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Polarized activation of *notum* at wounds inhibits Wnt signaling to promote planarian head regeneration

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Abstract

Regeneration requires initiation of programs tailored to the identity of missing parts. Head-versus-tail regeneration in planarians presents a paradigm for study of this phenomenon. Following injury, Wnt signaling promotes tail regeneration. We report that wounding elicits expression of the Wnt inhibitor *notum* preferentially at anterior-facing wounds. This expression asymmetry occurs at essentially any wound, even if the anterior pole is intact. *notum(RNAi)* animals regenerate an anterior-facing tail instead of a head, and double-RNAi experiments indicate *notum* inhibits Wnt signaling to promote head regeneration. *notum* expression is itself controlled by Wnt signaling, suggesting regulation of feedback inhibition controls the binary head-tail regeneration outcome. We conclude that local detection of wound orientation with respect to tissue axes results in distinct signaling environments that initiate appropriate regeneration responses.

How an organism determines what cells or tissues are missing for regeneration is poorly understood. Planarians are freshwater flatworms that can regenerate from nearly any injury (1). The head-versus-tail regeneration decision in planarians, known as regeneration polarity, is a paradigm for studying appropriate regeneration program specification (2). Wnt signaling controls regeneration polarity, with pathway components *β-catenin-1* (3–5) and *wnt1* (formerly called *wntP-1)* (6–8) required to prevent head regeneration and promote tail regeneration at posterior-facing wounds. *wnt1* expression is upregulated near both anterior- and posterior-facing wounds (6, 8, 9). Therefore, how *wnt1* and *β-catenin* act to promote tail formation only at appropriate wounds is unknown.

We sought factors that inhibit Wnt signaling at anterior-facing wounds to promote head regeneration, and identified a planarian homolog of *Drosophila notum* (*Smednotum*, Figure S1). Notum proteins are secreted α/β-hydrolase-family members (10, 11), cleave GPI anchors of cell-surface proteins (12), and can act on glypicans to modulate *Drosophila* Wnt signaling (10, 11, 13, 14). Glypicans are cell-surface, heparan-sulfate proteoglycans that participate in several signaling pathways (15). The roles of Notum proteins in development are unknown outside of *Drosophila*.

notum was expressed at the planarian anterior pole (Figure 1A). *wnt1*, by contrast, is expressed oppositely, at the posterior pole (3, 6). Early following head and tail amputation (6–24 hours, h), *notum* expression was highly upregulated preferentially near anterior-facing wounds (Figure 1B, Figure S2). *notum* expression was weaker, and initiated later, at posterior-facing wounds. Later following amputation (48–72h), anterior *notum* expression was coalesced at the pole, while posterior expression remained low (Figure 1C, Figure S2).

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notum was expressed in subepidermal cells (Figure 1D) that, at wounds, resemble *wnt1*-expressing cells (6). Indeed, *notum* and *wnt1* were co-expressed in some cells at anterior-facing wounds (Figure 1E).

To test whether wound-site *notum* expression is specific to head amputation, we incised animal sides without tissue removal. *notum* expression was detected specifically on the anterior-facing side of these sealed incisions (Figure 1F). Therefore, asymmetric *notum* expression following wounding (greater at anterior-facing than at posterior-facing wounds) does not require loss of large tissue regions, such as the anterior pole. Asymmetric wound expression also occurred at sealed incisions diagonal to the main body axis (Figure 1G) and was independent of anterior or posterior pole presence (Figure 1H), indicating local cues rather than signals from poles control *notum* expression asymmetry at wounds. We conclude that wounding elicits *notum* expression, dependent on wound-edge orientation with respect to the polarized primary body axis.

Posterior-facing wounds could be non-permissive, and/or anterior-facing wounds could be specifically instructive, for *notum* expression. We therefore examined *notum* expression between two closely opposed wounds. Regions neighboring only an anterior-facing wound had more *notum*-expressing cells than did regions bordering both anterior- and posterior-facing wounds (11.0 +/- 6.9 versus 1.6 +/- 2.4 cells, respectively, n=8 animals; Figure 1I). These data suggest posterior-facing wounds suppress wound-induced *notum* expression, providing expression asymmetry.

The specificity of strong *notum* expression for anterior-facing wounds suggested *notum* might control regeneration polarity. Following head and tail amputation, *notum(RNAi)* animals failed to regenerate a head with photoreceptors (47%, n=113) and regenerated posterior-facing tails apparently normally (Figure 2A). *notum(RNAi)* animals that did regenerate at least one photoreceptor did so aberrantly, possibly reflecting a weakly expressive *notum(RNAi)* phenotype (Figure S3). To characterize *notum(RNAi)* anterior blastemas lacking photoreceptors, we assessed axial marker expression (Figure 2B-F). *notum(RNAi)* anterior blastemas lacked cephalic ganglia and anterior-pole marker expression (*sFRP-1*) (Figure 2B-C). By contrast, *notum(RNAi)* anterior blastemas expressed the posterior markers *wnt1* and *frizzled-4* (Figure 2D-E). Furthermore, *notum(RNAi)* animals regenerated an anterior gut with posterior-specific morphology (two main branches; Figure 2F). We conclude that *notum* inhibition caused regeneration of an anterior-facing second tail following head and tail amputation. *notum* dsRNA delivery only after amputation also resulted in a regeneration polarity reversal (Figure S4), indicating a requirement for new *notum* expression following wounding.

wntP-2 expression is upregulated at posterior- and not anterior-facing wounds, and this requires *wnt1* and *β-catenin-1* (6). Therefore, *wntP-2* expression reflects an early readout (*i.e.*, prior to significant tissue formation) of *wnt1*/*β-catenin*-mediated polarity specification. *notum(RNAi)* fragments expressed *wntP-2* ectopically at anterior-facing wounds by 48 hours after injury (Figure 2G), indicating *notum* normally prevents activation of *β-catenin* targets at anterior-facing wounds.

The *notum(RNAi)* phenotype is similar to that caused by inactivation of APC, an intracellular β-catenin inhibitor (4). Additionally, *Drosophila notum* inhibits Wnt signaling in imaginal discs (10, 11). Therefore, we performed double-RNAi experiments to assess the candidate pathway of action involving *notum*, *β-catenin-1*, and *wnt1*. *β-catenin-1* and *notum* double RNAi resulted in a polarity phenotype identical to that of *β-catenin-1* RNAi alone—anterior- and posterior-facing head regeneration (Figure 3A). Similarly, *wnt1* inhibition suppressed the polarity phenotype caused by *notum* RNAi (Figure 3A). *notum* RNAi

efficiency was not reduced in double-RNAi animals (Figure S5), indicating that suppression of the *notum* phenotype by *wnt1* and *β-catenin-1* dsRNA is unlikely simply due to competition with *notum* dsRNA for RNAi. These data suggest that the *notum(RNAi)* phenotype requires *wnt1* and *β-catenin* genes, supporting a model in which *notum* normally inhibits *wnt1* and *β-catenin-1* function to allow head regeneration.

Hedgehog signaling impacts planarian regeneration polarity (9, 16), so we tested whether *notum* requires or influences Hedgehog signaling. *patched* RNAi overactivates Hedgehog signaling and increases *wnt1* wound expression; *hedgehog* inhibition reduces *wnt1* wound expression (9, 16). By contrast, *notum(RNAi)* animals displayed normal *wnt1* expression following amputation (Figure S6A), suggesting *notum* does not act in polarity by influencing Hedgehog activity. Second, *patched(RNAi)* animals regenerated anterior tails (n=3/10), but had normal asymmetric *notum* expression at wounds (n=8/8, Figure S6B), suggesting Hedgehog signaling does not act in regeneration polarity to drive asymmetric *notum* expression at wounds.

notum can function as a *wingless* (Wnt) feedback inhibitor in *Drosophila* (10, 11), so we tested whether Wnt signaling is required for wound-induced *notum* expression. *β-catenin-1* inhibition prior to amputation robustly reduced *notum* expression levels near wounds (Figure 3B, S7). Conversely, *APC* RNAi caused *notum* upregulation near wounds (Figure 3B, S7A-C). Therefore, Wnt signaling is necessary and can be sufficient at wounds for *notum* expression. Whether *Smed-notum* is a direct or indirect β-catenin transcriptional target is unknown, but *notum* is a direct target of Wnt signaling in cultured *Drosophila* and mammalian cells (17, 18). Wnt signaling perturbation impacted *notum* expression regardless of wound orientation (Figure S7A-B), so we propose that some other process ensures asymmetric *notum* expression at wounds. Specifically, in *APC(RNAi)* animals, *notum* was upregulated at wounds, but expression asymmetry remained. Because *Smed-notum* inhibits *β-catenin-1* activity and requires *β-catenin-1* for its effects, these results suggest regulation of feedback inhibition controls the regeneration polarity decision (Figure 3C).

Wnt signaling is used broadly in regeneration (19–23), and our results suggest that Notum proteins can be an important determinant of the outcome of Wnt expression in regeneration. Additionally, primary body axis development involves anterior Wnt inhibition in many animals (24); *notum* is an ancient gene present in many metazoans (Fig. S1), making it a candidate for controlling anterior identity broadly. Feedback inhibitors operate in many signaling pathways (25), and frequently simply attenuate pathway output. Here we present evidence that a target and inhibitor of Wnt signaling, the secreted hydrolase NOTUM, controls the switch-like behavior of the head-versus-tail regeneration decision in planarians. These results raise the possibility that control of whether feedback inhibition occurs could in general be used to mediate binary developmental decisions.

In principle, decisions of which tissues to regenerate could be accomplished only by sensing the absence of particular structures. By contrast, our results indicate that regeneration programs elicited at wounds can involve local responses to tissue orientation regardless of the identity of missing tissue. We conclude that initiation of correct regeneration programs involves responses to wounding that depend on local tissue polarization, such as along a body axis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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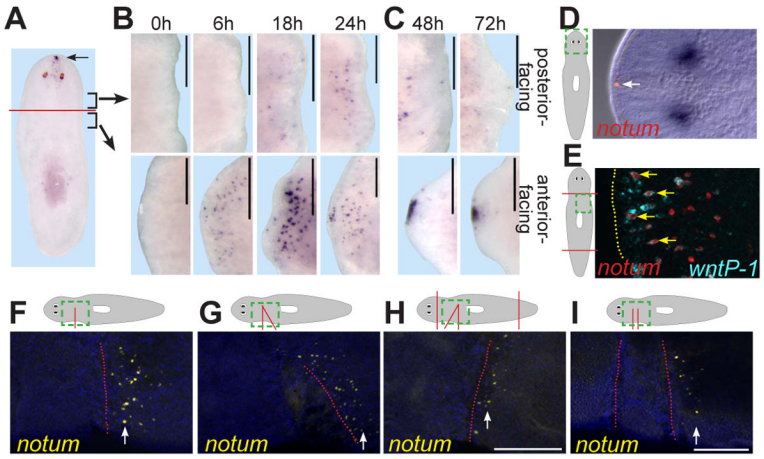


Figure 1. *notum* is expressed at anterior-facing wounds

(A-C) *notum in situ* hybridizations: intact animals (A); regenerating head and trunk fragments over time (hours, h) (B, C). (A) Brackets: regions imaged in (B, C). (D) *notum* is expressed in anterior-pole, subepidermal cells (arrow). (D, E) Dotted green line is enlarged in right panels. (E) Double-fluorescence *in situ* hybridization (FISH); *notum* (red) and *wnt1* (blue) are co-expressed (arrows) at an anterior-facing wound (dotted line) 18h after amputation. (F-I) FISH; *notum* expression 6h after incisions. Above, green box depicts region imaged. Red lines depict incisions. Below, red line shows sealed wound location. In (G, H) triangular tissue was removed and the wound allowed to seal; tissue between incision sites (~200 microns apart) in (I) was not removed. Anterior, left (B-I) or top (A). Dorsal view (A, 72h in C, D), or ventral view (all others). Images represent ≥4 of 5 animals per panel. Bars, 200 microns.

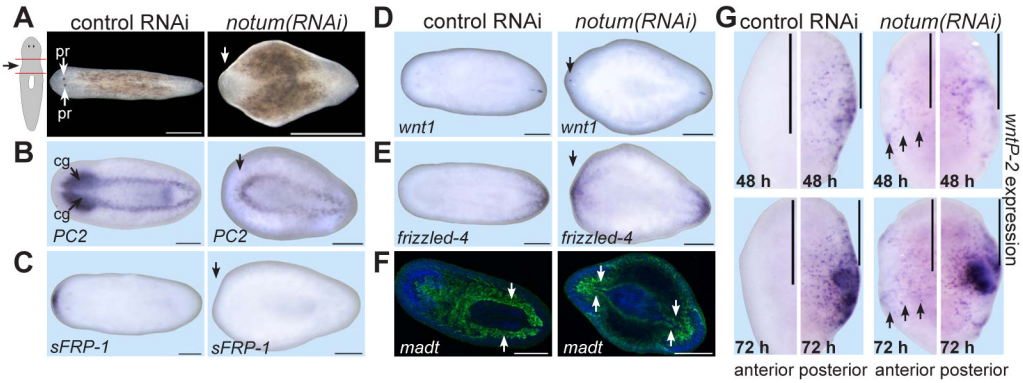
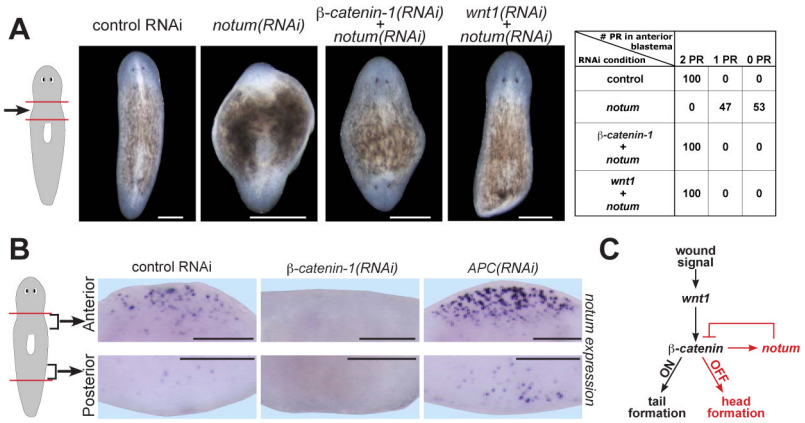


Figure 2. *notum* is required for head-tail regeneration polarity

(A) *notum(RNAi)* fragments failed to regenerate a head by 14 days following amputation (47%, n=133; controls were normal, 100%, n=101). (B-F) Control or *notum(RNAi)* regenerating animals lacking photoreceptors were probed for expression of (B) *PC2* (prohormone convertase 2, CNS marker), (C) *sFRP-1* (anterior-pole marker), (D) *wnt1* and (E) *fzd-4* (posterior markers), and (F) *madt* (gut marker, green). Blue, Hoechst. Arrows, lack of anterior marker (B-C), posterior marker presence (D-E), or posterior gut morphology (F), in *notum(RNAi)* animals. cg, cephalic ganglia; pr, photoreceptors. Images are representatives: (B) 9/11, (C) 8/25, (D) 7/24, and (E) 11/38 *notum(RNAi)* animals; other panels, 100%, n ≥ 7. (G) Anterior- or posterior-facing wounds, probed for *wntP-2* expression. *wntP-2* has been proposed to be Wnt11-related with name *wnt11-5* (8). Arrows, ectopic *wntP-2* expression. Images represent ≥5/6 animals per panel. Anterior, left. Bars, 500 microns (A) and 200 microns.



RNAi condition \ # PR in anterior blastema	2 PR	1 PR	0 PR
control	100	0	0
notum	0	47	53
β-catenin-1 + *notum*	100	0	0
wnt1 + *notum*	100	0	0

Figure 3. *notum* is a Wnt signaling-dependent Wnt inhibitor that controls regeneration polarity

(A) Double RNAi between *notum* and Wnt signaling components. Chart shows phenotypes (PR, photoreceptors) as animal percentages. *wnt1* RNAi can cause tail regeneration failure and/or head regeneration at posterior-facing wounds (6–8). Competition between *wnt1* and *notum* dsRNA likely accounts for tail regeneration failure rather than ectopic head regeneration in *wnt1(RNAi);notum(RNAi)* animals. (B) *β-catenin-1(RNAi)* reduced, and *APC(RNAi)* enhanced, *notum* expression 18h after amputation. *notum*-expressing cell numbers at anterior-facing wounds: controls, 102+/−17 cells; *β-catenin-1(RNAi)*, 17+/−23 cells ($p=6.5x10^{-8}$); *APC(RNAi)*, 186+/−37 cells ($p=8.1x10^{-6}$). Number of *notum*-expressing cells at posterior-facing wounds: controls, 9+/−5 cells; *β-catenin-1(RNAi)*, 1+/−3 cells (p=0.003); *APC(RNAi)*, 30+/−24 cells (p=0.014). Errors, standard deviations; p-values, 2-tailed T-tests. Anterior, top (A), or left (B). Bars, 200 microns. (C) Proposed pathway: selective feedback inhibition of wound-induced Wnt signaling by *notum* at anterior-facing wounds controls switch-like behavior of regeneration polarity.