, Inversin and Diversin, have been considered to function as molecular 'switches', by inhibiting one intracellular response and promoting another (Schwarz-Romond et al., 2002; Simons et al., 2005; Yan et al., 2001), and they too might be prime examples of nodes in the network that integrate different signaling inputs.

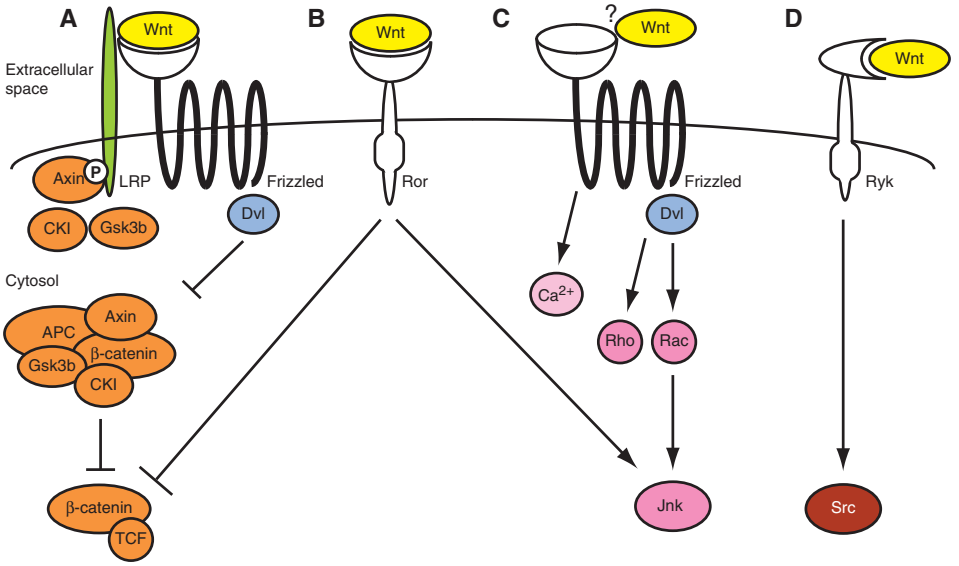

Table 1. Conservation of Wnt and Frizzled proteins across metazoan species

Hydra	*Nematostella**	*Drosophila*	*C. elegans*†	*Xenopus*‡	Mouse and human
Wnt proteins from major subfamilies					
HyWnt1	NvWnt1	Wg		XWnt1	Wnt1
HyWnt2	NvWnt2			XWnt2 XWnt2B	Wnt2 Wnt2B
HyWnt3	NvWnt3			XWnt3 XWnt3A	Wnt3 Wnt3A
	NvWnt4		CWN-1	XWnt4	Wnt4
HyWnt5	NvWnt5	Dwnt5	CWN-2	XWnt5A XWnt5B	Wnt5A Wnt5B
	NvWnt6	Dwnt6		XWnt6	Wnt6
HyWnt7	NvWnt7A NvWnt7B	Dwnt2		XWnt7A XWnt7B XWnt7C	Wnt7A Wnt7B
HyWnt8	NvWnt8A NvWnt8B			XWnt8A XWnt8B	Wnt8A Wnt8B
					Wnt9A Wnt9B
HyWnt10A HyWnt10B HyWnt10C	NvWnt10	Dwnt10		XWnt10A	Wnt10A Wnt10B
HyWnt11	NvWnt11			XWnt11	Wnt11
HyWnt16	NvWnt16		EGL-20		Wnt16
Orphan Wnt proteins (not related to one another)					
	NvWntA				
		Dwnt4 DwntD			
			LIN-44 MOM-2		
Frizzled proteins					
HyFz	NvFz1	Fz	MOM-5 LIN-17	XFz1 XFz2 XFz3 XFz7	Fz1 Fz2 Fz3 Fz6 Fz7
	NvFz2	Dfz2	CFZ-2	XFz5 XFz8	Fz5 Fz8
	NvFz3 NvFz4	Dfz3 Dfz4	MIG-1	XFz4 XFz9 XFz10A XFz10b	Fz4 Fz9 Fz10

Overview of the different Wnt proteins and Frizzled cell surface receptors found in several metazoan species. Orthologs are grouped together based on published phylogenetic analyses (Huang and Klein, 2004; Kusserow et al., 2005; Lengfeld et al., 2009; Minobe et al., 2000; Momose and Houliston, 2007; Prud'homme et al., 2002; Schubert et al., 2000).

*Of note, 11 out of the 12 Wnt subfamilies found in mammals are also found in the sea anemone *Nematostella vectensis*, which suggests that the high genetic complexity that was present in a common metazoan ancestor has been lost in *Drosophila* and *C. elegans*.

†The nomenclature of Wnt proteins and receptors in *C. elegans* (and, to a lesser extent, *Drosophila*) does not follow that of vertebrate species.

‡The *Xenopus* genome has not yet been sequenced completely; therefore, additional Wnt proteins and/or receptors might still be identified.

Wallingford et al., 2001), the distinction between the two groups of ligands appeared clean-cut: canonical Wnts bound to Frizzled and activated β-catenin/TCF, whereas non-canonical Wnts bound to Frizzled and activated small Rho GTPases, c-Jun N-terminal kinase (Jnk) and other β-catenin-independent signaling events. However, the intrinsic properties of individual Wnt proteins are only part of the story, and in light of today's knowledge, it seems incongruous to refer to a given Wnt as canonical or non-canonical.

First, members of the low-density lipoprotein receptor-related protein (LRP; Arrow in *Drosophila*) family have been identified as single-pass transmembrane co-receptors for Frizzled (Mao et al.,

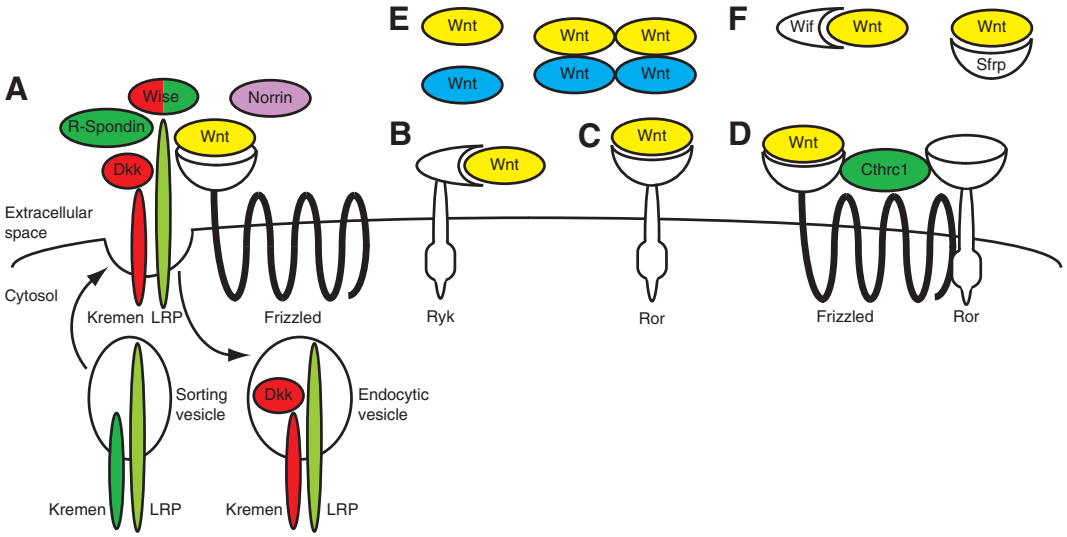

Fig. 1. Wnt proteins and their interaction partners. Depending on the developmental time-point, as well as on the tissue and cell type, Wnt proteins (with different family members depicted in blue and yellow) can encounter a multitude of different interaction partners that determine the response of the signal-receiving cell, with most Wnt signal transduction components not dedicated to a single intracellular response. Proteins that promote Wnt-Frizzled activity are depicted in green; proteins that impede Wnt-Frizzled activity are in red. See text for details. (**A**) Wnt proteins can engage with a variety of cell surface receptors, including members of the Frizzled seven-transmembrane-spanning receptor family. In order for β-catenin/TCF signaling to occur, the LRP co-receptor must be recruited into the Wnt-Frizzled complex. LRP availability is determined by the balance between its internalization and plasma membrane presentation (mediated by Dkk and Kremen). Other co-factors, such as R-Spondin and Wise, also affect Wnt-receptor complex activity, with R-Spondin competing with Dkk for binding to Kremen (Binnerts et al., 2007), and with Wise capable of both promoting and inhibiting Wnt signaling. Finally, non-Wnt proteins, such as the unrelated ligand Norrin, can compete for binding to Frizzled receptors (Xu et al., 2004). (**B**,**C**) Wnt proteins can also bind to single-pass transmembrane RTKs of the Ryk and Ror families. (**D**) In some cases, the Wnt-receptor interaction might require the recruitment of additional co-factors. For instance, Cthrc1 can promote the formation of a Wnt-Frizzled-Ror complex (see text for details). (**E**) Little is known about the active conformation of Wnt proteins. They might signal as monomers, or in homo-, hetero- or oligomeric complexes. (**F**) In the extracellular space, Wnt proteins can encounter secreted inhibitors, such as Wifs and Sfrps, both of which bind and inhibit Wnt proteins directly.

2001; Tamai et al., 2000; Wehrli et al., 2000) (Fig. 1A). In fact, the phosphorylation of LRP/Arrow, as well as its ability to form large aggregates or ‘signalosomes’, both of which require Frizzled function, have recently been shown to be indispensable for the accumulation of β-catenin upon Wnt stimulation in many species (Bilic et al., 2007; Tamai et al., 2004; Zeng et al., 2008). In addition, certain non-Frizzled receptors have been shown to be capable of transmitting Wnt signals. Single-pass transmembrane receptor tyrosine kinases (RTKs) of the Ryk and Ror families appear to function as genuine Wnt receptors, with Wnt interactions mediated by Wnt inhibitory factor (Wif) domains in the case of Ryk receptors and by CRD domains in the case of Ror receptors (Forrester et al., 2004; Green et al., 2007; Inoue et al., 2004; Lu et al., 2004; Mikels and Nusse, 2006; Oishi et al., 2003) (Fig. 1B,C). With the identification of this novel class of Wnt receptors, old questions have come to the fore again: what determines the specificity of Wnt-receptor interactions, and what dictates the downstream cellular response?

Context-specific responses to Wnt

Essentially all of the experiments on which the classification of Wnt proteins has been based were performed prior to the identification of LRP co-receptors or of the alternative Wnt receptors Ryk and Ror. With hindsight, these studies should therefore be viewed as reflecting receptor expression in the model system under study, as much as the activities of the Wnts themselves. For instance, in an effort to determine ligand-receptor specificity, Takada and colleagues studied the effect of mammalian Wnt3A (traditionally considered a canonical Wnt) and Wnt5A (the prototypical non-canonical Wnt) on a number of mammalian Frizzled receptors heterologously expressed in *Drosophila* S2 cells (Takada et al., 2005). Whereas Wnt3A clearly stabilized the levels of the *Drosophila* β-catenin homolog Armadillo in combination with specific Frizzled receptors, Wnt5A failed to do so in any of the conditions tested. However, rather than an intrinsic difference in the capacity of Wnt3A and Wnt5A to activate signaling through β-catenin, this outcome might instead reflect an inability of Wnt5A to recruit the LRP homolog Arrow. Indeed, Wnt5A is able to induce signaling through β-catenin/TCF in cells of the human 293 embryonic kidney cell line that express Fz4 and LRP5 (Mikels and Nusse, 2006). Similarly, Wnt5A can induce an ectopic axis in *Xenopus* embryos in a system in which Fz5 is expressed (He et al., 1997; Holmen et al., 2002). These studies underscore the fact that subtle differences in the affinities of individual Wnt proteins for different receptors and co-receptors can influence experimental outcomes.

One might argue that the ability of Wnt5A to induce β-catenin/TCF signaling through Fz4 or Fz5 is an experimental overexpression artifact: in a normal biological context, Wnt5A might never encounter the combination of receptors that is required for this activity. The fact that this perspective is an over-simplification is best illustrated by the more recent finding that Wnt11, which is also typically considered to be a non-canonical Wnt, indeed appears to fulfill a dual role during development. Wnt11 is clearly required for convergent extension movements during gastrulation in amphibians (Heisenberg et al., 2000; Marlow

et al., 2002; Smith et al., 2000; Tada and Smith, 2000), yet maternally contributed Wnt11 also initiates axis formation in the early *Xenopus* embryo by causing a local accumulation of β-catenin (Tao et al., 2005). Recent work suggests that maternal Wnt5A similarly activates β-catenin/TCF signaling during *Xenopus* axis formation, as the depletion of maternal Wnt5A phenocopies the loss of maternal Wnt11 (Cha et al., 2008). Of course, great care should be taken when generalizing the function of individual Wnts based on data obtained using vastly different model organisms. Thus, until we have mapped all of the Wnt-receptor interactions that occur in the context of an intact organism and across different species, we cannot rule out the possibility that Wnts have multiple activities during development, and current evidence supports the notion that they do. It is interesting to speculate, then, how Wnt signaling can generate such diverse outputs. In doing so, however, it is important to realize that in addition to species-specific differences, many of the observed responses are likely to be stage dependent and tissue specific. The outcome of Wnt-receptor binding will depend as much on the developmental history of the receiving cell as on the stimulus it receives. For instance, Wnt/β-catenin signaling has been shown to promote self-renewal and proliferation of various stem cells (Reya et al., 2003; Sato et al., 2004), but it regulates distinct cell fate decisions in neural crest stem cells (Lee et al., 2004).

Observations such as those above raise the question of why we need so many Wnt and Frizzled proteins at all. The mere fact that all animal species have retained a large number of different Wnt proteins throughout evolution suggests that their functions are not redundant. However, whereas the deletion of *wg* in *Drosophila* results in defective β-catenin/TCF signaling, deletion of the other *Drosophila* Wnt genes does not cause gross developmental defects. In fact, with the exception of *Wnt2* mutants, which have a gonadal phenotype, functions for the other Wnt genes have mostly been observed in the context of *wg* co-deletion (Kozopas et al., 1998; Llimargas and Lawrence, 2001). This could indicate that the other *Drosophila* Wnts merely serve as co-factors that fine-tune the activities of Wg, but experimental evidence to support this hypothesis is lacking.

Unfortunately, it has remained a challenge to map the precise differences in protein structure and receptor-binding properties of the different Wnt proteins. Efforts to crystallize them and to determine their three-dimensional structure have been unsuccessful, and their hydrophobic nature has, until recently, hampered purification efforts (Willert et al., 2003; Willert, 2008). Overall, studies that have directly addressed Wnt-Frizzled binding affinities are limited. Experiments that use membrane-tethered *Drosophila*, *C. elegans* or *Xenopus* Wnt proteins and soluble, secreted Frizzled CRD domains have revealed that certain Wnt-Frizzled combinations appear to be favored over others (Bhanot et al., 1996; Green et al., 2007; Hsieh et al., 1999; Rulifson et al., 2000; Wu and Nusse, 2002). However, it is still far from clear to what extent specific Wnt-receptor pairings truly have distinct functions. Moreover, these experiments have the caveat that they do not take into account any potential modulation of Wnt-receptor interactions by co-receptors or by modifications to the Wnts themselves, both of which might influence the responses that occur in the context of an intact, developing organism.

Context-specific functions for cell surface receptors

The cellular response to a given Wnt-receptor interaction appears to depend, at least in part, on the signaling specificity of the receptor. In particular, sequences in the C-terminus of the Frizzled protein affect the ability of the receptor to activate β-catenin/TCF signaling. In *Drosophila*, Fz is involved in the establishment of planar cell polarity (PCP). It has a lower affinity for Wg than does Fz2, which is involved in β-catenin/TCF signaling rather than PCP (Bhanot et al., 1996; Bhat, 1998; Tomlinson et al., 1997). Exchanging the C-terminal sequences of Fz and Fz2 reverses their behaviors (Boutros et al., 2000).

Although such distinct signaling activities are in line with a model in which the outcome of a Wnt signal transduction event is determined by the receptors that are encountered on the cell surface, there are now many examples demonstrating that it would be too simplistic to view the receptor as the sole determinant of Wnt signaling activation. For instance, *Xenopus* Fz7 has been shown to mediate multiple intracellular responses. Fz7 affects convergent extension movements in a β-catenin/TCF-independent manner that involves Dishevelled, Syndecan 4 and Cdc42, but it is also required for dorsoventral mesoderm specification upstream of β-catenin/TCF (Medina et al., 2000; Munoz et al., 2006; Sumanas and Ekker, 2001; Sumanas et al., 2000). Similarly, many studies have demonstrated a role for Ror2 in the transmission of a Wnt5A signal that ultimately inhibits β-catenin/TCF signaling. Ror2 appears to function as a genuine RTK in this case (Billiard et al., 2005) (Fig. 1C). Recent reports, however, suggest that in a different cellular context, Ror2 might function to potentiate β-catenin/TCF signaling induced by Wnt1 or Wnt3A (Li et al., 2008; Winkel et al., 2008). The latter does not appear to require Ror2 tyrosine kinase activity, raising the possibility that Ror2 could also function as a co-receptor that merely aids in presenting Wnts to Frizzled (Fig. 1D). Work in *C. elegans* suggests yet another possible function for Ror2. The *C. elegans* Ror2 homolog CAM-1 antagonizes β-catenin/TCF signaling non-cell-autonomously (meaning that it functions on cells other than the Wnt target cell) and independently of its cytoplasmic domain (Green et al., 2007). As CAM-1 is able to interact with multiple *C. elegans* Wnts, the authors propose a model in which CAM-1 functions as a sink to sequester Wnt proteins, thereby limiting their effective range and concentration. It remains to be determined whether Ror2 has a similar role in other organisms. As illustrated by these examples, just as Wnt proteins cannot be strictly divided into classes with specific activities, the function of Frizzleds and of other Wnt receptors and co-receptors is also context dependent.

Dynamic interactions determine the cellular response to Wnts

In addition to Wnt proteins having the potential to engage a variety of different transmembrane receptors, both Wnts and their receptors encounter a host of co-regulators in the intra- and extracellular space, many of which appear to fulfill dual roles. Below we discuss some of these context-dependent modifiers in more detail in order to illustrate how the cumulative interactions between these different factors allow for flexible and dynamic cellular responses to the presence of a Wnt signal. It is by no means an exhaustive list, but serves to underscore the dynamic interactions that are likely to exist between many of the players involved.

As mentioned above, the canonical β-catenin-dependent and non-canonical β-catenin-independent responses to Wnt stimulation have generally been viewed as separate pathways. Specifically, the recruitment of the LRP co-receptor appears to be a prerequisite for inducing β-catenin/TCF signaling (Tamai et al., 2000; Wehrli et al., 2000). Recently, however, LRP6 has also been implicated in convergent extension movements and in the establishment of tissue polarity in vertebrates (Bryja et al., 2009; Tahinci et al., 2007). The LRP co-receptor can be prevented from engaging the Wnt-Frizzled complex by Kremen, a single-pass transmembrane receptor, and by

Dickkopf (Dkk), a soluble secreted protein that, when bound to LRP, mediates its internalization (Fig. 1A). By helping to promote this Dkk-mediated internalization of LRP, Kremen negatively regulates Wnt/β-catenin signaling (Mao et al., 2002). In the absence of Dkk, however, Kremen instead promotes the cell surface localization of LRP (Hassler et al., 2007). As pointed out by Cselenyi and Lee (Cselenyi and Lee, 2008), the stimulatory activities of Kremen on the one hand, and its inhibitory activities on the other hand, would allow it to generate a biphasic response: in regions of lower Dkk concentration Kremen could augment Wnt/β-catenin signaling, whereas it might actively inhibit Wnt/β-catenin signaling once a critical Dkk concentration has been surpassed.

Similar to LRP6, Dkk has now been linked to canonical as well as to non-canonical signaling events. The depletion of maternal Dkk in *Xenopus* embryos results in the ectopic activation of β-catenin/TCF signaling, but also disturbs convergent extension movements in a β-catenin-independent fashion as a result of enhanced Jnk activity (Caneparo et al., 2007; Cha et al., 2008). These and other studies suggest that canonical and non-canonical responses might be far more intimately linked than previously envisioned.

The LRP6-binding protein Wise (Sostdc1) is another prominent example of a context-dependent modifier (Fig. 1A). Early experiments in *Xenopus* revealed both stimulatory and inhibitory activities for the protein with regard to Wnt/β-catenin signaling, as well as a role in the regulation of convergent extension movements (Itasaki et al., 2003). In a follow-up study, Wise was shown to inhibit the activities of some Wnt proteins, but to augment the activity of others. Furthermore, Wise was found to have extracellular as well as intracellular functions (Guidato and Itasaki, 2007). A mutant Wise protein that is retained in the endoplasmic reticulum resulted in reduced cell surface expression of LRP6. Although it is currently unknown whether endogenous Wise is produced in a secreted as well as in an intracellular form in vivo, it is important to note that some of the phenotypes observed upon overexpression or knockdown of wild-type Wise are better recapitulated by a secreted form of the protein, but others by an endoplasmic, reticulum-retained form.

The recent identification of collagen triple helix repeat-containing protein 1 (Cthrc1) as a modifier of Wnt signal transduction illustrates the importance of extracellular co-factors for the generation of a functional Wnt-receptor unit (Yamamoto et al., 2008). Functionally, Cthrc1 interacts with the core PCP protein Vangl2 in mice, which suggests a role in the establishment of tissue polarity. In overexpression experiments, Cthrc1 physically interacts with a number of Wnts (Wnt3A, Wnt5A and Wnt11) and Frizzleds (Fz3, Fz5 and Fz6) as well as with Ror2. Moreover, Cthrc1 specifically enhances Wnt-Fz-Ror2 complex formation at the expense of Wnt-Fz-LRP6 complexes – an activity that the authors speculate might result in the simultaneous inhibition of β-catenin/TCF signaling and activation of intracellular signaling events that contribute to the establishment of tissue polarity. Although Ror2 functions as a genuine Wnt receptor by virtue of its own CRD domain (Fig. 1C), the study by Yamamoto et al., suggests that Ror2 might also function as part of a larger complex in which it collaborates with a Frizzled receptor (Fig. 1D) (Yamamoto et al., 2008). This would be in agreement with previously published data that show a physical interaction between Ror2 and Frizzled (Oishi et al., 2003).

In light of the Wnt-Fz-Ror2 complex formation described by Yamamoto et al. (Yamamoto et al., 2008) and of reports on the dimerization of individual receptors (Carron et al., 2003; Liu et al., 2008), the presence of multiple Wnt receptors in a single complex raises the possibility that Wnt proteins act as multimers instead of monomers (Fig. 1E). A recent study by Heasman and co-workers demonstrates just that: both Wnt5A and Wnt11 were shown to form homodimers, which then interact to form a functional, oligomeric Wnt5A-Wnt11 complex (Cha et al., 2008).

Finally, an additional level of regulation comes in the form of secreted Wnt inhibitors, such as the Wif proteins, the Wnt-interacting domain of which is similar to that of the Ryk receptors, and the secreted frizzled-related proteins (Sfrps), which contain a CRD that is similar to the Wnt-interacting domains of both Frizzled and Ror receptors (Fig. 1F). Wifs and Sfrps can directly bind to Wnt proteins in the extracellular space, thereby affecting receptor occupancy and, ultimately, the cellular response (Bovolenta et al., 2008; Kawano and Kypta, 2003).

Challenges in studying Wnt signaling in development

Whereas in vitro studies and biochemical approaches are invaluable for dissecting the functions of individual Wnt signal transduction components, their relevance for development must ultimately be demonstrated in vivo. Here, the situation quickly becomes very complicated, as cells can receive multiple inputs at the same time, and these might function together to elicit the desired effect. For instance, in *C. elegans*, two opposing Wnt signaling events collaborate in orienting cell polarity (Green et al., 2008). On the one hand, Wnt/LIN-44 and Wnt/MOM-2, acting through Fz/LIN-17 and Ryk/LIN-18, induce signaling through β-catenin/TCF. On the other hand, Wnt/EGL-20 acts through Ror2/CAM-1, in collaboration with the PCP protein Vangl1/VANG-1. All of these inputs simultaneously direct the oriented cell divisions that are required to polarize cells properly during vulval development. When interpreting these results, however, we should bear in mind that the roles of different proteins and the specific cell behavior they elicit, are likely to have become diversified over the course of evolution. For instance, the function of Arrow in *Drosophila* appears to be limited to Wnt/β-catenin signaling. No apparent homologs of LRP/Arrow have been identified in *C. elegans*. Yet in vertebrates, LRP6 has been implicated in both β-catenin-dependent and -independent responses, as discussed above. Also, the establishment of tissue polarity in *Drosophila* appears to require Frizzled, but not Wnt (Chen et al., 2008). Conversely, Wnt proteins have been implicated in convergent extension and tissue polarity in vertebrates (Dabdoub et al., 2003; Heisenberg et al., 2000; Kilian et al., 2003; Qian et al., 2007).

Given the large number of Wnt and Frizzled homologs in higher vertebrates, it remains a daunting task to determine the role of each of them in a given developmental process. Even in *Drosophila*, exhaustive studies of this nature have yet to be performed. In *C. elegans*, however, Zinovyeva et al. have recently investigated the involvement of all five Wnt proteins and all four Frizzled receptors in embryonic and post-embryonic cell migrations (Zinovyeva et al., 2008). They found all of them to be involved, as was Ror2/CAM-1. Moreover, a comparison of the phenotypes observed in quintuple Wnt mutants and in quadruple Frizzled mutant worms suggests the existence of additional Frizzled-independent roles for Wnt, and Wnt-independent roles for Frizzled. As proposed by the authors, the simultaneous expression of multiple Wnt homologs in the same tissue could provide increased positional and directional information and help in the fine-tuning of the cellular responses.

In *C. elegans*, as well as in other animal species ranging from *Drosophila* to *Xenopus* and mice, Wnt proteins and their receptors are expressed in a tightly regulated spatiotemporal manner from

early development onwards. During the implantation of the mouse blastocyst, for instance, Wnts and Frizzleds are already expressed in very distinct and dynamic patterns, which suggest an equally dynamic regulation of downstream signaling events (Hayashi et al., 2009). Only recently have more advanced high-throughput approaches and quantitative analyses enabled the construction of a more detailed picture of the dynamic expression patterns of Wnt genes, their receptors and co-regulators during development. In the mouse limb, for instance, Witte and colleagues have performed a detailed analysis of the localization of transcripts that encode Wnt proteins and their secreted antagonists, revealing a dynamic distribution pattern (Witte et al., 2009). A similar situation was observed for expression of the different Frizzled receptor genes in the mouse brain (Fischer et al., 2007). Yet for many of the newly identified Wnt receptors and co-receptors, as well as for most anatomical sites, the expression characteristics remain largely undetermined. Public databases, such as the Gene Paint Atlas (http://www.genepaint.org) and the Edinburgh Mouse Atlas (http://genex.hgu.mrc.ac.uk/intro.html), will become increasingly informative with regard to Wnt signaling as these data accumulate.

Finally, more advanced techniques should allow us to find the answers to a wide range of pressing questions. What are the signals that regulate the expression of the different ligands and receptors themselves? What are the feedback mechanisms by which Wnt proteins might affect the expression of the receptor repertoire? In *Drosophila*, for instance, *fz3* is a target of Wg, and its upregulation in turn attenuates Wg signaling (Sato et al., 1999). In vertebrates, several negative regulators of Wnt signal transduction, such as *Axin2* and *Dkk4*, have also been identified as Wnt target genes (Bazzi et al., 2007; Jho et al., 2002). Such negative-feedback loops can have important developmental consequences. For instance, they can result in the stable oscillations that are observed during somitogenesis, when periods of high and low Wnt signaling activity alternate (Aulehla et al., 2003; Aulehla et al., 2008; Suriben et al., 2006), or in the regular spacing of hair follicles in the skin (Sick et al., 2006).

Do different signaling events really occur within the same cell? How do signaling events in neighboring cells, or even in cells that are further removed, affect the response of the tissue as a whole? As exemplified by the non-cell-autonomous role of Ror2/CAM-1 in *C. elegans* (Green et al., 2007), proteins may affect the response to Wnt by functioning in cells adjacent to, but different from, the Wnt target cell. How far do the different Wnt signals spread in vivo? Can we distinguish between direct responses and secondary effects? The latter is an important, yet often neglected, source of confusion. It is crucial to realize that the ultimate developmental read-out (i.e. the phenotype) and the direct, initial response to Wnt ligand stimulation (e.g. receptor complex formation, or the stabilization of β-catenin) are vastly separated in developmental time and should therefore be distinguished from one another.

Novel imaging tools should provide us with sufficient resolution to zoom in on Wnt signal transduction events in vivo. The C-terminal tails of the *Drosophila* Fz and Fz2 proteins, for instance, determine their subcellular localization (Wu et al., 2004). The Fz C-terminus directs apical localization in epithelial cells of the *Drosophila* wing imaginal disc, where it promotes PCP signaling but inhibits signaling through β-catenin/TCF. By contrast, the Fz2 C-terminus promotes a more basolateral localization, where it binds Wg and promotes signaling through β-catenin/TCF. This observation suggests that different intracellular responses might take place in distinct subcellular compartments. Likewise, the polarized

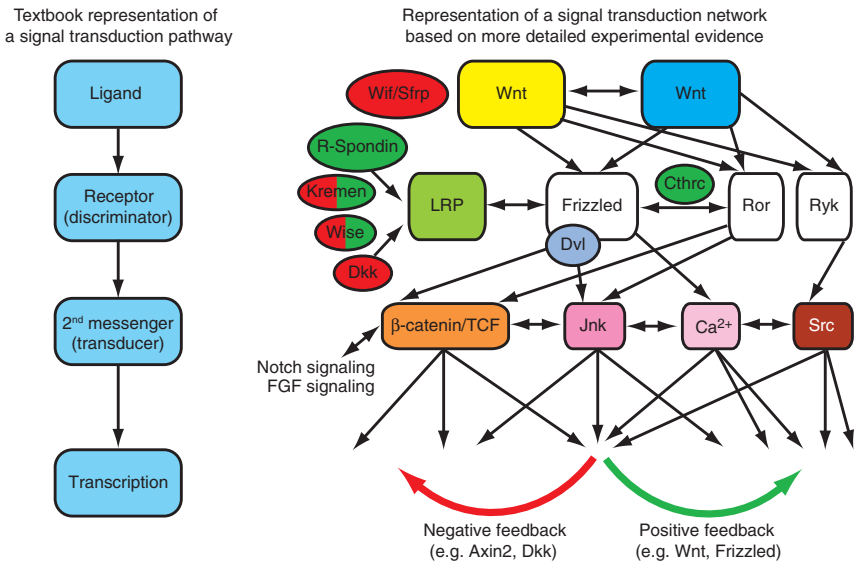

Fig. 2. Signal transduction networks. Textbook molecular biology ascribes that a signal transduction cascade begins with the binding of an extracellular ligand to a membrane receptor, after which cytoplasmic second messengers transduce the signal to the nucleus, resulting in the transcription of a given set of target genes. In reality, the situation is not that simple, and complications exist at practically every level. A wide variety of ligands exists. The mammalian genome, for instance, encodes 19 different Wnt ligands. The same holds true at the receptor level. Multiple receptor homologs (ten Frizzled receptor homologs in mammals), different receptor families and additional co-receptors further diversify the signal transduction cascade. To think of intracellular signaling events as linear pathways is another over-simplification. In reality, biology does not follow a straight line. Signals can branch off at virtually every step, modifiers can represent a parallel input that affects the outcome of signal transduction independently of extracellular ligand stimulation, intense cross-talk (depicted by double-headed arrows) exists between different signaling cascades, and feedback mechanisms provide an additional layer of control. So, although we often still think in very linear terms, we are actually dealing with ever-evolving and intertwined signal transduction networks in which multiple inputs are integrated at virtually every level.

distribution of LRP6 observed in cells that undergo convergent extension movements (Tahinci et al., 2007) suggests that close observation might be required to reveal subtle differences that could have important developmental consequences.

Conclusions

For many years, most of our efforts in studying Wnt signal transduction have focused on resolving the biochemical nature of downstream signaling events. In the past decade, however, attention has shifted back to the plasma membrane. How all the various pieces of information obtained from these studies fit together in the puzzle of a complete signal transduction network within the context of a living organism remains to be resolved. These studies have revealed the existence of extensive cross-talk between the numerous ligands, receptors, co-receptors and additional regulators, as well as between downstream intracellular messengers. As a result, the outcome of a given Wnt signal is highly unpredictable. As much as we still like to think of signal transduction pathways in terms of linear cascades and stable, well-defined interactions, reality teaches us that things are more flexible, dynamic and, as a result, more complicated (Fig. 2). This is especially true in an intact organism, where cells exist in the context of complex tissues in which numerous cell types communicate with one another. An important feature of a system that depends on the proper integration of multiple inputs is its sensitivity to small changes in the dose or gradient of extracellular factors, as well as to the absolute levels of the different receptors and co-receptors involved. This realization brings with it the requirement for a new way of thinking about signaling events that also integrates more advanced experimental analyses and novel bioinformatic modeling tools (Kestler and Kuhl, 2008).

As the response to a specific signal is cell type specific and context dependent, we urge caution in concluding which particular molecular components are recruited for this purpose within a cell. Whereas multiple tools exist to monitor the involvement of β-catenin/TCF signaling, many of the other downstream signaling events still lack specific read-outs. We strongly emphasize that we should no longer think of Wnt signal transduction in terms of linear pathways, either intracellularly or extracellularly. As helpful as breaking down the signaling cascade into different pieces has been for resolving the functions of its core components, it is now time to see Wnt signal transduction for what it truly is: a complex network of protein interactions, with multiple outcomes, cross-talk and regulatory inputs at practically every level.

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