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Review

Generating Cellular Diversity and Spatial Form: Wnt Signaling and the Evolution of Multicellular Animals

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There were multiple prerequisites to the evolution of multicellular animal life, including the generation of multiple cell fates ("cellular diversity") and their patterned spatial arrangement ("spatial form"). Wnt proteins operate as primordial symmetry-breaking signals. By virtue of their short-range nature and their capacity to activate both lineage-specifying and cell-polarizing intracellular signaling cascades, Wnts can polarize cells at their site of contact, orienting the axis of cell division while simultaneously programming daughter cells to adopt diverging fates in a spatially stereotyped way. By coupling cell fate to position, symmetry-breaking Wnt signals were pivotal in constructing the metazoan body by generating cellular diversity and spatial form.

When the first animals (metazoans) arose from their unicellular predecessors (some 600 million or more years ago), fundamental symmetry-breaking events must have reshaped aggregates of uniform eukaryotic cells into axially patterned bodies containing multiple cell types. Some eukaryotes display temporary multicellularity and as such are considered the closest living relatives to metazoans. By way of example, choanoflagellates and filastereans can form multicellular colonies, albeit without predetermined spatial organization (Dayel et al., 2011; Sebé-Pedrós et al., 2013; Wainright et al., 1993). So how did spatial pattern incipiently emerge in the earliest metazoa as they took shape and form from their unicellular ancestors?

The spatially organized body plan of animals is often ascribed to positional regulators including *Hox* genes. Nonetheless, an organized body plan likely predated *Hox* genes (Larroux et al., 2007; Martindale, 2005), implying a more ancient patterning signal at the dawn of metazoans. Genomic sequencing of the most ancient (so-called basal or early-branching) metazoans has provided insight into the origins of patterning. The genomic inventory of comb jellies (ctenophores, arguably the earliest extant metazoa [Moroz et al., 2014; Ryan et al., 2013]) suggests that they lacked *Hox* genes (Ryan et al., 2010) as well as familiar developmental signaling pathways including FGF (fibroblast growth factor) and Hedgehog. However, the Wnt and transforming growth factor β (TGF-β) pathways were apparently present in the developmental repertoire of these patterned animals (Moroz et al., 2014; Pang et al., 2010; Ryan et al., 2013) in addition to other pre-bilaterians including poriferans (Adamska et al., 2007), placozoans (Srivastava et al., 2008), and cnidarians (Hobmayer et al., 2000; Wikramanayake et al., 2003). Hence, the Wnt pathway was absent from unicellular organisms but arose concurrently with the first metazoans, being present in all extant metazoans: only organisms with a patterned body axis harbor a complete Wnt pathway.

Wnt proteins spatially organize multicellular organisms by implementing the primary body axis during embryonic development (variously referred to as the animal-vegetal, anterior-posterior, oral-aboral, or dorsal-ventral axis, depending on the animal in question) (Holstein, 2012; Holstein et al., 2011; Niehrs, 2010; Petersen and Reddien, 2009). Irrespective of species-specific idiosyncrasies, the primary body axis is the first axis in the nascent embryo that scaffolds the addition of subsequent lineages and tissues. Moreover, the pole of the primary body axis specified by high Wnt signals becomes the blastopore, from which the endoderm and mesoderm emanate during gastrulation (Martindale and Hejnol, 2009).

Here we argue that Wnt proteins were capable of acting as evolutionary symmetry-breaking signals due to two of their distinctive features: (1) their short-range nature and (2) their capacity to simultaneously activate lineage-specifying as well as cell-polarizing intracellular signaling pathways (which we refer to here as the "cell-fate" and "cell-polarity" cascades). Hence, a local source of Wnt can spatially polarize a cell, orienting it to divide asymmetrically and generating progeny of two different fates in a spatially arrayed fashion (Goldstein et al., 2006; Habib et al., 2013; Rocheleau et al., 1997). By simultaneously specifying the identity and position of a cell (through the cell-fate and cell-polarity pathways, respectively), Wnt proteins coupled the generation of lineage diversity with the definition of spatial form. This culminated in spatially coordinated specification of cellular identity—a key prerequisite for a patterned body plan.

Wnt: A Primordial Means of Short-Range Communication

The Wnt pathway (Figure 1) is a short-range extracellular communications system. In brief, it consists of a lipid-modified ligand (Wnt) produced by a secreting cell, its cognate

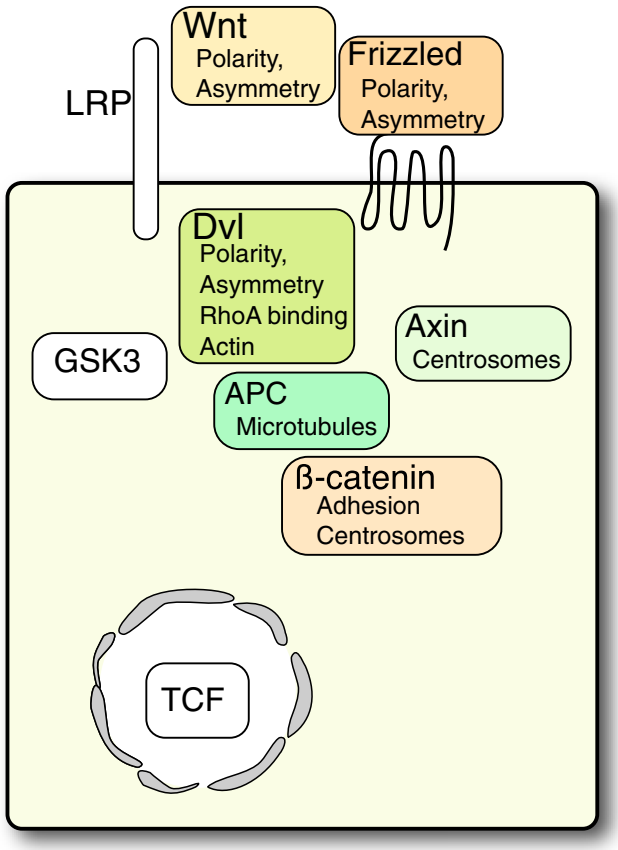

Figure 1. The Integrated Wnt Cell-Fate and Cell-Polarity Pathways
Components traditionally associated with the Wnt signaling pathway also have pivotal roles in the various aspects of cell polarity and asymmetry. When a Wnt protein engages a Frizzled receptor, Disheveled (Dvl) is intracellularly recruited to Frizzled. By genetic and cell biological evidence, each of these have been implicated in cell polarity and asymmetry (Wu et al., 2013; Sugioka et al., 2011). Many of the same components also have important roles in cell polarization in the Wnt cell-polarity cascade, including redeployment of the actin cytoskeleton via RhoA activation, among other proposed mediators (Habas et al., 2001; Witze et al., 2008). Several other Wnt signaling components, such as APC, Axin, and β-catenin, interact with the cellular organization and adhesion machinery (Nelson and Nusse, 2004; Fumoto et al., 2009; Barth et al., 2008). Hence, core Wnt pathway components often function both in cell-fate specification and cell polarity, indicating that the cell-fate and cell-polarity pathways are intertwined with respect to their molecular executors.

receptor/co-receptors (including Frizzled) on a receiving cell, and downstream transduction components (including Disheveled) that enable the receiving cell to functionally respond.

Among various developmental signals, Wnt proteins are somewhat idiosyncratic because they are mostly short-range signals (Alexandre et al., 2013; Farin et al., 2016). This might be attributable to their covalent lipid modification in the form of a palmitoleate group (Takada et al., 2006; Willert et al., 2003). This hydrophobic appendage might limit the diffusion of Wnt proteins in the hydrophilic extracellular space. The lipid is appended by a dedicated enzyme (Porcupine, which is evolutionarily as ancient as Wnt itself [Pang et al., 2010]) and is crucial for Wnt ligands to engage their cognate receptors and thus to signal (Janda et al., 2012; Takada et al., 2006).

Although Wnt proteins are often construed as long-range diffusible morphogens, recent work implies that they might largely constitute membrane-bound signals acting between neighboring cells that are touching each other. Strikingly, in *Drosophila melanogaster*, the signaling activity of the Wnt protein wingless can be largely afforded by a non-diffusible, membrane-tethered form of the protein (Alexandre et al., 2013). Likewise, in mouse intestinal organoid cultures endogenous Wnt3 protein only transfers between cells that are directly touching (Farin et al., 2016).

Hence, longer-range activities ascribed to Wnt may be secondary effects, caused by sequential signaling between Wnt target cells and their neighbors, migration of Wnt-emitting cells toward distal targets (Serralbo and Marcelle, 2014), staggered expression of multiple Wnt genes across a broad field of cells (Kusserow et al., 2005), or dilution of Wnt protein that is tethered to a Frizzled receptor-expressing cell as it moves and divides (Farin et al., 2016). Alternatively, membrane-tethered Wnt proteins might be transported over longer ranges by distribution through vesicles (Gross et al., 2012; Korkut et al., 2009) or conveyance on long-range cellular projections such as axons or cytonemes/filopodia (Korkut et al., 2009; Stanganello et al., 2015).

The emergent view is that short-range Wnt signals prevalently feature in diverse cellular venues. Consequently, when cells signal to each other at close range, the orientation of signaling on a micro-scale will have polarizing consequences for the target cells, in such a way that the cells become asymmetric along the axis of signaling, the evolutionary consequences of which we explore below.

Reconciliation of Wnt Cell-Fate and Cell-Polarity Pathways

Activation of Wnt receptors triggers a number of downstream signaling cascades. Often thought to be mutually exclusive, these cascades were variously referred to by divergent nomenclature in the past (e.g., "canonical" versus "non-canonical" Wnt signaling). For the purpose of this review, we broadly categorize these pathways into "cell-fate" or "cell-polarity" cascades (Figure 1), which need not be mutually exclusive or have opposing biological roles. Rather, they may work coordinately toward a singular biological objective.

The cell-fate pathway (alternatively referred to as the "Wnt/β-catenin pathway" or the "canonical Wnt pathway") is the best studied and is activated when Wnt ligands bind both the Frizzled (Fzd) receptor and the Lrp5/6 co-receptor and ligate them together, leading to propagation of Wnt signaling beyond the plasma membrane. The core cytoplasmic unit of this pathway is the so-called destruction complex, which controls the turnover of the armadillo-repeat protein β-catenin. In metazoans this complex contains two kinases (GSK3 and CKI) and is constitutively active, leading to the phosphorylation and thus continuous degradation of β-catenin. Upon Frizzled-Lrp5/6 receptor complex formation at the plasma membrane, Disheveled (Dvl) is recruited to Fzd, leading to inhibition of the destruction complex. This results in the stabilization of β-catenin, whereupon it enters the nucleus and, in conjunction with Tcf/Lef transcription factors, transcriptionally activates a Wnt target agenda, often leading to the specification and/or maintenance of cell fate.

Box 1. The Wnt Cell-Polarity Pathway

Different Wnt proteins display remarkable functional diversity, in terms of both biological effect and molecular signaling mechanisms. Dissecting the activities of individual Wnt proteins is complicated by the existence of multiple Fzd homologs and, like the *Wnt* genes themselves, these *Fzd* genes show dynamic spatiotemporal expression patterns in vivo. Moreover, at least some Wnt signaling activities are mediated through (or in concert with) non-Frizzled proteins, such as the Ryk and Ror receptor tyrosine kinases and/or in concert with Lrp co-receptors (Angers and Moon, 2009; Green et al., 2014; Ho et al., 2012; van Amerongen, 2012). Wnt cell-polarity signaling often proceeds independently of β-catenin, and subsumes a number of diverse intracellular effectors whose precise activities and interrelationships have not yet been fully resolved, owing to the lack of experimental systems enabling robust in vitro readouts. However, it is evident that Wnt cell-polarity signaling activities play crucial roles in vivo, most predominantly in controlling planar cell polarity (PCP), convergent extension (CE), and directed cell migration. For example, in the gastrulating amphibian embryo, Wnt5b and Wnt11 engage Ryk and Fzd7 to control convergent extension movements via small Rho GTPases RhoA and Def6, respectively (Goudevenou et al., 2011; Habas et al., 2001; Kim et al., 2008; Xu et al., 2014). In mice, Wnt5a and Ror2 control elongation of multiple tissues, including the embryonic limb and intestine (Cervantes et al., 2009; Gao et al., 2011; Ho et al., 2012; Yamada et al., 2010). Operation of the PCP pathway is evolutionarily ancient, being crucial for embryonic elongation even in cnidarians (Momose et al., 2012).

Therefore, Wnt cell-polarity pathway signaling responses are best studied in a complex, multicellular environment. As an example, the molecular mechanism underlying the establishment of PCP has been revealed in quite some detail by extensive genetic studies in both the fly wing and the mammalian inner ear (Montcouquiol et al., 2003, 2006; Qian et al., 2007). Like Wnt/β-catenin signaling, the central core of the PCP pathway also includes Fzd and Dvl (Figure 1). Rather than interacting with the GSK3/Axin/APC machinery that controls β-catenin turnover, however, Fzd and Dvl operate together with the dedicated PCP proteins Vangl/strabismus, Celsr/flamingo, and Prickle to coordinate cell orientation within the plane of the tissue.

Historically, individual Wnt proteins (and receptors, for that matter) were thought to exclusively activate either β-catenin-dependent signaling or β-catenin-independent responses. Yet it has become clear in the past decade is that individual Wnt proteins should no longer be classified as "canonical" or "non-canonical" to reflect their capacity to elicit a β-catenin-dependent or -independent response, respectively (van Amerongen et al., 2008; van Amerongen and Nusse, 2009). While some ligands (e.g., mammalian Wnt3a, the prototypical "canonical" Wnt) appear to be invariably associated with Wnt/β-catenin signaling, in most cases activity is determined by the receptor context. By way of example, Wnt5a (once the prototypic "non-canonical" Wnt) can elicit a variety of β-catenin-dependent and -independent responses, depending on whether it engages Ror2, Fzd4/Lrp5, or Fzd2 (Mikels and Nusse, 2006; Ring et al., 2014; Sato et al., 2010; van Amerongen et al., 2012). Taken together, the present experimental evidence thus suggests that the receptor context at the cell surface is largely responsible for dictating the signaling activity of a given Wnt protein. Which precise intracellular signaling events are activated in response to a Wnt signal is likely contingent on the exact Wnt protein and the complement of available receptors and co-receptors. This may additionally vary between tissues of a single species, and we thus propose that efforts to delineate individual distinct Wnt responses may ultimately prove futile in a complex developmental context.

The Wnt cell-polarity pathway (Box 1) is considerably more diverse in its intermediate effectors and final biological outcomes (which include orientation of cell division, planar cell polarity [PCP] [Wu et al., 2013], and/or convergent extension [CE]). However, we suggest the unifying term "cell-polarity pathway" because these biological outcomes converge on a common endpoint: the spatial polarization of a cell and the alignment of cell division, cellular migration, or cellular elongation (Gros et al., 2009) along that newly imposed spatial axis. In the cell-polarity pathway, Wnt engagement of Fzd similarly leads to Dvl recruitment through the same domain employed in the cell-fate pathway (Wu et al., 2008). Dvl action subsequently polarizes a cell through a multiplicity of intracellular pathways, independently of β-catenin. This can occur via the positioning of centrosomes at opposite ends of the cell (thus orienting cell division) (Habib et al., 2013), or through activation of downstream RhoA (Habas et al., 2001), JNK (Boutros et al., 1998), and/or Ca^{2+} signaling cascades (Angers and Moon, 2009), thus directing cell orientation and migration. While diverse in their exact molecular mechanism, these molecular intermediaries culminate in an important phenotypic outcome: spatial cellular polarization.

We assert that the historical division of Wnt signaling cascades into mutually exclusive "canonical"/β-catenin-dependent or "non-canonical"/β-catenin-independent pathways may be an artificial dichotomy, as it is clear that acting on a single cell, a localized Wnt signal can control both cell polarity and fate (Habib et al., 2013; Rocheleau et al., 1997) (discussed below; Box 2). This implies that Wnts can concurrently evoke both the cell-fate and cell-polarity pathways, concomitantly informing a cell of both its identity and position; this coupling may have been pivotal for the establishment of a patterned body plan. Indeed, the Wnt cell-fate and cell-polarity pathways share common components (most notably Fzd and Dvl) and thus appear intimately linked (Figure 1). This intertwined relationship was functionally necessitated by the fact that in a complex tissue, cells do not only need to acquire information with respect to their position and identity but are also required to properly orient themselves with respect to their neighbors, as we argue below.

Evolutionary Provenance of Core Wnt Pathway Components

Whence did the metazoan Wnt signaling pathway emerge? It seems to be a tapestry interwoven from multiple weaves, each with different origins (Figure 2): during the course of evolution, Wnt proteins appear to have commandeered existing signaling

Box 2. Unifying the Wnt Cell-Fate and Cell-Polarity Pathways

Although the prevailing view of Wnt signaling has grown to one that distinguishes between distinct molecular responses, there is no reason why either one or the other pathway should be exclusively activated. This is exemplified by how a Wnt signal can simultaneously activate both cell-fate and cell-polarity pathways in a single given cell (Habib et al., 2013; Rocheleau et al., 1997), and we hypothesize that complex metazoan tissues were able to arise precisely because these related Wnt signaling responses acted in concert. While various studies reported crosstalk or reciprocal inhibition between the Wnt/β-catenin and the PCP/CE pathways at the signal transduction level (mediated by Lrp6 [Allache et al., 2014; Andersson et al., 2010; Bryja et al., 2009; Gray et al., 2013; Tahinci et al., 2007], Inversin [Simons et al., 2005], or Ptk7 [Bin-Nun et al., 2014]), the phenotypic evidence that a localized Wnt signal can both spatially polarize a cell and concomitantly determine the fate of its daughters (Habib et al., 2013; Rocheleau et al., 1997) indicates that both cell-fate and cell-polarity pathways may be active in simultaneity. Indeed in early *Xenopus* development, the blastomere with the highest level of Wnt/PCP signaling also transduces the highest levels of Wnt/β-catenin signaling (Ohkawara and Niehrs, 2011). Finally and most importantly, the cell-polarity pathway need not always adhere to the definition of PCP/CE cascade, but also includes other β-catenin-independent signaling mechanisms (e.g., orientation of the plane of mitotic division) such as those employed in *C. elegans* asymmetric cell divisions (Rocheleau et al., 1997).

modules to take control of cell polarity and differentiation in an oriented fashion.

In order to signal, Wnt ligands physically engage the cysteine-rich domain (CRD) of their cognate Fzd receptors. They do so through two discrete contacts: a palmitoleate-modified "thumb" (the D1 domain) and an "index finger" (the D2 domain), which grasp opposite ends of the Fzd CRD (Janda et al., 2012). Additionally Wnt also simultaneously binds co-receptors including Lrp5/6, juxtaposing Frizzled and Lrp5/6 to activate Wnt/β-catenin signaling (Bilic et al., 2007; Kim et al., 2013; Metcalfe et al., 2010). The palmitoleate group is crucial for Wnt ligands to interact with Fzd, as it extends from the D1 "thumb" and inserts itself into the lipid-binding pocket of the Fzd CRD (Janda et al., 2012).

The key requirement of the lipid modification in receptor binding implies that ancestrally, Wnt proteins may originally have signaled via a lipid-sensing signaling pathway. Indeed, lipid signals are a primary means of communication in primitive organisms, including bacteria (Kearns and Shimkets, 2001) and choanoflagellates (Alegado et al., 2012). Although the Wnt ligand in toto is a metazoan innovation, it probably arose from a fusion of earlier precursors: the D2 "index finger" resembles various cytokines, whereas the palmitoleate-modified D1 "thumb" is ancestrally related to the saposin fold, an ancient lipid-binding domain (Bazan et al., 2012) (Figure 2A). As proposed by Bazan et al. (2012), an early Wnt may have been able to acquire a lipid through its inherently lipid-seeking D1 domain, and by further gaining the capacity to covalently bind a lipid (via Porcupine) the Wnt protein may have been able to appropriate a primordial lipid-responsive pathway for its own purpose. Interestingly, a sulfonolipid physiologically elicits choanoflagellate eukaryotic cells to form multicellular colonies (Alegado et al., 2012), intimating a relationship between an ancient lipid signaling pathway and multicellularity.

Fzd receptors themselves have an evolutionary origin that predates metazoans. The non-metazoan eukaryotic slime mold *Dictyostelium* harbors two Fzd-like G-protein-coupled receptors (FslJ and FslK; Figure 2A) (Prabhu and Eichinger, 2006), which harbor both a CRD domain and the KTXXXW protein motif, the latter of which is essential for Dvl binding and thus Wnt signaling in metazoa (Wong et al., 2003). Nonetheless, because no upstream Wnt-like molecules are known to exist in *Dictyostelium* (or, for that matter, Disheveled itself [Dillman et al., 2013]), these Fzd-like receptors were not subservient to Wnt and their functional roles remain enigmatic. In fact, the Fzd CRD domain has been suggested to have arisen from a cyclic AMP receptor early in the eukaryotic lineage, suggesting that it may have been an early directional sensor involved in chemotaxis/directed cell migration (Krishnan et al., 2012; Pei and Grishin, 2012).

Dictyostelium also harbors another ancestral subcomponent of the Wnt pathway: a GSK3/β-catenin subcircuit. While not directly a multiprotein destruction complex, this module in *Dictyostelium* contains a GSK3 homolog (GskA) (Plyte et al., 1999) that functionally controls a β-catenin homolog (Aar) (Grimson et al., 2000), thereby regulating cell "fate" (Figure 2A). In addition, Aar also functions in cell adhesion (Grimson et al., 2000), similar to β-catenin in metazoans. In fact, although *Dictyostelium* lacks cadherins, Aar is required for the establishment of epithelial (i.e., apical-basal) polarity together with an α-catenin homolog (Dickinson et al., 2011; Weis et al., 2013). Importantly, this suggests an early evolutionary origin of the β-catenin/α-catenin module in regulating epithelial cell polarity predating metazoans.

Taken together, we propose that upon emergence of Wnt proteins in a common metazoan ancestor, Wnt signals were able to seize Fzd receptors and in doing so commandeered pre-existing cellular machinery that controlled epithelial polarity and cell fate (GSK3/β-catenin), thereby providing cells with positional information and enabling formation of a complex body plan (Figures 2B and 2C). This notion might be testable through an "evolutionary re-engineering" approach, for instance by ectopically expressing a mammalian Fzd and Dvl in *Dictyostelium* and testing whether treatment with Wnt proteins leads to recruitment of the GSK3/β-catenin subcircuit, as assayed by redistribution of Aar/β-catenin from cell junctions (Grimson et al., 2000) to the nucleus.

Axial Patterning by Wnt: Emergence of Metazoan Asymmetry

The emergence of metazoa necessitated the genesis of multiple ($\geq$2) cell types and their organized spatial deployment along an embryonic axis, known as the primary body axis (Niehrs, 2010; Petersen and Reddien, 2009). Consensus holds that the first bodily asymmetry in metazoa was the separation of the body into a prospective "head" (anterior region) and "tail" (posterior

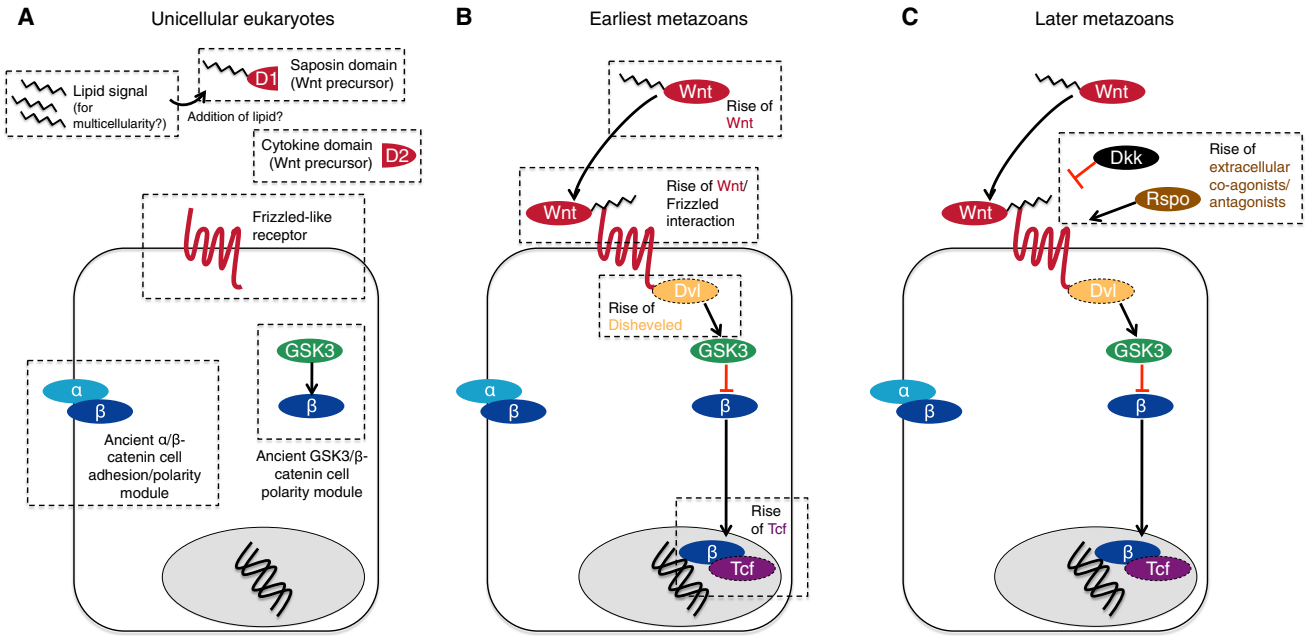

Figure 2. Ancestral Molecular Building Blocks of the Wnt Signaling Pathway

(A) Several independent fragments of the future Wnt signaling pathway existed in unicellular organisms: their ancestral remit was largely to control cell polarity and cell adhesion, before the emergence of animals. These isolated components included an ancient pairing of β-catenin with α-catenin that controlled cell adhesion and polarity (Dickinson et al., 2011; Weis et al., 2013); a subcomponent featuring GSK3, which likely activated β-catenin (Grimson et al., 2000) to control multicellular aggregation in *Dictyostelium* (Plyte et al., 1999); as well as a Frizzled-like receptor of unknown function, which already contained a lipid-binding CRD domain as well as a Disheveled-interacting domain (despite the lack of Disheveled). Lipid signals were important for extracellular communication and controlled multicellular aggregation (Alegado et al., 2012). A lipid-binding saposin domain (bearing resemblance to the D1 "thumb" of the future Wnt ligand) may have been able to acquire a lipid (Bazan et al., 2012) and therefore commandeer a lipid-sensing signaling pathway. Separately, the other half of the future Wnt ligand, "D2," likely arose from some type of cytokine molecule (Bazan et al., 2012).

(B) Contemporaneous with the rise of the first metazoa, the Wnt signaling pathway was instantiated as the whole of several previously disparate pieces. Gene fusion of the Wnt D1 and D2 domains generated a lipidated signaling molecule capable of binding Frizzled with high affinity (Bazan et al., 2012). With the rise of the Wnt-Frizzled interaction, Frizzled was now able to recruit the metazoan-specific Disheveled protein and in so doing, activate intracellular Wnt signaling responses. With the emergence of metazoan-specific Tcf protein(s) (Pang et al., 2010), β-catenin could exert transcriptional regulation in the nucleus.

(C) In higher metazoa, Wnt pathway activity was refined through the gain of multiple novel components, including diffusible co-agonists (e.g., R-spondins) as well as diffusible antagonists (e.g., Dkk1).

region) (Niehrs, 2010). Here we use the terms "head" and "tail" with liberty, as these structures vary considerably depending on the exact geometry of the animal in question. Although the primary body axis does not always formally correspond to the anterior-posterior axis of the future adult (Stern et al., 2006), the "posterior" pole of the primary body axis is the conserved site where gastrulation begins, namely, the location of the blastopore, the originator of the endoderm/mesendoderm germ layers (Martindale and Hejnol, 2009). It is now clear that the organization of a primary body axis is respected throughout all extant animals and was the ancient statute of bodily organization even in early pre-bilaterians (Niehrs, 2010).

Transcending morphological differences between animal species, in all extant metazoa it seems that Wnts are almost exclusively expressed at the developing posterior domain (Figure 3), where they specify the posterior fate and likely fulfilled the role of the primordial "symmetry-breaking" signal. In so doing they established the primary body axis (Holstein, 2012; Holstein et al., 2011; Niehrs, 2010; Petersen and Reddien, 2009). Wnts are invariantly first expressed at the posterior end of the body even in early pre-bilaterians such as the radially symmetric sponges (Adamska et al., 2007), *Hydra* (Hobmayer et al., 2000), and sea anemones (Kusserow et al., 2005), where the oral pole constitutes the posterior domain (Figure 3). Posterior Wnt expression along the primary body axis is sustained in bilateria, planarian tail (Petersen and Reddien, 2008), posterior spider blastomeres (McGregor et al., 2008), vegetal sea urchin blastomeres (Wikramanayake et al., 2004), dorsal *Xenopus* blastomeres (Tao et al., 2005) and finally the mouse posterior epiblast/primitive streak (Liu et al., 1999) (Figure 3).

Accordingly, nuclear β-catenin activity at the prospective posterior end of the embryo is often evident within the first few cell divisions of development in early blastomeres. Such localized nuclear β-catenin distribution is frequently the earliest sign of polarity within the body proper and presages the overt emergence of the primary body axis, as evinced in the anemone *Nematostella* (Wikramanayake et al., 2003), sea urchins (Logan et al., 1999), and sea stars (McCauley et al., 2015), as well as some vertebrates (Larabell et al., 1997). A striking example is *Platynereis*, wherein after virtually each mitotic cell division in the early embryo, nuclear β-catenin is consistently activated in the posterior daughter cell, indicating that Wnt activity is an iterative marker of posterior positional identity across developmental space and time in different lineages (Schneider and Bowerman, 2007).

The ancestral role of Wnt in early metazoa was to establish the posterior pole of the primary body axis, thus breaking symmetry,

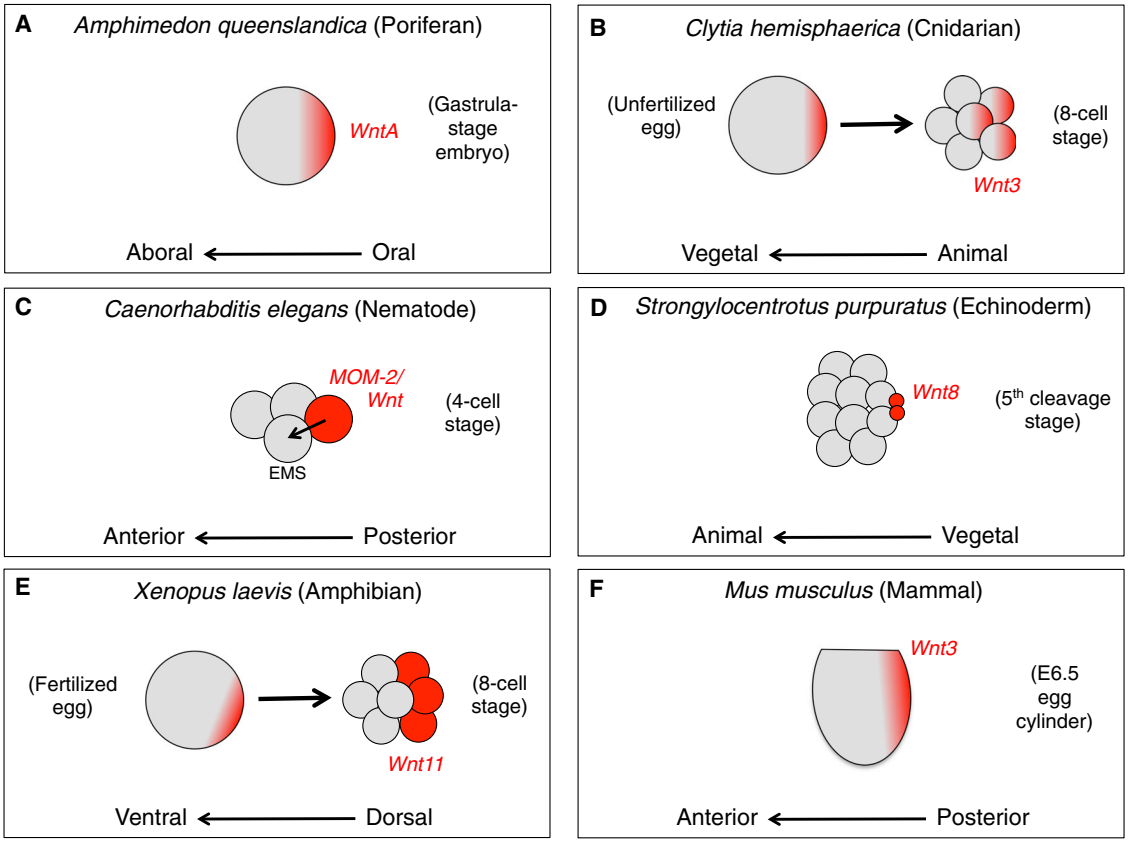

Figure 3. Posterior Symmetry Breaking by Wnt across Diverse Phyla

Asymmetric Wnt signaling becomes prevalent by the earliest stages of embryogenesis in diverse animal phyla. Often this is one of the earliest molecular asymmetries in the developing early embryo, and serves to set up the primary body axis.

(A) In the sponge *Amphimedon queenslandica*, the *WntA* gene is asymmetrically expressed along the primary body axis at the prospective oral (posterior) pole of the early gastrula-/gastrula-stage embryo (Adamska et al., 2007).

(B) Starting in the unfertilized egg of the cnidarian *Clytia hemisphaerica*, *Wnt3* mRNA is maternally localized to the prospective animal (oral) pole, and after cleavage divisions is inherited by animal blastomeres wherein it is necessary for establishing the primary body axis and future oral fates (Momose et al., 2008).

(C) At the 4-cell stage of nematode *C. elegans* embryogenesis, *MOM-2/Wnt* expressed by the P2 blastomere signals to the adjacent EMS blastomere and polarizes it such that it divides along the anterior-posterior axis. When it does so, the nearest Wnt-proximal daughter forms endoderm (E), whereas the further Wnt-distal daughter generates mesoderm (MS) (Rocheleau et al., 1997).

(D) At the 16-cell stage of development of the sea urchin *Strongylocentrotus purpuratus*, *Wnt8* is first expressed by the vegetal blastomeres (micromeres) (Wikramanayake et al., 2004).

(E) In the fertilized egg of *X. laevis*, maternal *Wnt11* mRNA is largely constrained to the dorsal-vegetal domain due to a combination of maternally pre-localized mRNA deposition and cortical rotation after sperm entry. As a consequence, *Wnt11* mRNA is preferentially inherited by dorsal-vegetal blastomeres, leading to nuclear β-catenin activity in dorsal blastomeres and prefiguring the primary body axis (Tao et al., 2005).

(F) Within the pluripotent epiblast of the embryonic day ~6.5 mouse embryo, *Wnt3* is exclusively expressed at the posterior pole of the primary body axis, where it induces posterior epiblast to differentiate into the primitive streak (the originator to endoderm and mesoderm) (Liu et al., 1999).

positioning the site of gastrulation, and ultimately permitting the emergence of a patterned body plan (Holstein, 2012; Holstein et al., 2011; Niehrs, 2010; Petersen and Reddien, 2009). Even in *Hydra*, an early pre-bilaterian, hyperactivation of Wnt causes ectopic tentacles (posterior tissue) to dramatically sprout from the body column (Broun et al., 2005). Similarly, upon head amputation planarian flatworms will typically regenerate a head, yet ectopic Wnt signaling will cause a tail to form in lieu of a head (Gurley et al., 2008), providing further evidence for the ubiquity of Wnt as a posteriorizing signal.

In amniotes, the earliest morphological sign of anterior-posterior polarity within the embryo proper is the formation of the primitive streak (blastopore) at the posterior end of the pluripotent epiblast, which is the site of endoderm/mesoderm formation. Mice mutant for *Wnt3*, *Lrp5*;*Lrp6*, or *β-catenin* fail to form a primitive streak (Huelsken et al., 2000; Kelly et al., 2004; Liu et al., 1999), lack endoderm and mesoderm as a consequence, and therefore enter immediate developmental arrest because a posterior fate cannot be imposed upon pluripotent cells. Therefore, Wnt is fundamentally important in breaking symmetry and organizing the primary axis, without which the body could not form. Wnt predates the "*Hox* code" (Larroux et al., 2007; Martindale, 2005) and other developmental patterning signals, and thus appears to constitute the original ancestral program for primary body axis specification.

Wnt as a Posterior Organizer Signal

The posterior patterning function of Wnt may have been availed by its lipid modification. Wnt signals evolutionarily predated their counterparts, diffusible Wnt antagonists including Dkk (Nichols

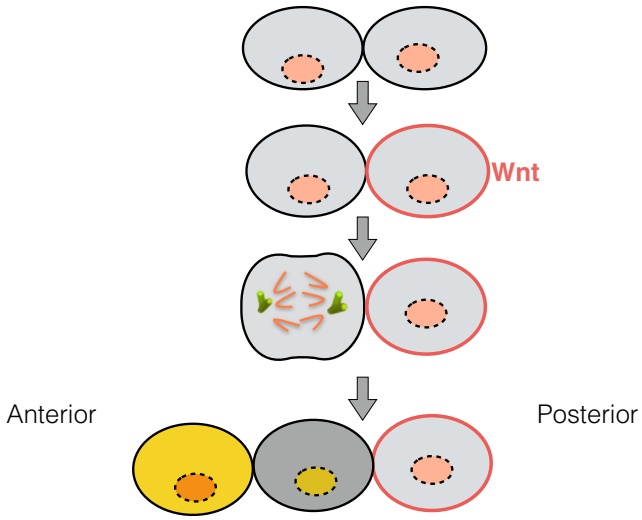

Figure 4. Coordinated Lineage Specification and Spatial Polarization by Wnt

In a generalized developmental venue, Wnt proteins are expressed by a presumptive posterior cell (depicted on the right) and signal to a directly adjacent cell, concordantly specifying the fates and positions of its daughters. At the point of contact with a Wnt signal, a cell becomes polarized and its centrosomes deployed to opposite poles of a cell, orienting its axis of mitotic division while simultaneously redistributing Wnt cell-fate pathway components in an asymmetric way. Therefore upon cell division, the two daughters attain different fates and do so in a spatially stereotyped way, relative to their proximity to the Wnt signal. In this fashion, cellular position is directly coupled with cellular identity.

et al., 2006; Ryan et al., 2013). If Wnts could be conveyed over long ranges (and initially went unopposed by Dkk), this would beg the question of why, within the confines of an early developing metazoan embryo, would not all cells be subject to Wnt and thus posteriorized? The short-range nature of Wnt ligands provides an expedient solution. By virtue of their restricted production, lipid modification, and limited range, localized Wnt signals concentrated at the posterior end of the embryo would only posteriorize nearby cells (Figure 4). Cells out of the spatial reach of Wnt could then adopt a separate, anterior fate. While the spatial distribution of Wnt proteins themselves in early embryos has yet to be rigorously established, the available evidence indicates that Wnt11 protein is asymmetrically distributed along the future primary (dorsoventral) body axis among distinct *Xenopus* blastomeres (Schroeder et al., 1999), corroborating the notion of a spatially constrained posterior Wnt signal.

Spatially localized pre-deployment of Wnt activity to one side of the developing embryo is therefore the decisive step in specifying the prospective "posterior" end of the embryo and establishing the primary body axis. Posterior deployment of Wnt signaling in the early embryo is often a maternally imposed developmental directive that pre-figures the future embryonic body axis. For instance, in the cnidarian *Clytia*, maternal *Wnt3* and *Fzd1* mRNAs are spatially confined to the animal pole of the unfertilized egg (corresponding to the posterior end of the future primary body axis; Figure 3B) (Momose et al., 2008; Momose and Houliston, 2007). Therefore, after fertilization and cleavage divisions, *Wnt3* and *Fzd1* mRNAs are principally inherited by blastomeres derived from the animal pole (Figure 3B), and activation of Wnt/β-catenin signaling in such cells leads these Wnt-responding blastomeres to subsequently form endoderm and thus the posterior territory of the primary body axis (Momose et al., 2008; Momose and Houliston, 2007). In summary, spatial confinement of Wnt activity to the prospective "posterior" territory is often a maternally imposed directive in diverse species (Lee et al., 2007; Miller et al., 1999; Tao et al., 2005; Weitzel et al., 2004), and the short range of Wnt signaling therefore enables spatial partitioning of an "anterior" versus "posterior" embryonic compartment along the primary body axis.

Once deployed to one end of the prospective body, Wnt behaves as a fundamental posterior organizer signal, as it is sufficient to initiate organization of complex posterior tissues composed of multiple cell types. For instance, an ectopic Wnt signal is sufficient to specify a fully-fledged tail in planaria (Gurley et al., 2008), to generate a tentacle in *Hydra* (Broun et al., 2005), and to induce a second, perfectly formed dorsoventral axis in *Xenopus* (McMahon and Moon, 1989). To induce a fully organized tissue, Wnt must be upstream of complex programs for lineage specification and spatial tissue morphogenesis and must coordinate (or initiate) these processes. For instance, in *Hydra* tentacle development Wnt both imposes a posterior (tentacle) fate upon epithelial cells (through β-catenin) and then subsequently, through JNK signaling, triggers the evagination and movement of these cells, leading to the morphogenetic emergence of a tentacle, in both fate and in shape (Philipp et al., 2009).

How a complex tissue of diverse lineages can be assembled by a singular signal (Wnt) remains to be fully resolved, but one emergent principle is that Wnt often induces the formation of so-called organizer tissue, cells that behave as signaling centers and autonomously self-organize nearby tissues to explicitly assume their proper fates. Indeed in *Hydra*, Wnt elicits the formation of a posterior organizer capable of coercing nearby cells to form a tentacle (Gee et al., 2010). Of particular significance, during early developmental specification of the primary body axis by Wnt, the Wnt-induced blastopore region is inextricably endowed with Spemann organizer activity (Martindale and Hejnol, 2009), enabling it to self-organize the assembly of the appropriate tissues at the posterior pole of the primary body axis (Niehrs, 2004). This therefore causally explains why ectopic *Wnt1* expression in *Xenopus* embryos can cause an axial duplication: it forms a supernumerary Spemann organizer and leads to the formation of a second primary body axis (McMahon and Moon, 1989).

Pattern Maintenance in Development and Regeneration

After Wnt expression incipiently begins at the posterior pole, a self-organizing program sustains Wnt expression at that domain to persistently maintain posterior patterning (Box 3), ensuring developmental fidelity. In the cleavage-stage sea urchin embryo, although *Wnt8* first appears at the future posterior pole, the expression of *Wnt8* soon expands anteriorly, progressively "sweeping" through the posterior domain of the embryo (Figure 5A). In this case, Wnt8 initially travels from the posterior-most tip to the next layer of cells, where it activates expression of the transcription factor *Blimp1*, which itself elicits *Wnt8* expression (Smith et al., 2007). Therefore Wnt8 is repetitively passed

Box 3. Wnt-Positive Feedback Signaling, Axial Elongation, and Evolution

A pivotal evolutionary innovation was extension of the metazoan body through "terminal addition." This entailed the maintenance of a pool of posteriorly located progenitors early in embryogenesis that serially deployed blocks of repeating trunk tissue along the anterior-posterior axis (Jacobs et al., 2005; Martin and Kimelman, 2009). This evolutionary innovation led to extension of the metazoan body axis and must have entailed a mechanism to sustain posterior progenitors and, in doing so, prolong axial extension. An ancient component of the Wnt-imposed posterior program is the transcription factor *Brachyury*, which is present in virtually all extant metazoa (Ryan et al., 2013) just like Wnt itself. In diverse animals ranging from *Hydra* (Nakamura et al., 2011) to mice (Arnold et al., 2000), Wnt directly activates *Brachyury* expression at the posterior pole of the primary body axis (by virtue of Tcf/Lef binding sites in its genomic regulatory elements): the domain of high Wnt activity/Brachyury expression frequently marks the blastopore (Martindale and Hejnol, 2009), and Brachyury is responsible for transcriptionally executing posterior development. However, in later species including chordates, *Brachyury* itself acquired the capacity to upregulate *Wnt* genes (Martin and Kimelman, 2008) and in doing so generated a self-sustaining cohort of *Brachyury*-expressing posterior progenitors that maintained themselves via autocrine Wnt signaling (Aulehla et al., 2008; Dunty et al., 2008). Therefore, innovation of a self-perpetuating *Wnt-Brachyury* program enabled terminal addition and, thus, axial elongation to occur in later metazoans (Martin and Kimelman, 2009). In accord, precociously terminating Wnt signaling in posterior progenitors leads to severely truncated bodies harboring a head but a much-shortened tail in vertebrates (Aulehla et al., 2008). Wnt signals may sustain proliferative posterior progenitors by fueling metabolism (Oberhofer et al., 2014).

like a baton from posterior cells to the immediately adjacent layer of cells wherein it induces its own expression, enabling this short-range signal to be sequentially propagated throughout the entire posterior domain (Smith et al., 2007) (Figure 5A). In a similar example of self-organizing posterior Wnt expression in *Hydra*, Wnt3 is expressed by the posterior organizer (Hobmayer et al., 2000). Here, Wnt3 directly upregulates its own expression by virtue of Tcf binding sites within its promoter element (Broun et al., 2005; Nakamura et al., 2011), while simultaneously downregulating the expression of *Dkk* (Guder et al., 2006). Therefore, the posterior organizing center self-maintains itself as a domain of high Wnt activity.

Wnt not only crucially acts to establish an embryonic pattern, but also persistently maintains this pattern after embryogenesis. This is particularly important during homeostatic tissue turnover and regeneration after injury, when pattern maintenance (or rather, re-establishment) is pivotal such that the original anatomy may be correctly reconstituted after repair (Clevers et al., 2014). This is clearly evinced when *Hydra* are dissociated into single cells and reaggregated: remarkably, within days the cells spontaneously self-organize and re-establish their original anatomic pattern (Gierer et al., 1972). Presumably within these otherwise homogeneous aggregates, small numbers of Wnt-expressing cells instruct nearby cells to express *Wnt3* while suppressing *Dkk* expression (Guder et al., 2006), locally establishing and stabilizing a Wnt signaling center (Hobmayer et al., 2000; Technau et al., 2000) that then acts as an organizer to assemble nearby posterior tissue (Figure 5B). Similarly, otherwise homogeneous aggregates of mouse embryonic stem cells self-organize themselves into asymmetric "embryoid bodies" with the spontaneous emergence of a Wnt-expressing area that then leads to symmetry breaking along a given axis (ten Berge et al., 2008).

In another example, if the tail of a planarian flatworm is amputated, it will regenerate within days. This regenerative capacity again can be ascribed to the posterior organizing activity of the Wnt/β-catenin pathway, which provides positional information pivotal for proper posterior tissue regeneration. Accordingly, if *β-catenin* is silenced, a head will regrow instead of a tail, forming a "two-headed" animal (Gurley et al., 2008; Petersen and Reddien, 2008). In the most extreme cases of *β-catenin* suppression, planarians not only lose their tail, but ectopic heads replete with eyespots begin sprouting around the periphery of the body, eventually forming almost a circular organism whose perimeter essentially consists of multiple heads (Iglesias et al., 2008) (Figure 5D). The key roles of Wnt in pattern establishment and maintenance rely on the coordination of two distinct cellular processes, discussed below.

Coupling Cell Identity with Cell Position through Asymmetric Division

Axial patterning involves not only cell-fate specification but also the dispensation of such cells in a spatially organized fashion. This requires the developmental coordination of cell identity and cell position. To this end, Wnt proteins have the unique property of being able to specify cell fate while simultaneously orienting the direction of cell division, in essence specifying both a cell's identity and position through a single signal.

In early embryogenesis, a local pulse of Wnt signaling can polarize a cell to divide asymmetrically, causing the proximal Wnt-responding and distal Wnt-unresponsive cells to adopt diverging fates, simultaneously distributing them along a spatial axis and in doing so coordinating lineage specification with spatial morphogenesis. In 4-cell *Caenorhabditis elegans* embryos, the EMS blastomere divides to form two daughters that respectively form endoderm and mesoderm. This division is controlled by the directly adjacent, Wnt-expressing P2 blastomere: the Wnt signal orients the EMS blastomere to divide asymmetrically by orienting the plane of cell division, such that the proximal cell (under the influence of Wnt) differentiates into endoderm whereas the distal one (beyond the range of Wnt) commits to mesoderm (Rocheleau et al., 1997) (Figure 3C). By analogy, pluripotent mouse embryonic stem cells (mESCs) become polarized when exposed to a localized Wnt signal in cell culture, which distributes the centrosomes at opposing poles of the cell and sets up the mitotic spindle such that after division, the Wnt-proximal cell remains a mESC whereas the Wnt-distal cell commences differentiation

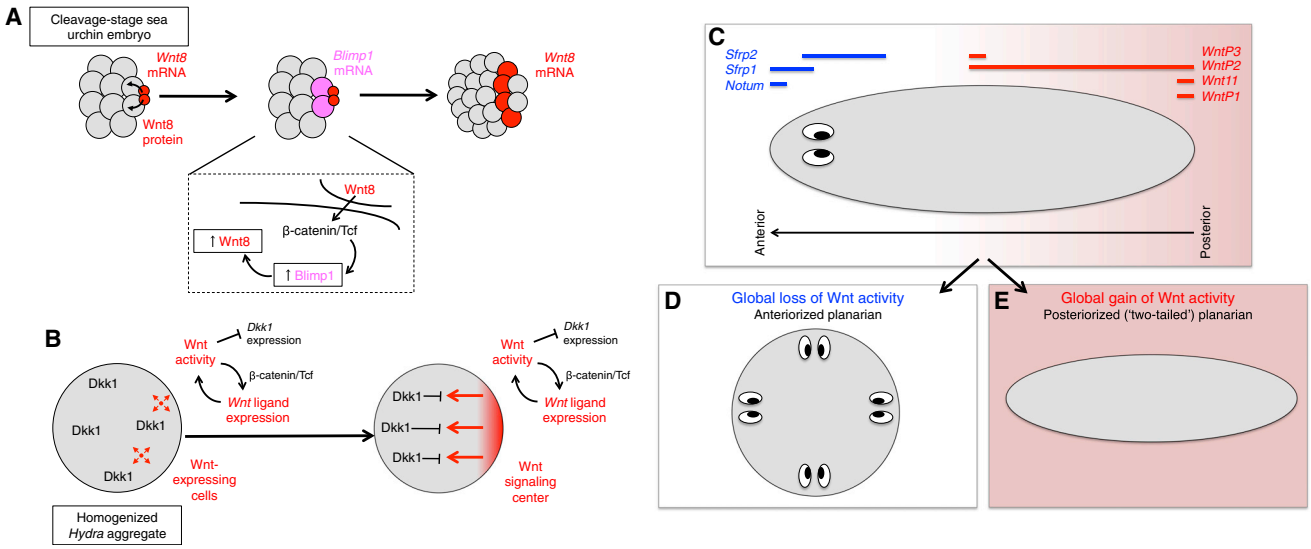

Figure 5. Wnt Activity and Pattern Maintenance

Wnt multiply functions not only in pattern formation during embryogenesis but also persistent maintenance of this spatial pattern in adulthood.

(A) Wnt8 is first produced by the vegetal-most blastomeres in sea urchin embryogenesis. As a short-range signal, it diffuses to the immediately adjacent tier of cells, wherein it activates its own expression through transcription factor Blimp1 (see main text for details) (Smith et al., 2007). Therefore, *Wnt8* expression progressively "sweeps" anteriorly through the posterior pole of the primary body axis.

(B) Homogenized aggregates of *Hydra* cells can spontaneously reorganize into a perfectly formed animal. In these aggregates, small numbers of Wnt-expressing cells induce *Wnt* expression in adjacent cells while simultaneously inhibiting expression of a Wnt antagonist (*Dkk*), leading to the generation of a Wnt signaling center that locally orchestrates posterior (e.g., tentacle) tissue development (Guder et al., 2006; Hobmayer et al., 2000; Technau et al., 2000).

(C) In adult planarian flatworms, Wnt pathway components (only some of which are visualized here) show staggered expression domains along the anterior-posterior axis, such that Wnt antagonists are broadly found at the anterior pole (the head), whereas Wnt ligands are largely expressed toward the posterior pole (the tail) (Petersen and Reddien, 2008; Reddien, 2011). This anterior-posterior gradient of Wnt activity is required to continuously anchor the adult body plan.

(D) Global abrogation of Wnt signaling (by *β-catenin* RNAi) either in steady-state or regenerating planarians leads to the relinquishment of posterior identities, and can generate radially transformed "hypercephalized" animals replete with ectopic heads sprouting along the perimeter (Iglesias et al., 2008).

(E) Global hyperactivation of Wnt signaling (by *Apc* RNAi) in regenerating planarians leads to the loss of anterior fates, and can generate "two-tailed" animals in which the head has essentially been converted into a tail (Gurley et al., 2008).

(Habib et al., 2013). Therefore, while conventional long-range morphogens are perceived to couple cell position with lineage in an indirect way (in that cell fate is specified as a consequence of cell position in a graded signaling field), Wnt directly couples cellular position with fate by orienting the plane of cell division and distributing daughter cells of different fates in distinct spatial territories.

The above examples illustrate that Wnt, acting on a single cell, can simultaneously control its polarity and identity, and in doing so coordinately specifies both the position and fate of its daughters. The underlying mechanism in *C. elegans* seems to be that a local Wnt signal induces accumulation of Fzd receptors as well as Dvl at the point of contact with the Wnt ligand, thereby actively polarizing the cell (Goldstein et al., 2006) while simultaneously distributing inhibitory pathway components Axin and adenomatous polyposis coli (APC) to the opposite end of the cell (Sawa, 2012). This subsequently leads to asymmetric partitioning of β-catenin in the nuclei of the two daughters (controlling cell fate) while concordantly orienting the plane of the mitotic spindle by positioning the centrosomes and controlling microtubule deployment (and hence cell polarity) (Sugioka et al., 2011). Indeed, proteins controlling mitotic spindle orientation (Anderson et al., 2016)—and strategies for controlling asymmetric cell division in general—are evolutionarily ancient and predate metazoans. Hence, Wnt signals may have appropriated pre-existing asymmetric cell division machinery to intertwine cell fate with position in early metazoans.

Cooperative action of the Wnt cell-fate and cell-polarity cascades is seen not only at the level of single cells (Habib et al., 2013; Rocheleau et al., 1997) but also at the level of whole developing tissues. Although this is seemingly at odds with reported cross-antagonism between Wnt/β-catenin and Wnt/PCP pathways (Box 2), this is not always the case in vivo (Ohkawara and Niehrs, 2011); moreover, the Wnt cell-polarity pathway extends beyond PCP/CE responses and broadly subsumes β-catenin-independent mechanisms through which cells become polarized, migrate, or divide. By way of example, in the above example of Wnt-driven *Hydra* tentacle development it is clear that Wnt specifies a tentacle fate through β-catenin and then acts through JNK to coordinate tentacle cell position (Philipp et al., 2009). Therefore, deployment of posterior tissue by Wnt depends equally on β-catenin-dependent and -independent signaling, which coordinately specify tissue identity and shape, respectively (Philipp et al., 2009). By analogy, in *Nematostella* primary body axis specification, it seems that at the posterior (animal) pole, Wnt/β-catenin signaling acts in parallel with PCP signaling to respectively specify endoderm identity and then trigger its morphogenetic migration (Kumburegama et al., 2011). In *Xenopus* ectoderm, there is a similar coupling of Wnt/PCP and Wnt/β-catenin signaling, and their unified action permits proper outer-inner patterning (Huang

and Niehrs, 2014). In the three above in vivo examples, it has yet to be determined whether Wnt cell-polarity and cell-fate signaling occurs in the same single cells, as has been formally shown in *C. elegans* and mice (Habib et al., 2013; Rocheleau et al., 1997). Nevertheless, the emergent view is that imposition of cellular identity (orchestrated by the Wnt/β-catenin cell-fate pathway) has become intimately coupled with specification of cellular position and directional migration (controlled by the Wnt cell-polarity pathway in the broadest sense), and by acting in synchrony these mechanisms permitted metazoan tissues to assume their identity and shape during the course of evolution.

Perspectives

By coupling the generation of lineage diversity (through the cell-fate cascade) with that of spatial form (through the cell-polarity cascade), Wnt signals were instrumental for the speciation of patterned animals from their unicellular predecessors. Although disparate pieces of the Wnt pathway were extant in the "pre-history" of animals within unicellular organisms, the pathway in its entirety only emerged contemporaneously with the first multicellular animals. In metazoan antiquity, primordial symmetry-breaking Wnt signals specified a primary body axis in pre-bilaterians (Holstein, 2012; Holstein et al., 2011; Niehrs, 2010; Petersen and Reddien, 2009), establishing an organized body plan and predating other major developmental regulators. Later, in the evolution of bilaterians, the BMP (bone morphogenetic protein) and TGF-β pathways were respectively exploited to create dorsal-ventral and left-right axes that ran perpendicularly to the primary body axis (Holstein et al., 2011; Niehrs, 2010), begetting greater morphological complexity.

Because Wnt can simultaneously specify cell identity while orienting the axis of cell division, it can distribute cells of different fates along a spatial axis, contemporaneously specifying both cell fate and position. This is of fundamental importance for axial patterning during embryogenesis, because it means that cell specification and tissue morphogenesis are inseparably linked and executed by the combined activities of β-catenin-dependent and -independent Wnt responses. In this regard, the direction from which Wnt signals are conveyed is crucial in polarizing cells to divide and differentiate in a spatially ordered fashion, as shown in multiple *C. elegans* cell types: if Wnt is presented to a cell but from the opposite side, a specified cell type will be formed but at the inappropriate position, with disastrous consequences for ensuing development (Goldstein et al., 2006). Because a local Wnt ligand polarizes a cell at its point of contact by concentrating Fzd receptors (Goldstein et al., 2006; Habib et al., 2013), the crucial proviso for this proposed model of combined cell polarization and lineage assignment is that Wnt predominantly acts as a short-range signal (Alexandre et al., 2013) and thus is capable of asymmetrically polarizing a cell at its contact surface. This supplies an interesting insight—that in contrast to more traditional "morphogens" that acted over long ranges, an explicitly short-range Wnt signal may have been uniquely capable of polarizing cells and thus establishing a body axis in early metazoa.

AUTHOR CONTRIBUTIONS

Conceptualization, K.M.L., R.v.A., and R.N.; Writing – Original Draft, K.M.L., R.v.A., and R.N.; Writing – Review & Editing, K.M.L., R.v.A., and R.N.

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