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Wnt signaling in planarians: new answers to old questions

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ABSTRACT Wnts are secreted glycoproteins involved in a broad range of essential cell functions, including proliferation, migration and cell-fate determination. Recent years have seen substantial research effort invested in elucidating the role of the Wnt signaling pathway in planarians, flatworms with incredible regenerative capacities. In this review, we summarize current knowledge on the role of canonical (β-catenin-dependent) and non-canonical (β-catenin-independent) Wnt signaling in planarians, not only during regeneration, but also during normal homeostasis. We also describe some of the preliminary data that has been obtained regarding the role of these pathways during embryogenesis. Models are proposed to integrate the different results which have been obtained to date and highlight those questions that still remain to be answered.

KEY WORDS: *planarian, Wnt signalling, regeneration, development, axial polarity*

Introduction

Wnt ligands are a family of secreted glycoproteins involved in cell-cell communication events that control every major developmental process, including cell-fate determination, cell proliferation, polarity, adhesion, motility, apoptosis, and hence, patterning and morphogenesis (reviewed in Mikels and Nusse 2006). Historically, Wnt ligands have been classified as canonical or non-canonical, depending on whether or not they lead to the activation of β-catenin. However, this classification may be artificial and the function of a specific Wnt ligand should not be understand as an intrinsic property of the ligand, but rather as a context-specific result of its interaction with different receptors (reviewed in van Amerongenen and Nusse, 2009). Although the best known Wnt receptors belong to the Frizzled (Fz) family, other receptors, such as the tyrosine kinases Ror2 and RYK, have begun to be identified (Angers and Moon 2009).

The best understood Wnt signal-transduction cascade is the canonical pathway, in which binding of Wnt proteins to Frizzled/LRP5-6 receptors causes the recruitment and activation of Dishevelled, responsible for the disassembly of the β-catenin destruction complex, mainly composed of Axin, APC and GSK-3. As a consequence, β-catenin is stabilized and enters the nucleus, where it associates with Lef/Tcf transcriptional repressors, causing the derepression of transcriptional targets (reviewed in Huang and He, 2008). Thus, canonical Wnt signaling directly targets the nucleus, and it is broadly used to regulate cell fate, proliferation and self-renewal of stem and progenitor cells in any tissue and at any stage of metazoan life.

Despite the broad range of activities, β-catenin signaling is a strikingly conserved mechanism to pattern the antero-posterior (AP) axis in nearly all animals examined (reviewed in Petersen and Reddien, 2009). It controls specification of posterior identities in most bilaterian embryos (reviewed in Niehrs, 2010), and this role is conserved in adult stages during regeneration, as demonstrated in cnidarians (Hobmayer *et al*., 2000) and in our current model, planarians.

By definition, Wnt signaling that does not lead to β-catenin activation is referred to as non-canonical or β-catenin independent. A number of non-canonical Wnt signaling pathways exist, including the planar cell polarity (PCP) pathway and the calcium pathway. PCP refers to the coordinated polarization of cells or structures in the plane of a tissue (Goodrich and Strutt, 2011). For instances, in *Drosophila* wing imaginal discs, where this phenomenon was first discovered, Dvl is recruited by a Fz receptor, promoting the asymmetric localization of the PCP core proteins within the cell, becoming the Fz-Dvl-Diversin/Diego (Div/Dgo) complex localized oppositely to the Strabismus/Van-Gogh (Stbm/Vang)-prickle (Pk) complex. The disruption of these protein asymmetries is manifested by the miss-orientation of wing hairs. Nowadays, β-catenin-independent pathways are linked to a highly diverse set of processes in both vertebrates and invertebrates. These include assembly and patterning of neural circuitry, convergent extension movements during gastrulation, orientation of cell division and planar polarization of cells and tissues (reviewed in Goodrich and Strutt, 2011). Although all these events occur in different biological contexts and they can

Abbreviations used in this paper: ap, antero-posterior; Fz, Frizzled; pcp, planar cell polarity.

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be viewed as independent from each other, their basis may be common: a concerted intracellular rearrangement controlled by regulating actin filaments, which models cell and tissue architecture.

The planarians are a particularly interesting model in which to explore the role of Wnt signaling in events as diverse as cell proliferation and patterning. These freshwater flatworms can regenerate a whole animal from almost any piece of their body. Although other species such as *Dugesia japonica*, are used by several research groups, *Schmidtea mediterranea* is now the most widely studied planarian species since its genome has been sequenced and several EST and transcriptomic databases have been generated. Since technology is also available for functional analysis using methods such as RNA interference (RNAi), it is well suited to experiments designed to unravel the molecular control of processes such as the re-establishment of tissue polarity during regeneration. In this review, we present the current data on the roles of the different planarian Wnt signaling elements in development and regeneration and propose models to integrate the findings that have been obtained to date.

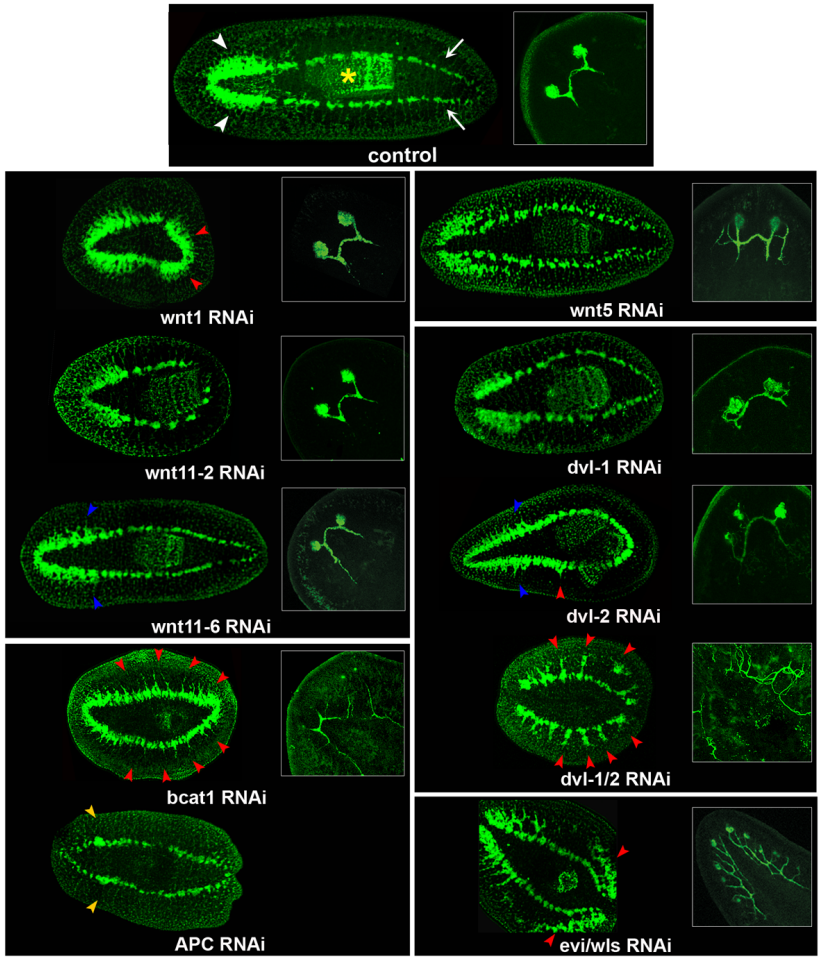

Fig. 1. Comparative analysis of the phenotypes generated after silencing different Wnt signaling elements. Immunohistochemistry with anti-synapsin (CNS) and anti-arrestin (VC-1) (visual system, within white squares) shows the CNS organization and paths of visual axons after different genetic ablations. *In the control animal, the brain and the ventral nerve cords are labeled with white arrowheads and arrows, respectively. The pharynx is labeled with a yellow asterisk. In the silenced animals, the ectopic brain is labeled with red arrowheads, the expansion towards posterior of the brain is labeled with blue arrowheads and the two ectopic brain primordia are labeled with orange arrowheads. All animals correspond to trunk pieces after 12-20 days of regeneration. All images are confocal z-projections. Anterior is shown to the left.*

Canonical/β-catenin-dependent Wnt signaling specifies posterior identity in adult planarians

Before the molecular era, generation of two-headed planarians was not a strange event. Morgan and Child observed that when a planarian is cut into extremely thin fragments, 'two-headed' or 'janus headed' planarians often develop (Morgan 1898; Child 1911). These observations were central to their proposal of a gradient, which Child supposed to be a 'metabolic gradient', responsible for AP axis patterning. According to this hypothesis, when the size of the sectioned planarian is very small, the difference in the activity of the 'metabolic gradient' between both edges is insufficient to instruct different fates in the two edges (reviewed in Blackstone 2006). The idea of a gradient underlying the bipolar anatomy of planarians has remained broadly accepted since its initial proposal by Morgan and Child (reviewed in Meinhardt 2009; Adell *et al.*, 2010). This parallels development in experimental embryology, where gradients have long been thought to be responsible for regulating axial development. It has now been demonstrated that the patterning of the AP axis (primary axis in prebilaterians) involves Wnts and β-catenin activity in almost all metazoans (reviewed in Petersen and Reddien, 2009; Niehrs, 2010).

The first and the most striking evidence for the essential role of canonical Wnt signaling in the specification of the planarian AP axis came from experiments involving silencing of *βcatenin-1*. The *S. mediterranea* genome contains at least three β-catenins. *βcatenin-1* seems to be both necessary and sufficient to transduce the canonical Wnt signal (Gurley *et al.*, 2008; Petersen and Reddien 2008; Iglesias *et al.*, 2008), whereas *βcatenin-2* functions exclusively in cell-cell adhesion (Chai *et al.,* 2010). Although other organisms such as *C. elegans* and vertebrates also have genomic duplications of β-catenin, only planarians are known to exhibit a clear functional specialization between different paralogs. Thus, silencing of *βcatenin-1* results in the complete loss of posterior and central identities and the generation of a fully anteriorized animal (Fig. 1 and 2). The planarians generated by *βcatenin-1* silencing were referred to 'radial-like hypercephalized' animals because of their almost radial symmetry. Such a striking transformation of the body plan as a consequence of targeting a single gene had no precedent. Consistent with the role of *βcatenin-1* as an effector of a morphogenetic signal, decreasing doses of *βcatenin-1* dsRNA generate a range of AP phenotypes. The strongest phenotype is the 'radial-like hypercephalized' planarian, followed by 'two-headed' planarians with multiple ectopic eyes, 'two-headed' planarians without ectopic eyes, and finally 'tailless' planarians, in which the tail has a rounded shape and the animals have two ventral nerve cords (VNCs) that fuse with a rounded morphology instead of connect-

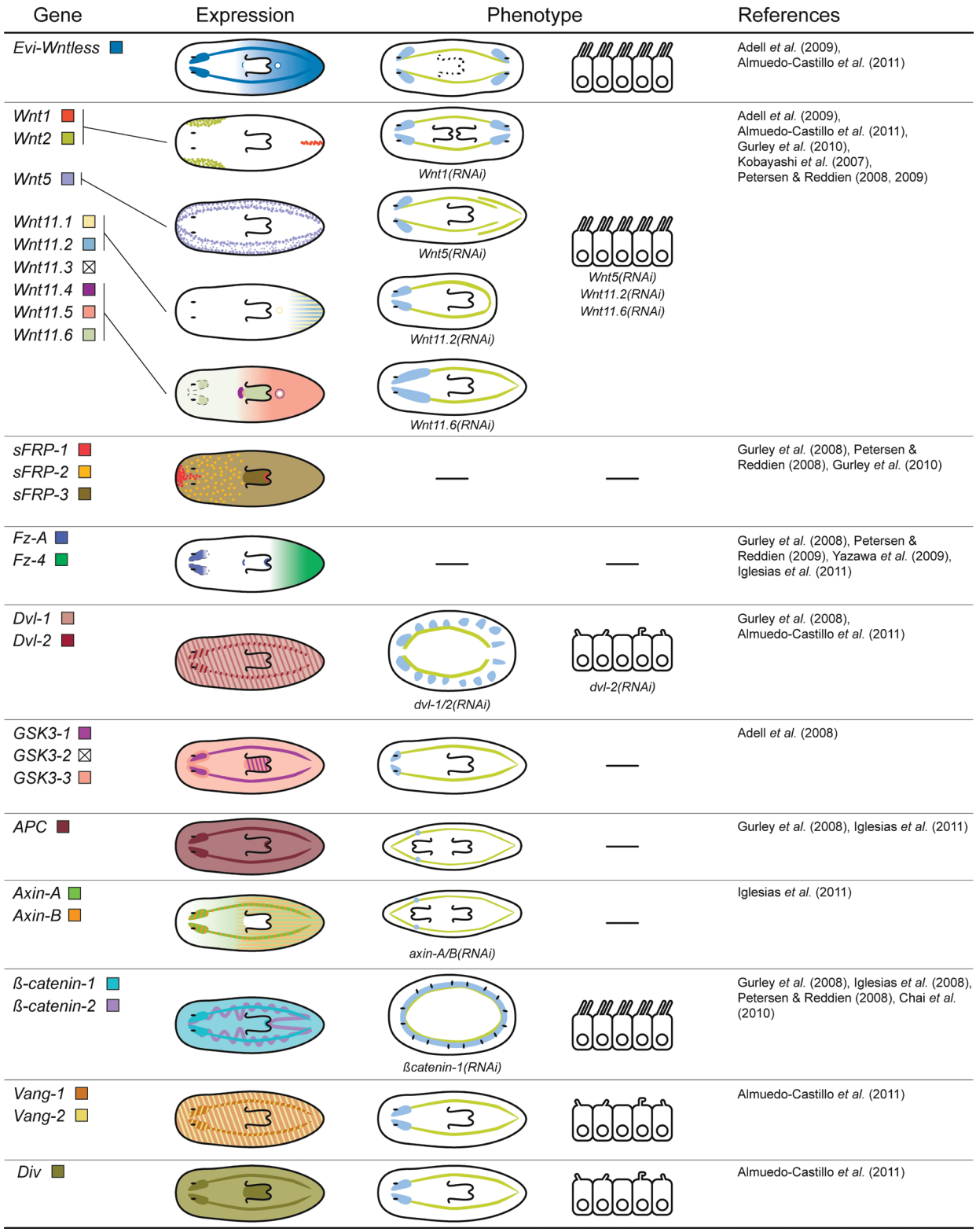

Fig. 2. Schematic summary of the expression patterns of Wnt elements and the phenotype generated after they are silenced. *Gene expression is depicted in stripes when different paralogs are expressed in the same region. The features displayed in the phenotypes are the pattern of the CNS (brain in blue and ventral nerve cords in green), the eyes, the number and position of the pharynx and the organization of the epidermal cilia (apically located and well oriented, as in a wild-type situation, or internalized and with less density, after knockdown of components of the planar cell polarity pathway). In the case of the Wnt ligand family, only the ones for which a phenotype is obtained upon silencing are shown. The phenotype of* vang-1/2 *and* div *RNAi planarians corresponds to a control phenotype apart from the positioning of the cilia.*

ing in the tip of the tail (Iglesias *et al.,* 2008; Adell *et al.,* 2010). Silencing of both *S. mediterranea dvls (dvl-1* and *-2*) also results in the complete loss of posterior identity (Gurley *et al*., 2008; Almuedo-Castillo *et al*., 2011). Moreover, both paralogs seem to be functionally specialized, and only *dvl-2* is clearly involved in β-catenin-dependent signal transduction (Almuedo-Castillo *et al.,* 2011) (Fig. 1 and 2). Furthermore, silencing of *S. mediterranea APC* or *axins* (*axin-A and -B*), two elements of the β-catenin destruction complex, leads to posteriorized or 'two-tailed' planarians (Gurley *et al.,* 2008; Iglesias *et al.,* 2011) (Fig. 1 and 2).

The *S. mediterranea* genome contains a large Wnt family comprising 9 members (Petersen and Reddien 2008; Adell *et al*., 2009; Gurley *et al*., 2010). Although their phylogenetic relationship has not been definitely solved, 6 of them have been classified in the Wnt11 subfamily (*wnt11-1* to *-6*) and the other three each belongs to a different subfamily: Wnt-1 (*wnt1*); Wnt-2 (*wnt2*), and Wnt-5 (*wnt5*). With the exception of *wnt11-3* (formerly known as *wntP-4*), for which expression has never been detected, all of the *S. mediterranea* Wnts display a very specific expression pattern (Fig. 2). *wnt11-6* (formerly *wntA*, based on homology to the first reported *D. japonica* WntA) is expressed in the posterior part of the brain and in the pharynx, and causes posterior expansion of the brain when silenced (Kobayashi *et al.,* 2007; Adell *et al.,* 2009). *wnt11-4* (formerly *wntP-3*) is expressed in clusters of cells in the esophagus and also in isolated cells in the posterior part of the animals (Petersen and Reddien 2008; Gurley *et al.,* 2010), and *wnt-2* is detected laterally in the head region. However, no phenotype has been reported after silencing of these genes. *wnt5* is expressed from the most external region of the CNS to the lateral edges of the planarian (Adell *et al.,* 2009; Gurley *et al.,* 2010; Almuedo-Castillo *et al.,* 2011). Its role will be discussed in the corresponding section, as it clearly functions as a non-canonical Wnt in our model.

In this section we will focus on the 'posterior' Wnts, expressed in the posterior part of the intact planarians. *wnt1* (formerly *wnt-P1*) is expressed in a row of discrete cells exclusively in the posterior dorsal midline (Petersen and Reddien 2008; Gurley *et al.,* 2010), *wnt11-1* and *2* in a broader domain in the tail (Petersen and Reddien 2008; Adell *et al.,* 2009), and *wnt11-5* (formerly *wnt-P2)* as a gradient from the tail to the prepharyngeal region (Petersen and Reddien 2008; Petersen and Reddien 2009) (Fig. 2). Interestingly, during regeneration, their mRNA appears gradually. The first Wnt expressed is *wnt1* in individual cells in both anterior and posterior blastemas. In fact, it has been shown to be activated in response to any wound as early as 6 hours after amputation (Petersen and Reddien 2009; Gurley *et al.,* 2010). At 48 hours, its expression is restricted to the posterior midline, resembling the pattern in the intact animals. *wnt11-1* and *2* are not detected until 48 hours after amputation, when they appear exclusively in the posterior blastema (Adell *et al.,* 2009; Gurley *et al.,* 2010). *wnt11-5* expression is known to be triggered by *wnt1* and β*-catenin* at posterior wounds at 24 h post-amputation (Petersen and Reddien 2009). As is already expressed in the trunk region of intact animals, it does not disappear after amputation but moves posteriorly as regeneration proceeds, in parallel to the morphogenetic changes and establishment of new AP identities (Fig. 3). It is interesting to note that the wound induced *wnt1* expression and the dynamics of *wnt11-5* expression are not affected after ablation of neoblasts by irradiation, suggesting that, although proper morphallactic remodeling cannot take place, the first global response to the new positional identities are stem cell independent (Petersen and Reddien 2009; Gurley *et al.,* 2010).

Functional analysis of the 'posterior' Wnts by RNAi demonstrates that *wnt1* signals through a canonical pathway, since its silencing produces 'two-headed' planarians (Fig. 1 and 2). However, the loss of polarity is achieved in a very low percentage of animals, and after silencing of *wnt1*, most of the animals appear with the same 'tailless' phenotype already reported to be generated after low-level silencing of β*catenin-1* and *dvl-2* (Adell *et al.,* 2009; Almuedo-Castillo *et al.,* 2011). Planarians in which *wnt11-2* has been silenced also display the 'tailless' phenotype, but never a reversal of polarity (Fig. 1 and 2). No phenotype is reported after individual silencing of the other posterior Wnts. Based on their expression pattern and functional data, it has been suggested that synergistic effects of several posterior Wnts account for the graded activation of β-catenin along the planarian AP axis, as it has also been proposed in cnidarians (reviewed in Guder at al., 2006). Specifically, *wnt1* is thought to activate *wnt11-5* through β*catenin-1* and both would be responsible for patterning the posterior end. This relationship is supported by the observation that silencing of *wnt11-5* together with *wnt1* increases the proportion of 'two-headed' planarians (Peteresen and Reddien 2009). It has also been proposed that *wnt11-2* could be a target of β*catenin-1* (Yazawa *et al.,* 2009), but synergistic effects have never been demonstrated. Remarkably, *wnt11-5* expression appears just after *wnt1* and before *wnt11-1/2* during regeneration of head fragments, which normally do not express posterior Wnts (Peteresen and Reddien 2009). Thus, current data suggest that *wnt1* and *wnt11-5* act through β*catenin-1*. However, the canonical role of *wnt11-1* and *2* remains to be demonstrated. After silencing of *wnt11-2*, genes expressed in the midline of planarians fail to properly localize along the posterior midline of the animal, but are detected as dispersed cells around the rounded tail. It has therefore been proposed that *wnt11-2* is required for recruitment of midline cells rather than for establishment of posterior identity (Gurley *et al*., 2010). It is possible that *wnt11-2* controls the cell migration or the oriented cell division required to extend the posterior axis, similar to the non-canonical function of Wnt-11 and Dishevelled in AP elongation of the epiblast during vertebrate gastrulation (Gong *et al.,* 2004).

Interestingly, 'radial-like hypercephalized' planarians are never generated when *wnts* are silenced alone or in combination (Adell *et al.,* 2009; Petersen and Reddien 2009). To explain this observation it could be proposed that inputs from different Wnts could control activation of β*catenin-1*. Although some Wnt ligands are more relevant than others in targeting a specific response, receptors are the key components to initiate the different cascades (van Amerongen and Nusse, 2009). We can thus expect some degree of rescue by the other Wnt ligands that preferentially signal through different receptors. An alternative explanation is that RNAi targeting of a single intracellular element, which probably has a short half-life, is much easier than silencing several more stable secreted proteins. Finally, the available data also suggest that the position within the pathway at which the silenced gene acts can be decisive. For instance, silencing of *S. mediterranea* dishevelleds, an intracellular element common to every Wnt branch and that directly controls β-catenin degradation, produces a rapid and complete anteriorization of treated planarians (Fig. 1 and 2). In contrast, silencing of *evi/wls*, a transmembrane protein required for secretion of Wnts, only produces bipolar 'two-headed' animals with multiple eyes, even when two rounds of injection/regeneration

are performed (Adell *et al.,* 2009; Almuedo-Castillo 2011) (Fig. 1 and 2). As expected, silencing of those elements also produces β-catenin independent defects, which will be discussed in the corresponding section.

Canonical Wnt activation is responsible for posterior specification during regeneration but also during homeostasis in intact planarians. Injection of β*catenin-1* or *dvl-1/2* dsRNA generates fully anteriorized animals even when no injury has been induced (Peteresen and Reddien 2008; Iglesias *et al*., 2008; Almuedo-Castillo *et al*., 2011). This is not surprising if we consider that the mechanisms of pattern formation should always be active in planarians, which are continuously renewing their tissues and setting them to new proportions, for instance when adjusting their size to food availability. However, silencing of other elements of the pathway such as *wnt1* during homeostasis does not generate "two-headed" planarians, supporting that during regeneration, the early posterior-fate choice of *wnt1* takes place just in response to a wound. Amputation forces the regeneration of new tissues and, thus, de novo synthesis of proteins. In general, quicker and stronger effects are observed in regenerating than in intact animals. However, we should bear in mind that homeostasis and regeneration, particularly during early stages, are unlikely to involve the same molecular inputs and responses. During regeneration, cells must adopt completely different fates with respect to their original position and, thus, very early signals must re-direct their fate in a context of high rates of apoptosis and proliferation. In contrast, the changes in tissue fates during homeostasis are more progressive and follow already established patterns. For that reason it is important to pay attention to the expression pattern of genes that show a polarized expression at specific time points. The current data will be discussed bellow in an attempt to propose a general model for the re-establishment of AP pattern during regeneration.

Alternative mechanisms involved in planarian antero-posterior axis specification

Although canonical Wnt signaling is essential for patterning the AP axis in adult planarians, other pathways, such as the hedgehog (Hh) pathway, are known to be involved in establishing and maintaining polarity (Rink *et al.,* 2009; Yazawa *et al.,* 2009). Silencing of the planarian homologs of Patched, a Hh receptor with inhibitory activities, generates 'two-tailed' planarians, whereas inhibition of the planarian homologs of Hh, Gli-1 or Smo induces anteriorization (Fig. 3). However, inhibition of Hh signaling generates posterior heads in only a few animals, and the effect is only more penetrant when two of the elements are silenced simultaneously or two rounds of regeneration are induced (Yazawa *et al*.2009). In most animals, *Hh* silencing leads to a 'no-tail' phenotype (Fig. 3) that differs from the 'tailless' phenotype generated after β*catenin/wnt1/wnt-11-2* RNAi (Fig. 2). In fact, after *Hh* silencing, the posterior marker Fz4 does not disappear, demonstrating that posterior identity is not completely abolished (Rink *et al.,* 2009). The essential difference between the 'tailless' and the 'no-tail' phenotype is that in the first the VNCs and gut do differentiate but do not end at a posterior tip, while in the second the structures fail to differentiate. Those differences could reflect a secondary role for Hh in proliferation and growth, in addition to the one in fate determination.

Simultaneous silencing of *patched* and β*catenin-1* produces anteriorized animals, demonstrating that Hh acts upstream of β-catenin (Rink *et al.,* 2009). The expression patterns of the components of the Wnt pathway, together with the observation that Hh transcripts are found not in the posterior blastema cells but in the preexisting VNCs, led Yazawa *et al.,* (2009) to propose a model in which Hh might be transported posteriorly along axons in the VNCs. According to that model, Hh would then be responsible for the activation of *wnt1*, which would lead to the activation of β*catenin-1* and *wnt11-2*. This model is very suggestive, since, after amputation, planarians would regenerate according to the existing AP polarity of the fragment. If a directional signal exists, this is inherent to the existing CNS, and the fact that, after amputation, adjacent cells adopt different fates according to each blastema would be easily explained. As argued by the authors, a recent report demonstrating that voltage-gated Ca^{2+} channels are involved in the acquisition of proper polarity during regeneration (Noggi *et al*., 2009) would agree with this model, as Ca^{2+} channels are essential for microtubule assembly and axonal transport (Rajnicek and McCaig, 1997). However, it should be noted that, at least at

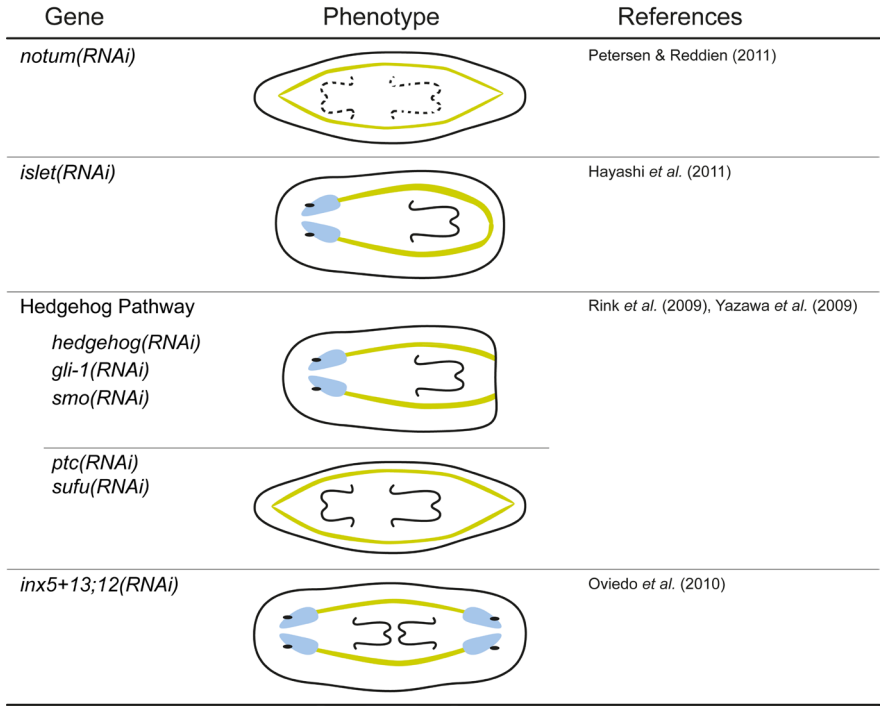

Fig. 3. Schematic representation of the antero-posterior defects generated after modulating "non-Wnt" elements. *The features displayed in the phenotypes are the pattern of the CNS (brain in blue and ventral nerve cords in green), the eyes, and the number and position of the pharynx. Silencing of* notum *or inhibitory elements of the Hh pathway induce "two-tailed" planarians. Silencing of activators of the Hh pathway or of* Hh *itself produces posterior regeneration defects. RNAi of* islet *leads to 'tail-less' regeneration phenotype. Downregulation of innexins generates 'two-headed' planarians. The pharynges of* notum *RNAi planarians are represented by dots because the exact position and number have not been precisely determined.*

the mRNA level, *ptc* expression does not show any asymmetry between 3 and 48 hours (Rink *et al.,* 2009).

Perhaps more interesting is the recent study by Beane *et al.,* (2011), who demonstrated that membrane depolarization is sufficient to drive anterior regeneration, even in posterior wounds, and that calcium signaling is at least one of the target effects of the depolarization. Taken together, these data highlight the essential role of ion transport as an early signal to regulate differential gene expression and to control polarity and morphogenesis.

The existence of long-distance communication mechanisms to rapidly inform cells of changes occurring during planarian regeneration is an attractive model. Further support for this mechanism comes from the work form Oviedo *et al.,* (2009) linking gap junctional communication with the integrity of the VNCs and the transport of signals such as Hh (Oviedo *et al.*, 2009). Gap junctions are plasma membrane channels involved in cell-cell communication via the direct transfer of small molecules between adjacent cells. They are known to be involved in syncytial communication between cell groups and to regulate many aspects of embryonic development (Levin *et al.*, 2007). The *S. mediterranea* genome contains a large family of innexins, the gap junctional components found in invertebrates, (Nogi and Levin 2005), and when their function is blocked, 'two-headed' planarians are generated (Nogi and Levin 2005; Oviedo *et al.,* 2009) (Fig. 3). Oviedo *et al.,* performed multiple experiments to block either innexins or the continuity of the VNCs during the process of regeneration and analyzed the polarity of the regenerated animals. Interestingly, they found that fragments containing the brain always regenerated a normal tail, even if the VNCs were disrupted or gap junctions blocked. However, when the VNCs were disrupted alongside blockade of gap junctions, the AP identity of the blastema was abnormal and ectopic posterior heads developed even in fragments containing the brain. Those results demonstrate an essential role of the pre-existing CNS in polarity specification and suggest that blastemas receive the information by at least two mechanisms: gap junctions and VNCs. Interestingly, both signals act very early and rapidly, as they are critical during the first 3-6 hours.

The strong phenotypes generated when modulating canonical Wnt signaling, suggest that the integration of information essential for AP patterning in adult planarians—irrespective of whether this occurs through long-range signals or local cues—acts at the level of *βcatenin-1* activity, and that other signals or mechanisms involved in AP patterning would function by regulating this activity. The interaction between the Hh and the Wnt pathway is an established mechanism for pattern formation during development. For instance, the absence of Shh signaling in vertebrates results in direct inhibition of β-catenin activity by Gli3R (Ulloa *et al.,* 2007). These findings are clearly consistent with the proposed cross-talk between Wnt and Hh signaling in planarians. On the other hand, it is not known whether Wnts can travel through gap junctions. However, both Innexins and Connexins, in invertebrates and vertebrates, respectively, appear to be regulated by Wnt signaling (reviewed in Bauer *et al.,* 2005). Further studies will now be necessary to determine how these different mechanisms work together to control AP patterning.

Early fate decisions and the role of locally secreted wound-activated signals: proposing models for antero-posterior axis establishment

Based on gene expression pattern and kinetics, we have grouped the molecular events underlying regeneration into three theoretical

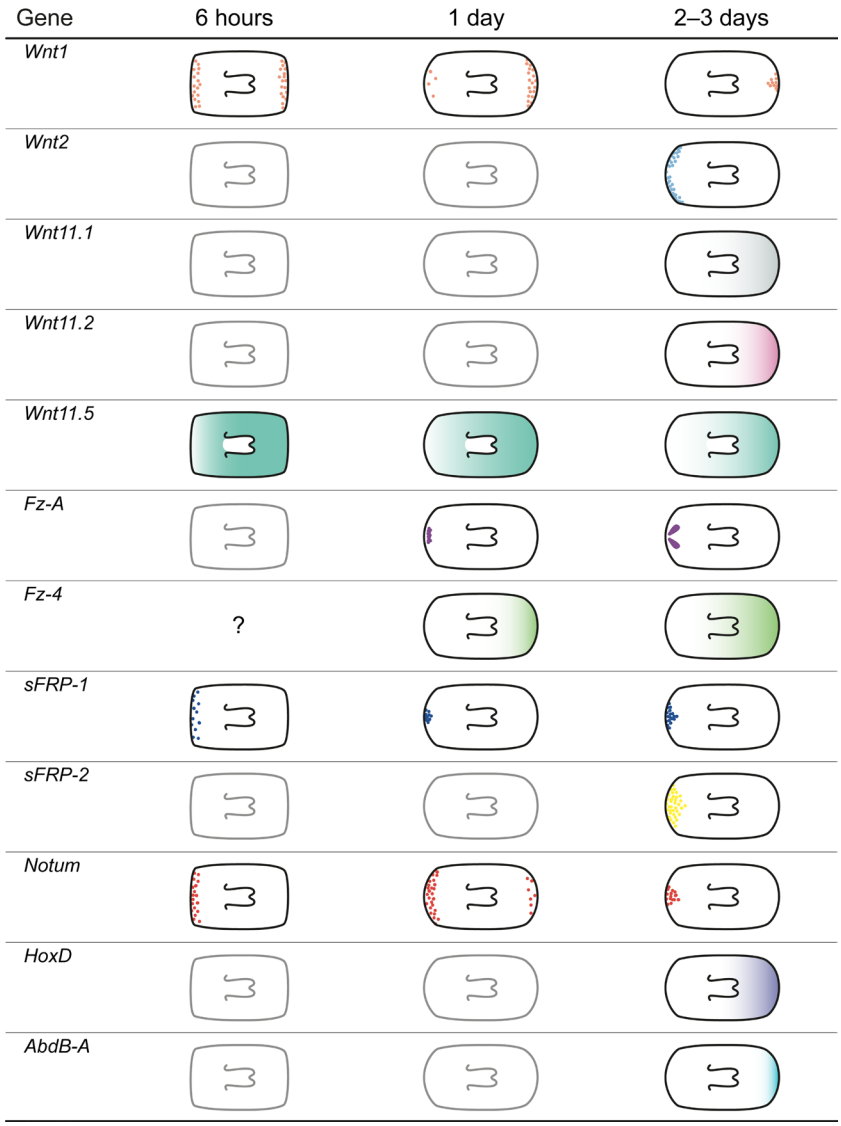

Fig. 4. Schematic representation of polarized gene expression during early regeneration. *The first genes appearing in response to the wound are* wnt1, sFRP-1 *and* notum, *although only* sFRP-1 *exhibits permanent polarize expression in the anterior wounds. One day after amputation,* fzA *and* fz4 *appear in the anterior and in the posterior wounds, respectively. At 3 days, all the known polarized genes are already detected, showing their anterior or posterior location, as well as* wnt1 *and* notum, *which show their final polarized pattern.* wnt11.5 *expression gradually moves posteriorly, in parallel to the re-patterning of the planarian fragment.*

stages. The earliest stage extends from wound healing to 18-24 hours after amputation. During this time, the expression pattern of several genes is "reset" and expression is either undetectable or found in regions that differ from those seen in adult planarians. It is important to note that AP decisions and cell-fate remodeling at this time are independent of stem cell proliferation (Peteresen and Reddien 2009; Gurley *et al*., 2010; Hayashi *et al*., 2011). The second stage is from 18-24 hours to 3 days. During this period, most genes begin to be expressed in a definitive pattern. During the last stage, from 3 days to the end of regeneration, genes are clearly expressed in the primordia of the differentiating tissues and organs.

Currently available data suggest that the critical decisions influencing the establishment of axial polarity and appropriate cell fates are taken during the first stage. At that time, *wnt1* is activated at any wound in a set of isolated cells before becoming restricted to the most posterior dorsal midline during the second stage. The recently identified planarian homolog of Notum, a secreted hydrolase that modulates the range of action of Wnt ligands, is also critical for the acquisition of AP polarity. *notum* is highly upregulated preferentially in anterior wounds as soon as 6 hours after amputation, and later on, at around 2 days, accumulates in the most anterior tip of the blastema; in intact animals it is exclusively expressed in the anterior-dorsal end (Petersen and Reddien 2011) (Fig. 4). Importantly, like *wnt1*, *notum* is expressed in any wound but always preferentially in the anterior edge. Supporting its role in preventing posterior specification, silencing of *notum* generates 'two-tailed' planarians (Fig. 3). *sFRP-1*, one of the three *S. mediterranea* secreted Frizzled-related proteins (sFRP), is the earliest gene to be detected in anterior blastemas, after only 3 hours (Fig. 4). No phenotype has been observed after silencing of *sFRP-1*, however. These wounding-response factors are perfect candidates to mediate the local control cell reprogramming after amputation and specifically launch the different regeneration programs.

A very recent report nicely shows that *D. japonica islet*, a LIM homeobox transcription factor, is essential to maintain *wnt1* expression during the second stage of regeneration, but does not have any effect on the wound-induced *wnt1* expression. During the first two days of regeneration, *islet* mRNA appears in the posterior dorsal midline, like *wnt1* mRNA. Moreover, *islet* RNAi planarians display the 'tail-less' phenotype, similar to the one caused by *βcatenin/wnt1/wnt-11-2* silencing (Fig. 3). The authors suggest that Islet would be inducing stem cells to differentiate into Wnt1-secreting cells. Accordingly, the first stage related *wnt1*-expression is independent of both Islet function and stem cell proliferation (Hayashi *et al*., 2011).

Except for *wnt11-5*, whose expression depends on the early posterior wound-induced *wnt1* expression (Petersen and Reddien 2009; Gurley *et al*., 2010), the other 'posterior' Wnts (*wnt11-1* and *11-2*) are detected later on, during the second stage of regeneration, and are stem cell dependent (Gurley at al., 2010). Similar patterns are observed for the only two Fz receptors analyzed to date, out of the 10 found in the *S. mediterranea* genome (Kobayashi *et al*., 2007; Gurley *et al.,* 2008). *fzA*, is expressed in the anterior blastema at 1 day of regeneration with a pattern directly related with the differentiation of the brain primordia. Likewise, *fz4* is expressed in the posterior blastema also after 1 day of regeneration (Rink *et al*., 2009) (Fig. 4). A second sFRP, *sFRP-2*, is detected in the anterior blastema, but not before the second day (Gurley *et al*., 2010) (Fig. 4). It could be hypothesized that the decisions taken in the first stage are permanently established during the second stage. Interestingly, the Hox genes *AbdbA* and *HoxD* are detected specifically in the posterior blastema around 2 days after amputation (Iglesias *et al.,* 2008) (Fig. 4), suggesting a possible role as canonical Wnt targets. From about 3 days after amputation, not only the Wnt elements but most of the genes are expressed in patterns clearly resembling the ones observed in adults. For instance, genes with polarized expression patterns are clearly concentrated at one tip of the animal, genes expressed in the brain appear as two bi-lateral domains in the anterior blastema and genes expressed in the pharynx appear in its primordium.

With all the available data, an integrative and coherent model for early AP fate decisions can be proposed in which wound-induced *wnt1* expression is responsible for activating *βcatenin-1* and its targets, such as *wnt11-5* and *notum* (Petersen and Reddien, 2011) (Fig. 5A). Notum is described as a target of β-catenin in other models, and in fact, its expression in planarians disappears or is upregulated after *βcatenin-1* or *APC* silencing, respectively. Moreover, evidence for an epistatic relationship is strengthened by the observation that silencing of *βcatenin-1* or *wnt1* together with *notum* produces the same phenotype as silencing *βcatenin-1* or *wnt1* alone, that is, 'two-headed' or 'tailless' planarians (Petersen and Reddien, 2011). Thus, in anterior wounds, inhibition of *wnt1* by anterior-specific signals such as *notum* and *sFRP-1* would allow the differentiation of a head. In posterior wounds, *wnt1* expression would be maintained and other *βcatenin* targets such as *wnt11-1/2* (Petersen and Reddien 2009; Yazawa *et al*., 2009), the posterior Hox genes, and probably *wnt1* itself (Petersen and Reddien 2011) would be activated (Fig. 5A). The observation that notum is transiently expressed in the posterior blastema suggests that an unknown factor must target its inhibition.

Activation of wound-induced *wnt1* expression is known to be independent of *βcatenin-1* and stem-cell proliferation (Petersen and Reddien 2009; Gurley at al. 2010). One possibility is that it is under the control of a general signal that is released by the cells around the injury and is linked to ion transport and depolarization of cell membranes. Once activated in the posterior blastema, maintenance of *wnt1* expression does depend on *βcatenin-1* and very probably on Hh activation (Yazawa *et al.,* 2009). Interestingly, the 'two-tailed' planarians generated after over-activation of Hh have normal levels of *notum* expression (Petersen and Reddien 2011). This capacity of Hh to induce tail differentiation in the presence of *notum,* indicates that Hh is not involved in generating the asymmetric expression of notum and raises the possibility of a late role of Hh in posterior specification. The role of Hh in early regeneration is still an important question to be elucidated. Over-activation of Hh by *ptc* RNAi increases early *wnt1* expression in anterior and posterior wounds, which would agree with the expression of at least *ptc* at that early time point in the wounds (Rink *et al*., 2009). The function of Wnt1 and Hh in anterior wounds also remains unknown, since no defect in anterior regeneration is observed after silencing *wnt1* or *Hh*. Different options can be considered, namely the expression of *wnt1* detected in anterior wounds does not imply a functional readout, or there is an early function of Wnt1 independent of posterior specification that we are not able to detect.

Since modulation of *βcatenin-1* leads to such striking AP defects, it has been widely assumed that its activation must be specific to posterior blastemas. However, this model predicts that *βcatenin-1* must also be active at least transiently in anterior tissues, for instance

to activate notum as early as 6 hours after amputation. In fact, *βcatenin-1* mRNA is detected in any wound, anterior or posterior, although not before 12 hours after amputation (Gurley *et al.,* 2008). Taking into account that βcatenin function is post-transcriptionally regulated, it could be that at very early time points the pre-existing protein levels are sufficient to activate early targets such as *notum*. The possibility that *βcatenin-1* is in fact active in any blastema at any time would explain the high levels of mRNA detected for this gene in both blastemas at all stages of regeneration. This is often overlooked in the literature but is consistent with the essential role of β-catenin in the control of fundamental cell processes. Although the canonical Wnt signal has been conserved for posterior specification, it is also clearly involved in the patterning of any tissue and organ at any developmental stage. For example, in vertebrates, β-catenin is responsible for posterior specification of the embryo, as well as for specifically patterning the liver, the gut (reviewed in Ladde and Monga 2011) and the brain (Kiecker at al., 2001). It therefore seems highly unlikely that the only function of *βcatenin-1* would be in AP axis specification, and evidence suggests that it is also a general mechanism necessary for the proliferation and differentiation of neoblasts at later stages of regeneration (Iglesias *et al.*, 2011). In our opinion, the fact that silencing of β*catenin-1*, *axins* or *APC* only appears to result in AP axis defects in planarians merely reflects the crucial importance of *βcatenin-1* activity during the first few hours after amputation. 'Two-headed' planarians, which appear to have two well-patterned brains and a well-differentiated gut, may well occur because the downregulation of *βcatenin-1* is mild or only occurs for a short period of time (or both). However, it remains to be determined whether 'radial-like hypercephalized' planarians have a well-patterned brain or a well-differentiated digestive system. We would argue that *βcatenin-1* is essential in the first few hours of regeneration to re-establish the posterior organizer, and that this explains why we mostly see an anteriorized phenotype after it is silenced, but that it is also essential for patterning of adult tissues such as the CNS. This would explain the ubiquitous expression of *βcatenin-1* in any blastema, throughout intact animals and both in differentiated cells such as neurons and in proliferating cells (Iglesias *et al.,* 2011) (Fig. 2).

Altogether, patterning of the planarian AP axis can be divided into two main phases. The first one, in response to wound healing and independent of stem cells, where AP fate choice takes place; and the second one, which involves stem cell proliferation, growth and identity maintenance (Petersen and Reddien 2009; Yazawa *et al.*, 2009; Gurley *et al.*, 2010; Petersen and Reddien 2011 and Hayashi *et al.*, 2011) (Fig. 5A). Accordingly, only the silencing of proteins that directly act in early posterior identity specification, such as *wnt1* or *βcatenin-1*, leads to the formation of a posterior head, while *wnt 11-1, 11-2* or *islet* RNAi interference cause defects in patterning and elongating posterior tissues, but never inversion of polarity.

Is the planarian antero-posterior (AP) axis also patterned by β-catenin activity during embryogenesis?

The role of the Wnt/β-catenin pathway in the establishment of the animal-vegetal (prospective AP) axis during early embryogenesis has been demonstrated not only in vertebrates but also in spiralians. In the nemertean *Cerebratulus lacteus*, as in most metazoans studied, β-catenin is restricted to the most vegetal cells and is required for proper endomesoderm development (Henry *et al.*, 2008). In the annelid *Platynereis dumerilii*, β-catenin is involved in establishing asymmetric sister-cell fate along the animal-vegetal axis (Schneider and Bowerman, 2007). Interestingly, this mechanism is also functional within Ecdysoazoans, in the nematode *C. elegans* (Kaletta *et al.*, 1997).

To date, only two reports have presented data on the expression of Wnt elements in planarian embryos, specifically in the planarian species *Schmidtea polycroa*, which is closely related to *S. mediterranea* (Martín-Durán *et al.*, 2010; Martín-Durán *et al.*, 2011). Despite their phylogenetic position as spiralians, planarians

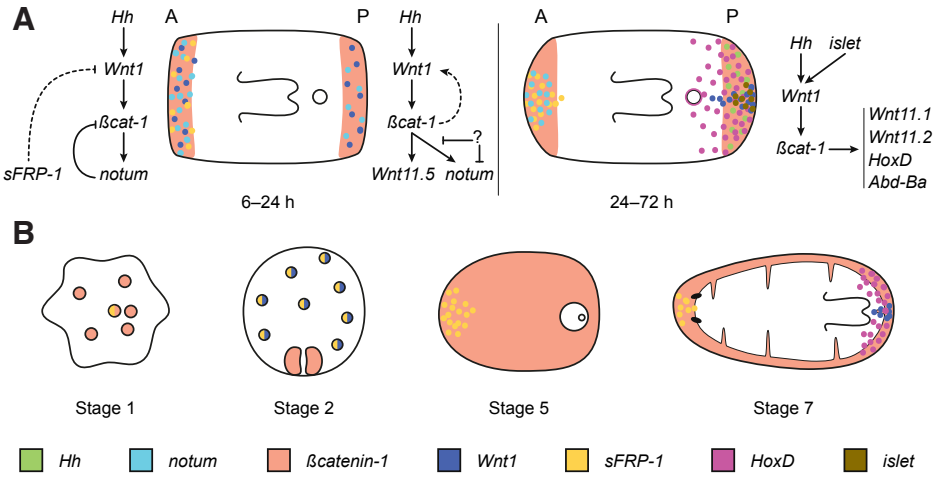

Fig. 5. Proposed model for establishment of posterior identities after amputation and comparison with the expression pattern of Wnt pathway components during embryogenesis. (A) *Proposed epistatic model for antero-posterior patterning during planarian regeneration. After wound-healing,* wnt1, *which is detected during the first few hours in any kind of wound, would activate* βcatenin-1, *which would in turn activate very early targets, such as* wnt11-5 *and* notum. *Notum, which normally modulates the range of Wnt activity, could inhibit the local function of* β*catenin-1 in the anterior wounds, but not in posterior ones, where an unknown signal should inhibit its function. Although no functional data have been reported, sFRP1 is predicted to inactivate Wnt1 in the anterior wound. Hh is known to modulate wound-induced* wnt1 *expression. In a second phase, differential programs are already launched in each blastema, but canonical Wnt activation needs to be maintained posteriorly. At this stage, around 2 days after amputation, the effectors of canonical Wnt signaling, such as the posterior Wnts and the posterior Hox genes, are activated. A indicates anterior; P, posterior.* **(B)** *Schematic representation of the expression of the Wnt elements analyzed during* Schmidtea polychroa *embryogenesis.* βcatenin-1 *is detected as early as stage 1, but only by RT-PCR. At stage 2,* βcatenin-1 *is expressed in the embryonic pharynx, and* sFRP1 *and* wnt1 *in the many blastomers around the embryo. At stage 5,* βcatenin-1 *is detected throughout the embryo, but the rest of the elements are not. At stage 7, the Wnt elements show a pattern clearly resembling that found in adults. Note that the expression dynamics of these Wnt elements during regeneration and during embryogenesis share some interesting features, for instance the early co-expression of* wnt1 *and* sFRP1 *and their subsequent polarization.*

show an ectolecithal embryo without any recognizable cleavage pattern and with the early formation of transient structures required to introduce the maternal nutrients. During cleavage, or stage 1 (the first 36 hours), blastomeres are not attached and remain dispersed within the yolk-derived syncytium. At stage 2, a recognizable organization emerges. Some blastomeres move towards the periphery of the syncytium and form a thin embryonic epidermis. Other blastomeres migrate to a point in the syncytium, which may or may not be prespecified by an existing signal, and differentiate to form a provisional embryonic pharynx that will ingest yolk cells (Martín-Durán *et al*., 2010). The embryonic pharynx is the only discernible external feature in planarian embryos until stage 6, when the definitive pharynx and the rest of the organs and tissues clearly differentiate.

βcatenin-1 is expressed at stage 2 exclusively in the embryonic pharynx of the yolk-feeding embryo. At stage 3 and 4, expression disappears, and at stage 5 it is detected ubiquitously in the germ band. By stage 7, embryos display the same expression pattern as that found in the adult, that is, widespread expression in the parenchyma and the nervous system (Fig. 5B). *wnt1* is also detected at stage 2 but in a completely different domain, that is, in the outlying blastomeres but not in the embryonic pharynx. Thereafter it is not detected until stage 7, in the juvenile, in a small cluster of cells at the posterior definitive tip, clearly resembling the adult pattern (Fig. 5B). *sFRP-1* is detected at stage 2 in the outlying blastomeres but not in the pharynx, the same as *wnt1*, but this expression is maintained during stages 3 and 4. At stage 5 it becomes restricted to discrete cells in the germ band on the opposite side to the degenerating embryonic pharynx. This asymmetric distribution is observed until the end of development, when *sFRP-1* is expressed in the anterior tip and in the definitive pharynx in the hatching embryo, again resembling the pattern found in the adult (Martín-Durán *et al*., 2010) (Fig. 5B).

Together, these data suggest that the definitive mechanisms for patterning of the AP axis, involving canonical Wnt signaling, emerge at stage 5, that is, after yolk ingestion and proliferation of the blastomeres in the germ band, and when the embryo begins to flatten and the definitive structures appear. Accordingly, *hoxD* is not detected until stage 6, initially in a strip of cells at the embryonic pole containing the definitive pharynx and spreading later on from the pharynx to the posterior end, resembling the adult pattern (Iglesias *et al*., 2008). Before stage 5, planarian embryos are undergoing patterning as well as differentiating a transient pharynx and beginning to actively feed on the external yolk. Due to its extremely divergent development, it is difficult to discern not only the role of canonical Wnt signaling but also whether axial polarity is established during the early stages of development. For instance, it is unclear whether the expression of *βcatenin-1* in the pharynx at stage 2 indicates a polarizing function of the whole embryo or is simply required for the differentiation of such a complex structure. However, the expression of β-catenin during differentiation of the transient pharynx could reflect an early role in endomesoderm specification, which is linked to posterior fates in most spiralian embryos analyzed (Martín-Durán *et al*., 2010). Nevertheless, the co-expression of *sFRP-1* and *wnt1* in the outlying blastomeres at stage 2, the maintenance of *sFRP1* but not *wnt1* at stage3, and the later restriction of their expression to the anterior and posterior tip of the embryo, respectively, strikingly resembles the situation found during regeneration in the anterior blastema (Fig. 5). However, the relationship between those genes and *βcatenin-1* in both situations is not clear at all, at least from the data available on mRNA expression. It is interesting to note that, despite *βcatenin-1* only being expressed in the pharynx during initial embryonic stages, it has been shown by RT-PCR to be highly upregulated during the first few hours of development. This raises the interesting question of whether in planarians, like in most metazoans, maternal β-catenin is essential for the first polar decisions of the embryo. In most bilaterians, the main body axes are established before gastrulation, and later on they are only refined (reviewed in Niehrs, 2010). If we understand gastrulation as the induction of extensive cell movements in order to differentiate the embryonic germ layers, we would expect that the first axial polarity is established in planarians during stage 1, before differentiation of the transient pharynx. It will be very interesting to analyze Wnt activation and *βcatenin-1* activity during this period to determine whether their roles are conserved not only for refining and re-patterning the AP axis but also for its establishment.

Do planarians have anterior and posterior organizers?

An organizer is often defined as a group of cells with the ability to induce organized cell fates in the surrounding tissue, even when ectopically transplanted. In planarians, like in *Drosophila* imaginal discs or regenerating amphibian limbs, the dorsoventral (DV) boundary seems to act as an organizing region. Interaction between dorsal and ventral epidermal cells is an essential step for planarian regeneration and, moreover, grafting experiments demonstrate that ectopic DV confrontations generate outgrowths with an organized AP pattern (Schilt 1970; Kato *et al.,* 1999; Agata *et al.,* 2007). Those observations suggest the presence of organizing regions at specific positions along the DV boundary, similar to the apical ectodermal ridge, which is responsible for proximo-distal patterning of vertebrate limbs during regeneration (reviewed in Fernandez-Teran and Ros, 2008).

These findings beg the question of whether an anterior and a posterior organizer are also required during planarian regeneration. Current data suggest the existence of a posterior organizer as a source of Wnts and an anterior one as a source of Wnt inhibitors (Adell *et al*., 2010; Meinhardt, 2009), and both of them would be very quickly re-established after amputation. However, this raises two important questions. Firstly, are those organizers also responsible for maintenance of the AP axis in intact animals? Secondly, is the anterior organizer also a source of neural activators?

The induction of different fates along the AP axis is likely to be the result of diffusion of the morphogens (Wnts) and their antagonists (sFRPs) or modulators (notum). However, given the large size of planarians (1- 10 mm), it seems unlikely that so few molecules could account for the patterning of the whole animal. Even during later stages of regeneration, when planarian fragments undergo extensive morphallaxis that requires complete cell re-specification, the range of apical morphogens will need to be very long. The observation that several Wnts and sFRPs are expressed in nested domains along the entire AP axis does support the hypothesis that their integrated activity would account for a graded activation of *βcatenin-1*. The 'intercalary model' of regeneration (Chandebois 1979; Chandebois 1980; Agata *et al.,* 2007) perhaps re-defines the mechanism of patterning more accurately. According to this model, the activity of the signals from the apical organizers would

be restricted to a number of cell diameters and would instruct the identity of tissue edges in the planarian. Intercalary regeneration can function as a general mechanism to re-arrange tissues and organs during regeneration and accounts for the re-specification of central structures. In our opinion, this model is also applicable to homeostasis in planarians. Of course, these models are not mutually exclusive, and canonical Wnt signaling could play an essential role in the process of intercalary regeneration, or 'continuous intercalary re-specification' in the case of intact animals. Nevertheless, it should be noted that the existence of a real gradient of β-catenin activity in planarians, such as the one reported in *Xenopus* embryos (Kiecker *et al.,* 2001), has never been demonstrated.

The potential role of the anterior organizer in the induction of neural fates is an intriguing problem, particularly the question of whether anterior identity can be uncoupled from neural differentiation. Although this issue is far from being resolved, some very interesting preliminary results have been obtained. It has been known for some years that silencing of *nou-darake* (*ndk*), an FGF receptor with inhibitory activities, induces brain differentiation along the length of the planarian body without affecting the AP identities (Cebriá *et al.*, 2002). Interestingly, *ndk* RNAi animals also differentiate a pair of 'brain primordia' in the posterior blastema (Cebriá *et al.*, 2002; Iglesias *et al.*, 2011). Thus, it could be that FGF activation induces neural fates in planarians, as has been described in models of embryonic development (reviewed in Stern, 2005). Very recently it has been reported that the 'two-tailed' animals generated after *APC* or *axins* RNAi also develop a pair of 'brain primordia' in ectopic posterior blastemas, which would correspond to the anterior wounds (Iglesias *et al.*, 2011). Although differentiation of those brain primordia cannot progress in the presence of high *βcatenin-1* activity, this observation demonstrates that neural differentiation can take place in 'posterior' fated tissue. Taken together, these results suggest that early brain determination can be uncoupled from blastema polarity, and that FGF could function as a 'brain activator' secreted from the anterior organizer (Adell *et al.,* 2010; Iglesias *et al.*, 2011). Further evidence that brain differentiation can be uncoupled from positional identify comes from functional analysis of *prep*, a TALE class homeodomain protein that is expressed specifically in the head region (Felix and Aboobaker 2010). Although this gene is required for head formation and anterior markers are dramatically diminished when it is silenced, the ectopic brain induced after *ndk* silencing does not require *prep* function.

Finally, the different phenotypes generated after canonical Wnt signal modulation suggest that the range and nature of the anterior and posterior organizers are essentially different (Meinhardt, 2009). *βcatenin-1* downregulation easily induces completely anteriorized animals with a continuous brain and without any central structures such as the pharynx. In contrast, upregulation of *βcatenin-1* never completely posteriorizes planarians. Instead, it generates 'two-tailed' animals with corresponding pharynges that have opposing orientations. Thus, suppression of the posterior organizer allows continuous differentiation of anterior structures, while induction of an ectopic posterior organizer is only possible at wounds and exerts a short-range effect. More data are required on the activity of Wnt pathway components and other factors that act in wounds and during homeostasis in order to explain these differential responses.

S. mediterranea wnt5 acts through a non canonical/β-catenin-independent pathway to control neural connectivity

Very little is known about the involvement of the β-catenin-independent pathway in the course of planarian regeneration. So far, the non-canonical Wnt pathway has been implicated in two different processes. Firstly, it is known to be involved in controlling neural connectivity during nervous system regeneration and, secondly, it plays a role in the apical docking and planar polarization of the cilia (Almuedo-Castillo *et al.*, 2011). *wnt5*, the only non-canonical Wnt described in planarians, is thought to function as a signal to restrict nervous system growth along the medio-lateral axis (Adell *et al.*, 2009; Gurley *et al.*, 2010). Secretion of Wnt5 is thought to require the transmembrane protein evi/wntless and probably also signals through both Dvls. This is based on the observation that loss of function of these former genes leads to similar defects in terms of lateral displacements and disconnections of the CNS, as well as aberrant projections of the visual axons (Adell *et al.*, 2009; Almuedo-Castillo *et al.*, 2011) (Fig. 1). However, the molecular mechanisms underlying wnt5-dependent positioning of neural structures remain to be defined. Two different options can be considered, namely that wnt5 establishes medio-lateral patterning

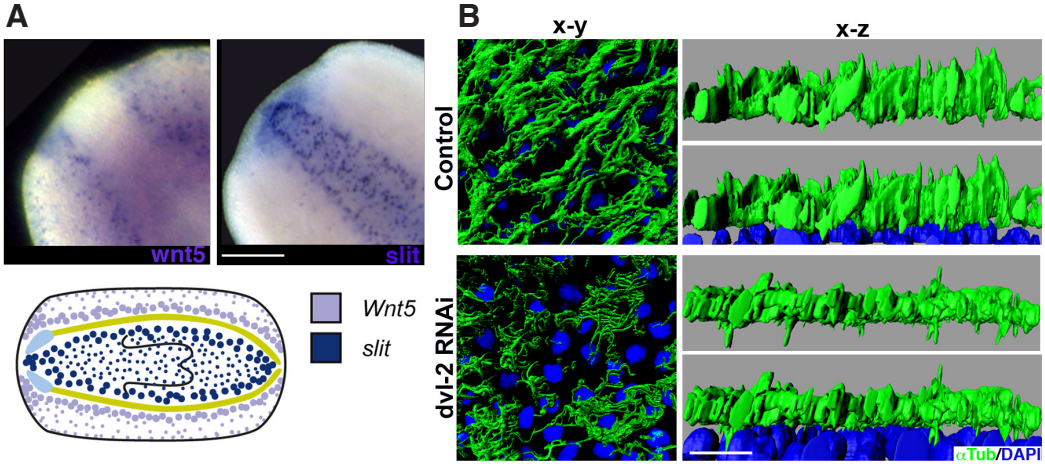

Fig. 6. The non-canonical Wnt pathway during planarian regeneration. (A) *The expression patterns of* wnt5 *and* slit *suggest a shared role in positioning the planarian nervous system along the medio-lateral axis. Animals correspond to trunk pieces 3 days after amputation. Anterior is shown to the left.* **(B)** *Anti-α-tubulin (αTub) immunostaining of control and* dvl-2 *RNAi planarians. Epidermal cilia of* dvl-2*-silenced planarians fail to localize apically and appear disorganized, shorter and embedded in the nuclear layer. x-y and x-z confocal projections are shown. DAPI nuclear staining is shown in blue. A region located approximately 1 mm from the anterior end of the planarian was chosen for imaging ventral cilia. Confocal sections were deconvolved using Huygens Deconvolution Software (Scientific Volume Imaging) and processed using Imaris Software (Bitplane) for 3D reconstructions. Scale bar, 10 μm.*

of the blastema (Gurley *et al*., 2010) or that it restricts the positioning of the regenerating nervous system by regulating migration of the neural precursor cells or axonal growth (reviewed in Endo *et al*., 2007). The second possibility would be consistent with its evolutionarily conserved role as a repulsive cue for growing axons. Notably, *Wnt5* and *slit,* another conserved signal involved in repelling axon growth, show complementary expression patterns in which *Wnt5* is expressed from the outer part of the CNS to the planarian margin and *slit* is expressed from the internal part of the CNS to the midline (Marsal *et al*., 2003; Cebriá *et al*., 2007; Adell *et al*., 2009; Gurley at al., 2010) (Fig. 2). Moreover, the silencing of slit in planarians leads to collapse of the CNS at the midline (Cebrià *et al*., 2007), a phenotype that could be considered opposite to the one generated after silencing *wnt5*, at least in relation to nervous system regeneration. All this may indicate that *wnt5*, together with *slit*, restricts the positioning of the newly differentiated nervous tissues along the medio-lateral axis of the blastema (Almuedo-Castillo *et al*., 2011) (Fig. 6A). Whether or not these two molecules have reciprocal roles in regulating and restricting the expression of the other remains to be determined.

A planar cell polarity network is required for ciliogenesis and docking of apical basal bodies in the planarian epidermis

Planar cell polarity refers to the ability of cells to become asymmetrically organized within the plane of the tissue. This capacity is commonly controlled by a non-canonical branch of the Wnt pathway known as the PCP cascade, which involves the accumulation of specific components to different regions of the cell. Some of the key components of the PCP cascade are known to be involved in ciliogenesis. For instance, the docking and polarization of cilia basal bodies within the epithelial sheet appears to involve a combination of hydrodynamic forces and signaling through PCP proteins (reviewed in Wallingford and Mitchell, 2011).

In planarians, we have found that components of the evolutionarily conserved core PCP pathway—planarian orthologs of dishevelled (*dvl1/2*), van-gogh (*vang1/2*) and diversin (*div*)—are involved at least in the apical positioning of the cilia basal bodies (Almuedo-Castillo *et al*., 2001). In a control situation, cilia emerge from the apical side of the cells, and the actin filaments are located on top of the nucleus (Fig. 6B). Silencing of *dvl-2*, *vang-1* and *-2*, or *div* leads to an aberrant arrangement of the cilia network, both at the level of the plane of the epithelium and at its apical-basal axis (Almuedo-Castillo *et al*., 2011). Epithelial cilia appear at a lower density and are shorter and disorganized. In addition, the basal bodies of the cilia fail to reach the most-apical part of the epithelial cells (Fig. 6B). Interestingly, this loss of polarization is also observed in the epithelial actin network and a high concentration of empty vesicles and isolated basal bodies is found to be embedded in the cytoplasm of *dvl-2* RNAi planarians. Consistent with this observation, it has been shown that the positioning of the basal bodies in *Xenopus* embryos relies on the functional interaction of Dvl and the actin-remodeling Rho-GTPase, RhoA, in directing vesicle-containing basal bodies towards the apical side of the epithelial cells by active actin cytoskeleton rearrangements (Park at al., 2008). These observations prompt to us to conclude that the defective apical positioning of the cilia observed after silencing core components of the PCP pathway in planarians could be a consequence of abnormal assembly of the actin filaments, leading to aberrant vesicle trafficking of the cilia basal bodies.

Given the phylogenetic position of planarians within the Lophotrocozoa superphylum, the presence of a functional PCP network in these organisms has evolutionary significance. The relationship between PCP and ciliogenesis has been demonstrated in vertebrates (reviewed in Wallingford and Mitchell, 2011), but not in invertebrates, possibly because the most common models, such as *Drosophila*, do not have a ciliated epidermis. The presence of this network within the spiralians thus suggests that it may be an ancient feature in bilaterians. Interestingly, while the role of Wnt ligands as master orientating cues for planar polarity is broadly accepted in vertebrates, there is no evidence for such a role in *Drosophila* (Chen *et al*., 2008; Gao at al.,2011). When we consider that no defects related to cilia docking are found after silencing any Wnt ligand in planarians (Almuedo-Castillo *et al*., 2011) (Fig. 2) these results together suggest that orchestration of planar polarity by a Wnt gradient may be a vertebrate innovation (reviewed in Goodrich 2011).

Finally, although it might be predicted that interference with core components of the PCP would affect processes such as cell migration of different cell types during regeneration, no such defects are observed. In fact, the only defects that have been described are those relating to ciliogenesis. Although we tend to think of all PCP proteins as a complex, however, it has been demonstrated that many features are context specific and vary between the different tissues and organisms under study (reviewed in Goodrich and Strutt, 2011). Thus, further studies are required in order to identify new functions of the PCP network during planarian regeneration.

Concluding remarks

Current evidence from planarians supports an evolutionarily conserved role for canonical Wnt signaling in specifying posterior identity not only during embryogenesis but also in adults. In planarians and cnidarians, the two classical models with extreme regenerative capacities, this mechanism is active both during regeneration and homeostasis. Although very little is known about the activation of canonical Wnt signaling at the adult stage of non-regenerating models, it is tempting to speculate that the constant maintenance of these 'embryonic' signals could be the key to regenerative capacity, as recently discussed in Reddien (2011). A second feature of regenerative animals is the existence of pluripotent and totipotent stem cells. The neoblast population of planarians possesses pluripotent/totipotent stem cells, able to differentiate into any cell type (Wagner *et al*., 2011). It may be that the maintenance of these stem cells in adult animals is possible thanks to the continued activation of the signaling centers. In planarians, the BMP signaling pathway also has an evolutionarily conserved role, as it is required for re-establishment and maintenance of the DV axis during regeneration and homeostasis, respectively (Molina *et al*., 2007; Orii *et al*., 2007; Reddien *et al*., 2007; Molina *et al*., 2011; Gaviño *et al*., 2011). Thus, in adult planarians, as in most metazoan embryos, Wnts and BMPs establish the main body axes that specify a spatial coordinate system to pattern the body. The establishment of both the AP and DV axis must be interdependent because they are always perpendicular. In planarians, it is not known whether a unique organizer could share both functions or, as proposed by Meinhardt (2009), the DV interaction triggers

the formation a first organizer, which establishes DV identities, and then the AP organizer arises to pattern the AP identities in a perpendicular direction.

In contrast to the β-catenin-dependent pathway, the molecular components and interactions that underlie the specific activation of the different non-canonical branches of the Wnt signaling pathway are still far from clear. Interestingly, results in planarians support a conserved role for Wnt5 as a β-catenin-independent input. It remains to be determined whether it regulates cell fate, cell migration or functions as an axon guidance cue. Perhaps more relevant, however, is the discovery that PCP elements have a conserved role in apical positioning of the cilia in planarians, as this had only been reported previously in vertebrates. In planarians, like in *Drosophila*, PCP appears not to depend on Wnt ligand, and components of the PCP pathway appear to exert local effects that do not require morphogenetic signals. Nevertheless, the possibility that Wnts act as modulators of this pathway, possibly through Frizzled binding, cannot be completely ruled out.

Recent years have seen substantial advances in our understanding of the role of the Wnt pathway during planarian regeneration and homeostasis, and evidence has accumulated that its function is conserved from the embryo to the adult. However, as usual, with new information come new questions. Many elements of the pathway remain to be characterized in planarians, for instance the receptors and co-receptors, and the nuclear transcription factors. More importantly, we still do not know which cell types express the different Wnt elements and how they control neoblast differentiation and migration. To advance research in these areas, technical developments will also be required, for instance to obtain reliable readouts of the different branches of the Wnt pathway or analyses at the protein level, such as protein localization, protein-protein interaction, over-expression and live-imaging. Of course, all these methodologies rely on the successful implementation of transgenesis in planarians. Furthermore, it is clear that much more effort should be invested in studying non-standard models across the entire phylogenetic tree and at different developmental stages in order to identify common and derived traits and discover the essential mechanisms that govern cell behavior. Planarians are currently established as an invaluable model for integrative research into body patterning, tissue regeneration and stem cell biology. We therefore expect many of the questions raised in this review to be answered in the coming years.

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