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# Expression of secreted Wnt pathway components reveals unexpected complexity of the planarian amputation response

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ABSTRACT

Regeneration is widespread throughout the animal kingdom, but our molecular understanding of this process in adult animals remains poorly understood. Wnt/β-catenin signaling plays crucial roles throughout animal life from early development to adulthood. In intact and regenerating planarians, the regulation of Wnt/β-catenin signaling functions to maintain and specify anterior/posterior (A/P) identity. Here, we explore the expression kinetics and RNAi phenotypes for secreted members of the Wnt signaling pathway in the planarian *Schmidtea mediterranea*. *Smed-wnt* and *sFRP* expression during regeneration is surprisingly dynamic and reveals fundamental aspects of planarian biology that have been previously unappreciated. We show that after amputation, a wounding response precedes rapid re-organization of the A/P axis. Furthermore, cells throughout the body plan can mount this response and reassess their new A/P location in the complete absence of stem cells. While initial stages of the amputation response are stem cell independent, tissue remodeling and the integration of a new A/P address with anatomy are stem cell dependent. We also show that WNT5 functions in a reciprocal manner with SLIT to pattern the planarian mediolateral axis, while WNT11-2 patterns the posterior midline. Moreover, we perform an extensive phylogenetic analysis on the *Smed-wnt* genes using a method that combines and integrates both sequence and structural alignments, enabling us to place all nine genes into Wnt subfamilies for the first time.


## Introduction

Most animal phyla contain species that regenerate tissues lost to injury with various degrees of success and some of these animals display extraordinary regenerative capacities (Brockes and Kumar, 2008; Holstein, 2008; Poss et al., 2002; Reddien and Sánchez Alvarado, 2004). Despite sharing a similar genetic toolkit with regeneration-competent animals, mammalian regeneration pales by comparison. Why such disparities in regenerative abilities exist across metazoan phyla is presently unknown.

The interrogation of animal development in recent decades has revealed a deep conservation of intercellular signaling pathways that allow cells to communicate and coordinate embryonic processes such as axis formation, cell division, differentiation, organogenesis, and tissue patterning (Pires-daSilva and Sommer, 2003). Some of these pathways are re-activated during regeneration, but little is known about how signaling is coordinated during a regenerative response or whether differences in regenerative abilities stem from differences in signaling pathway recruitment (Galliot and Ghila, 2010; Gurley and Sánchez Alvarado, 2008; Stoick-Cooper et al., 2007a).

Planarians provide an attractive model system to study the role of cell signaling during regeneration because their genome encodes major signaling pathway components (Adell et al., 2009; Gurley et al., 2008; Iglesias et al., 2008; Petersen and Reddien, 2008; Rink et al., 2009; Yazawa et al., 2009) and because they display an incredible ability to tolerate a wide variety of amputations (Morgan, 1898, 1900). Even small fragments removed from the flank of the body can regenerate entire worms of proper proportion (Randolph, 1897). This remarkable plasticity relies on the presence of adult somatic stem cells that are broadly distributed throughout the body plan, divide to constantly replenish cells lost to tissue turnover, and give rise to all tissues including the nervous, gastrovascular, muscular, and excretory systems (Newmark and Sánchez Alvarado, 2000; Pellettieri and Sánchez Alvarado, 2007; Reddien and Sánchez Alvarado, 2004).

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Planarian stem cells located anywhere along the anterior/posterior (A/P) axis have the intrinsic ability to regenerate a head or tail (Morgan, 1904). This choice depends upon the cell's position in the freshly amputated fragment (Morgan, 1898). Thus, communication between stem cells and the surrounding pre-existing tissue is critical for proper fate choice. However, the extent to which differentiated cells respond to amputation or to their new relative location independent of stem cells is poorly understood. It was recently shown that normal amputation-induced organism-wide apoptotic responses still occur in the absence of stem cells (Pellettieri i et al., 2010), but we have only begun to understand which signaling pathways are involved in the initial phases of regeneration and how these pathways are coordinated to facilitate a regenerative response.

We and others have demonstrated that Wnt/β-catenin signaling is essential to guide proper regeneration in planarians (Adell et al., 2009; Gurley et al., 2008; Iglesias et al., 2008; Petersen and Reddien, 2008, 2009). Wnt ligands define a deeply conserved family of secreted glycoproteins that have diverse effects on cell function through β-catenin dependent or independent pathways. Depending on context, Wnts influence cell proliferation, fate choice, migration, survival, and even maintenance of multipotency (Clevers, 2006; van Amerongen and Nusse, 2009; Veeman et al., 2003). In adult humans, Wnt pathway misregulation can lead to disease and cancer (Clevers, 2006; Logan and Nusse, 2004; Moon et al., 2004).

In planarians, Wnt/β-catenin signaling is a critical molecular switch that controls the choice to regenerate a head or tail. Specifically, increased Wnt/β-catenin activity specifies posterior fate and elicits tail regeneration (Gurley et al., 2008; Rink et al., 2009), while decreased Wnt/β-catenin activity specifies anterior fate and triggers head regeneration (Adell et al., 2009; Gurley et al., 2008; Iglesias et al., 2008; Petersen and Reddien, 2008, 2009). Interestingly, silencing *Smed-βcatenin-1* in intact planarians causes widespread anteriorization and ectopic head formation (Gurley et al., 2008; Iglesias et al., 2008; Petersen and Reddien, 2008), suggesting that as in humans, β-catenin signaling is active and highly regulated in intact adult planarians.

Consistent with a role for β-catenin in specifying posterior fate, numerous *wnt* genes are expressed in the posterior end of intact planarians (Gurley et al., 2008; Petersen and Reddien, 2008). Likewise, two of the three secreted frizzled related proteins (sFRPs), which are frequently assumed to be inhibitors of Wnt signaling (Mii and Taira, 2009), display anterior-specific expression (Gurley et al., 2008; Petersen and Reddien, 2008). After amputation, small fragments such as tails radically reorganize the A/P axis and coordinately modify wnt and sFRP expression to re-establish the proper adult patterns. It is unknown to what extent this process depends on the regeneration of new tissue. Two previous reports suggested that pre-existing differentiated tissues can respond to amputation and reorganize the A/P axis in the absence of stem cells (Ogawa et al., 2002; Petersen and Reddien, 2009). However, these analyses were both limited to the expression of one or two genes and did not address how newly generated tissues integrate with pre-existing tissues during later stages of regeneration and tissue remodeling.

To gain further insight into the regeneration response of planarians, we characterized the phenotypes resulting from *wnt* gene silencing, and explored the expression of eight *wnt* and three *sFRP* genes during regeneration. Our expression studies provide valuable insights into the dynamic response of planarian tissues to amputation and to the interplay between pre-existing tissues and stem cells during regeneration. We show that cells throughout the animal assess their new position along the A/P axis in the complete absence of stem cells. However, both the remodeling of existing organ systems and the proper integration of A/P location with the anatomy is stem cell dependent. Additionally, our extensive phylogenetic analyses placed all nine *Smed-wnt* genes into Wnt subfamilies for the first time. Finally, we report on phenotypes resulting from *Smed-wnt5 (RNAi)* and *Smed-wnt11-2(RNAi)*. WNT5 functions reciprocally with SLIT to organize the mediolateral axis, while WNT11-2 patterns the tail midline.

## Materials and methods

### *Planarian maintenance*

The CIW4 clonal line of *Schmidtea mediterranea* was maintained as previously described (Cebrià and Newmark, 2005; Sánchez Alvarado et al., 2002). 1–2 week starved animals were used for all experiments.

### *Gene sequences*

Human protein sequences were used to find planarian homologs of secreted Frizzled-related proteins: *Smed-sFRP-2* (Gurley et al., 2008), GenBank accession number HM751831; and *Smed-sFRP-3* (Gurley et al., 2008), GenBank accession number HM751832, from the *S. mediterranea* genome database (smedgd.neuro.utah.edu) (Robb et al., 2008) via BLAST (Fig. S1). Planarian homologs were then used for reciprocal BLAST against the human refseq database to verify homology. Protein domains were predicted using InterPro (Hunter et al., 2009). All sequences were cloned from cDNA obtained from an 8-day regeneration series as described (Gurley et al., 2008). Complete sequences and accession numbers have been previously reported for all nine *Smed-wnt* genes, in addition to *sFRP-1* (Gurley et al., 2008; Petersen and Reddien, 2008), *porcn-1 (porcn-a*, Gurley et al., 2008, EU130791), *PC-2* (Gurley et al., 2008), and *slit* (Cebrià et al., 2007).

### *Phylogenetic methods*

#### *Sequence alignments*

The Wnt sequences of the planarian *S. mediterranea* were aligned using the integrated alignment approach that combines sequence and structural alignment (Lengfeld et al., 2009).

#### *Phylogenetic trees*

Phylogenetic trees were reconstructed using maximum likelihood (ML) and Bayesian methods using the WAG model (Whelan and Goldman, 2001) assuming rate homogeneity or assuming rate heterogeneity with 4 discrete Gamma rate categories (Yang, 1993). Missing parameters are estimated from the data and option set to default settings if not otherwise stated. Maximum likelihood trees were constructed using IQPNNI 3.3 (Minh et al., 2005; Vinh le and Von Haeseler, 2004) applying the stopping rule after a minimum of 200 iterations and a maximum of 2500. ML bootstrap trees/values from 100 bootstrap trees were computed with the same parameters but using the bootstrap option (-bs) of IQPNNI 3.3 and summarized using a relative majority consensus (Schmidt, 2003) as implemented in TREE-PUZZLE 5.3 (Schmidt and von Haeseler, 2007). Puzzling trees and puzzle support values have been constructed with TREE-PUZZLE 5.3. For Quartet Puzzling (QP) and/or SuperQP trees puzzling trees and puzzle support values have been constructed with TREE-PUZZLE 5.3 (Schmidt and von Haeseler, 2007) applying either Quartet Puzzling voting scheme (QP, cf.) (Strimmer et al., 1997; Strimmer and Von Haeseler, 1996) or the Superquartet Puzzling scheme (SuperQP) (Schmidt, 2003) summarizing with a relative majority consensus (Schmidt, 2003). Bayesian trees were computed using MrBayes (Ronquist and Huelsenbeck, 2003) performing four runs with two chains running for 30 Mio generations each. Every 200th tree was sampled from the cold chains after a burn-in of 5 Mio generations. The results were checked for convergence artifacts with Tracer 1.4.1 (http://tree.bio.ed.ac.uk/software/tracer/).

### *RNAi*

RNAi feedings were performed as described previously (Gurley et al., 2008) with the following modifications: soft-serve RNAi food for all genes was prepared 2–4 times more concentrated. *Smed-wnt(RNAi)* animals were fed 4–9 times every 2–3 days prior to a single amputation. Long-term *Smed-wnt(RNAi)* intact animals were fed RNAi food 1–2 times per week until the indicated fixation day. For Fig. S18, *APC(RNAi)* animals were fed 3–4 times and *βcatenin(RNAi)* animals were fed twice before amputation. For all RNAi experiments, animals were cut 3–5 days after the last feed.

### *Gamma irradiation*

100 Gy (10,000 rd) of γ-irradiation was delivered to animals as previously described (Eisenhoffer et al., 2008). Animals were then amputated 3–5 days after irradiation as specified in the text. These are time points when markers for proliferation, neoblasts, and immediate division progeny have already been lost (Fig. S16A) (Eisenhoffer et al., 2008).

### *In situ hybridization and immunostaining*

Fluorescent and colorimetric in situ hybridizations were performed as previously described (Pearson et al., 2009). Anti-α-Tubulin AB-2 mouse monoclonal antibody from Fisher Scientific was used at 1:300 to detect the cephalic ganglia, nerve cords, and pharynx (Robb and Sánchez Alvarado, 2002). VC-1 mouse monoclonal antibody, a kind gift from Dr. Kiyokazu Agata, was used at 1:10,000 to detect photoreceptors and the visual axons (Agata et al., 1998). Anti-phospho-histone H3 (ser10) MC463 rabbit