Supporting Online Material (SOM)

Methods and Materials

Gene sequences

Smed-notum was identified by BLAST searches on an assembly of the *S. mediterranea* genome (http://genome.wustl.edu). 5′ and 3′ RACE was performed to identify the full-length sequence of *Smed-notum* using the FirstChoice RLM-Race kit (Ambion). Complete sequences for *Smed-notum* were deposited with NCBI. Unless otherwise noted, dsRNA and riboprobes were made from a 1969-bp fragment of *notum* (primers 5′-AAAATTTCTGAGGATCGAAAAA-3′, 5′-TGAAGCTAGATTTATGTGAAAAACCA-3′; nucleotides 43-2012). In Figure 3A and S5, *notum* dsRNA was prepared from a 1438-bp fragment (primers 5′-TCGAGTGATTTGTGGTCTGG-3′, 5′-TGAAGCTAGATTTATGTGAAAAACCA-3′, nucleotides 575-2012). Unless otherwise noted, control dsRNA was synthesized from a 1527-bp fragment of *Photinus pyralis luciferase* from the pGL3-control vector (Promega) (primers 5′-TATCCGCTGGAAGATGGAAC-3′, 5′-CGGTACTTCGTCCACAAACA-3′). *wntP-1* (*S1*), *wntP-2* (*S1*), *sFRP-1* (*S1*), *frizzled-4* (*S1*), *PC2* (*S1*), and *madt* (*S2*) riboprobes were described previously.

Fixations, in situ hybridizations, and immunostainings

Unless otherwise noted, fixations and *in situ* hybridizations were performed as described previously *(S3)*. Animals were killed in 5% N-acetyl-cysteine in 1xPBS for 5 minutes at room temperature followed by fixation in formaldehyde. Animals were bleached in 6% hydrogen peroxide overnight and stored in methanol. Digoxigenin- or fluorescein-labeled riboprobes were synthesized as previously described (*S4*). Nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) colorimetric or fluorescence *in situ* hybridizations were performed as previously described (*S3*). For double labeling, HRP-inactivation was performed between labelings in 4% formaldehyde. In Figure 1A-C and Figure S2, animals were killed in 9% N-aceteyl-cysteine in 1xPBS for 3 minutes at room temperature, followed by fixation in Carnoy's

solution, as described previously (*S1*). Subsequent *in situ* hybridizations were performed as described previously (*S1*).

RNAi

For RNAi by injection, dsRNA was prepared from *in vitro* transcription reactions (Promega) using PCR-generated templates with flanking T7 promoters, purified by phenol extraction and ethanol precipitation, and annealed after resuspension in water. Unless noted otherwise, intact animals were injected with 4 x 30 nL dsRNA on three consecutive days, amputated transversely to create a prepharyngeal fragment, and this fragment was injected with 2x13 nL dsRNA two hours after surgery. In both Figures S3 and S4, freshly amputated prepharyngeal transverse fragments were injected with 3x13 nL dsRNA 1 hour and 3 hours after surgery. In Figure 3B, animals were fed a mixture of liver and bacteria expressing dsRNA every 3-4 days for 21 days prior to amputation of heads and tails.

Realtime PCR

Notum mRNA was detected by realtime PCR using SYBR Green and quantified using the standard curve method. For Figure S5 and S6a, total RNA was isolated from three biological replicates of three regenerating fragments each. For Figure S7C, total RNA was separately isolated from 8 individual prepharyngeal fragments. Total RNA was isolated by mechanical homogenization in Trizol (invitrogen). RNA samples were DNAse-treated using DNA-free (Ambion), and cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen). *Notum* mRNA was detected with primers 5′-TATTTGGTTTTTATTCCAGGATCA-3′ and 5′-ATCCATTGATCTTCAATAGGCTCA-3′, and *gapdh* mRNA was detected with primers described previously (*S5*).

Figure S1

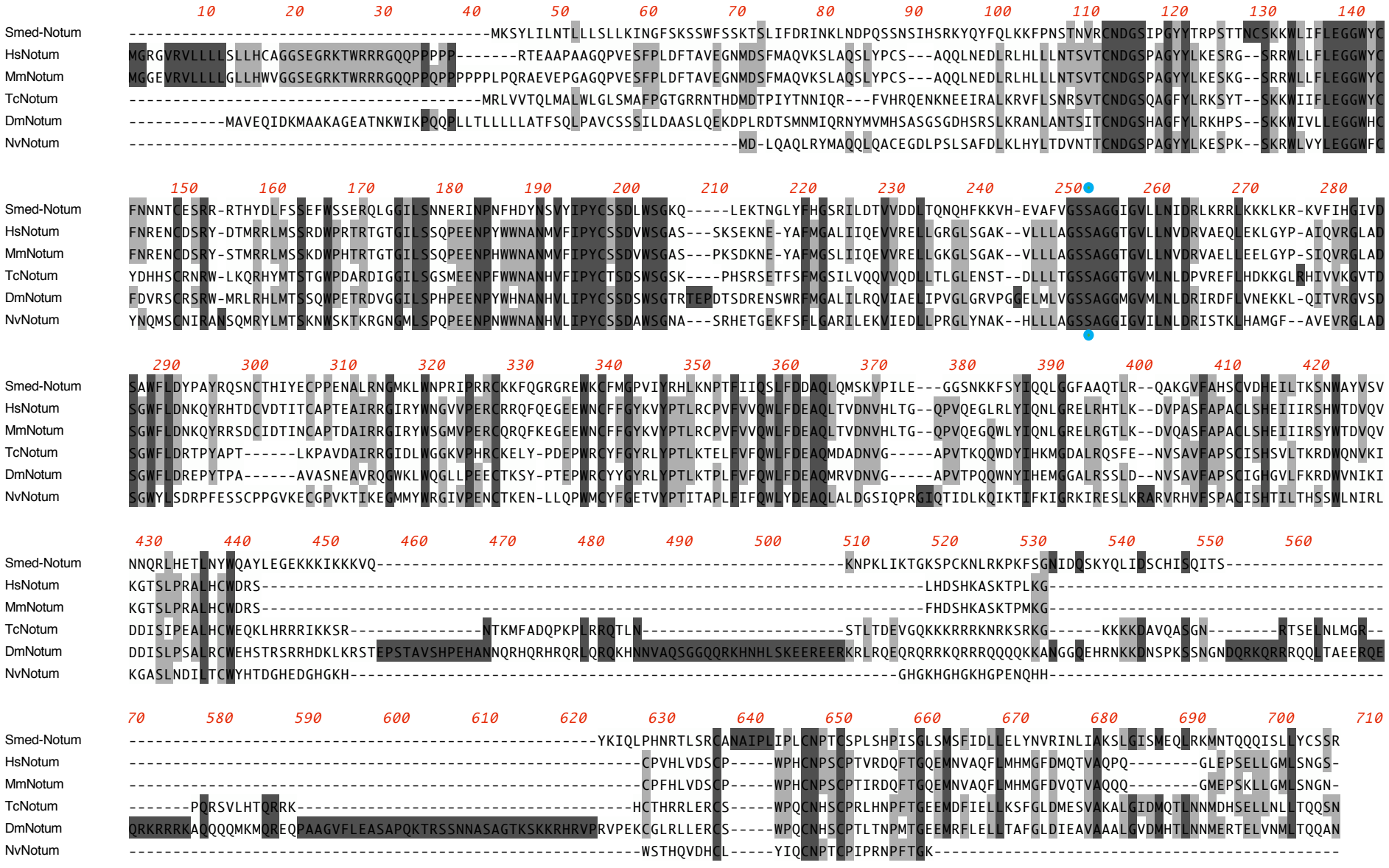


Figure S2

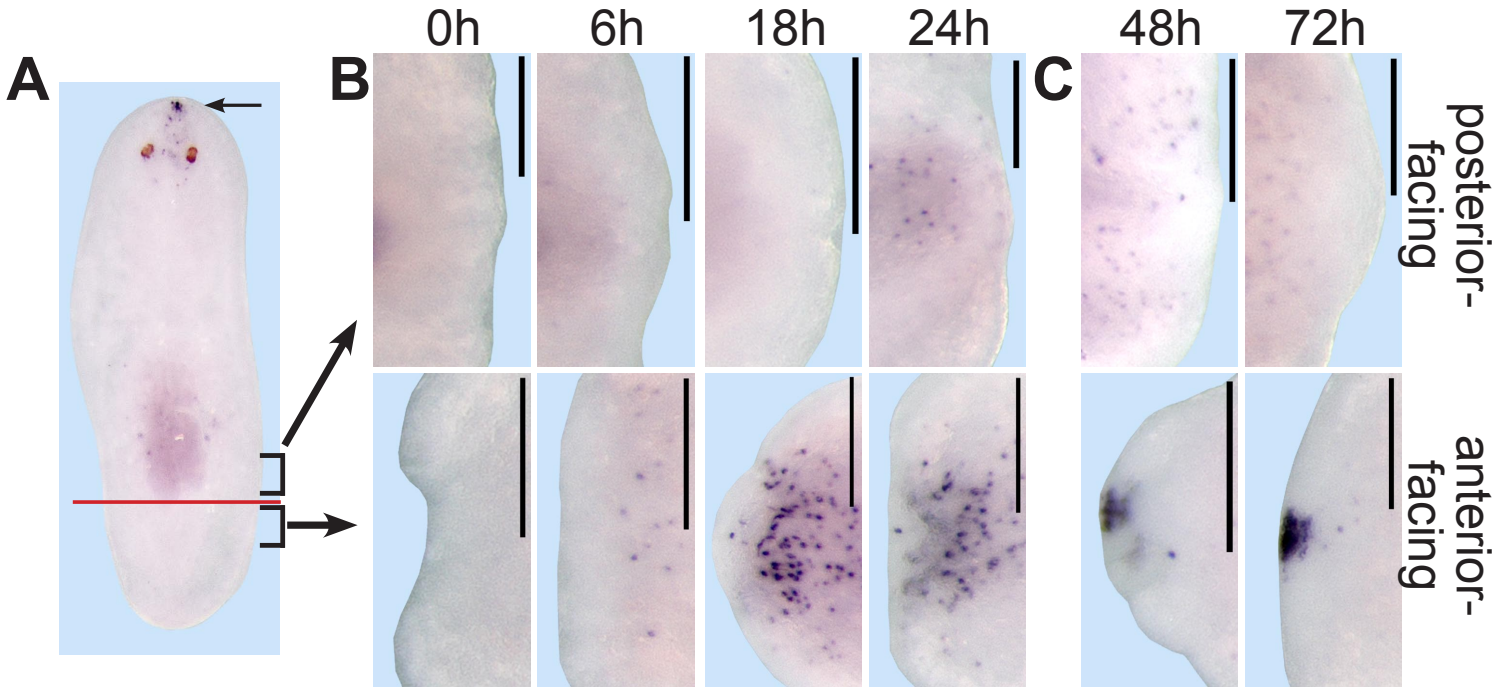


Figure S3

Figure S4

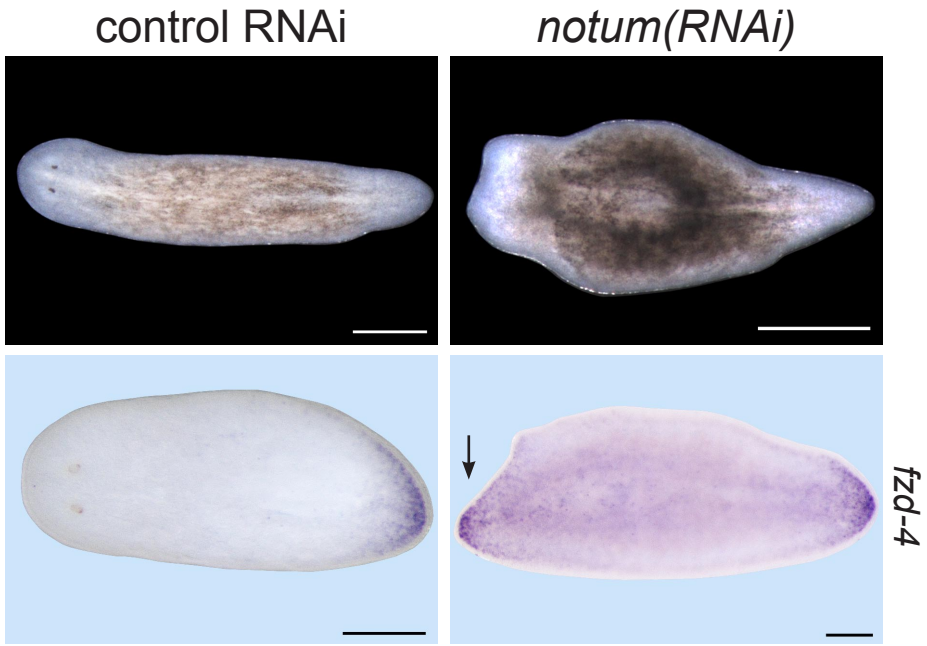


Figure S5

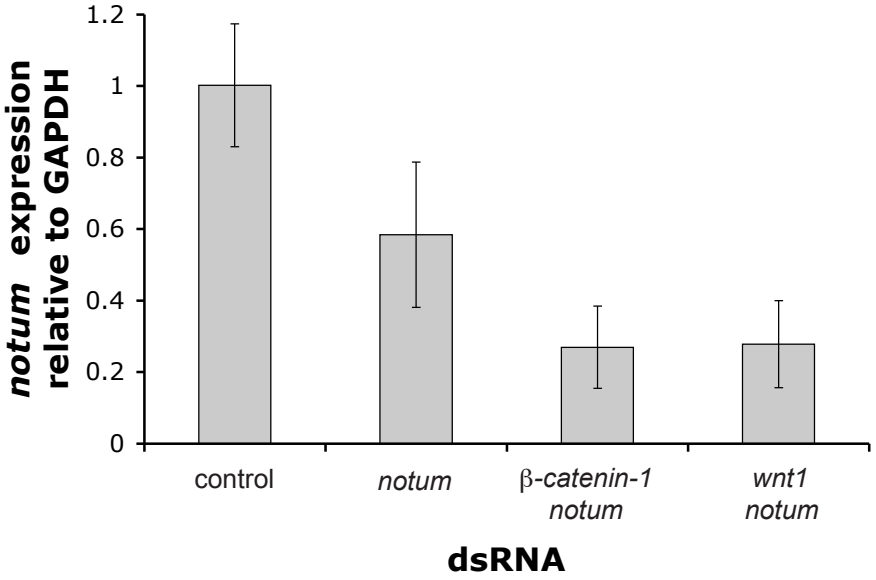
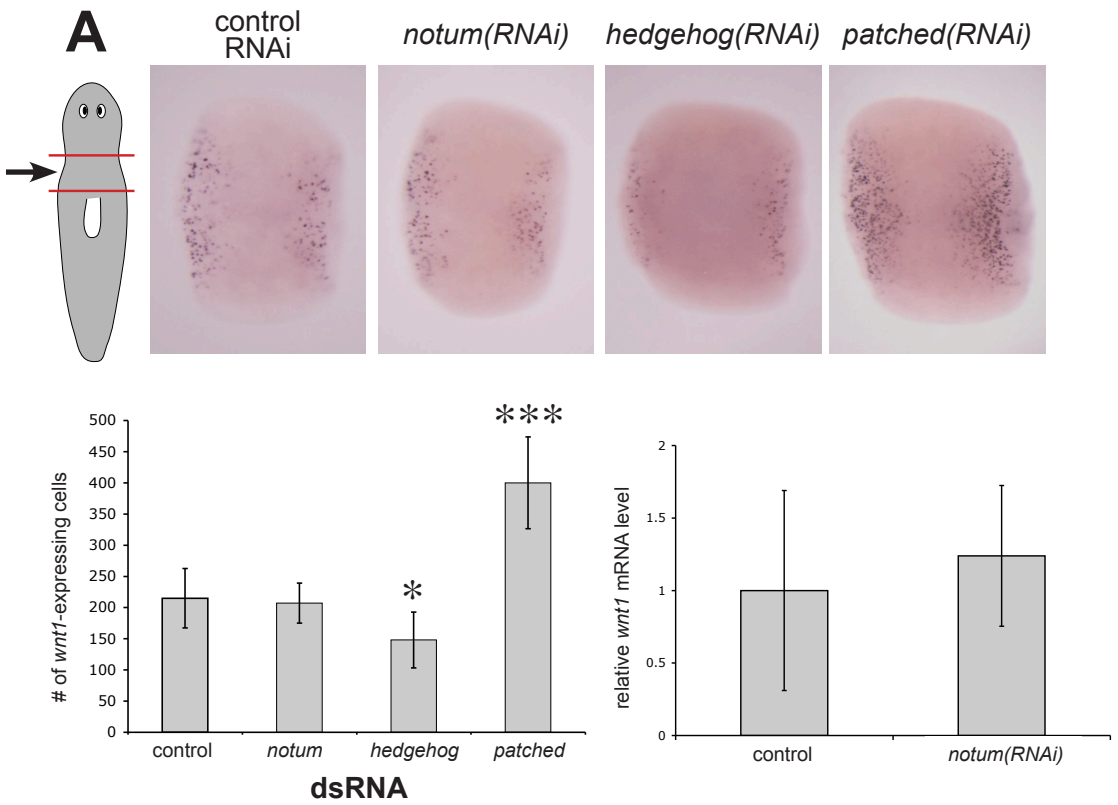

1.2
1
0.8
0.6
0.4
0.2
0
notum expression relative to GAPDH
control
notum
β-catenin-1 notum
wnt1 notum
dsRNA

Figure S6


A
control RNAi
notum(RNAi)
hedgehog(RNAi)
patched(RNAi)
# of wnt1-expressing cells
500
450
400
350
300
250
200
150
100
50
0
control
notum
hedgehog
patched
dsRNA
***
*
relative wnt1 mRNA level
2
1.5
1
0.5
0
control
notum(RNAi)

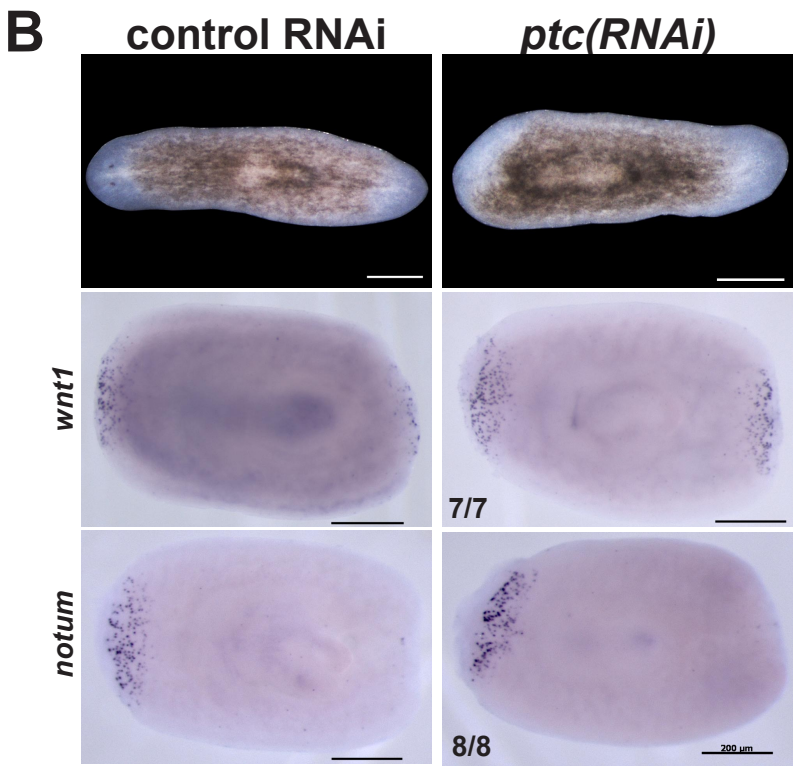

B
control RNAi
ptc(RNAi)
wnt1
notum
7/7
8/8
200 µm

Figure S7

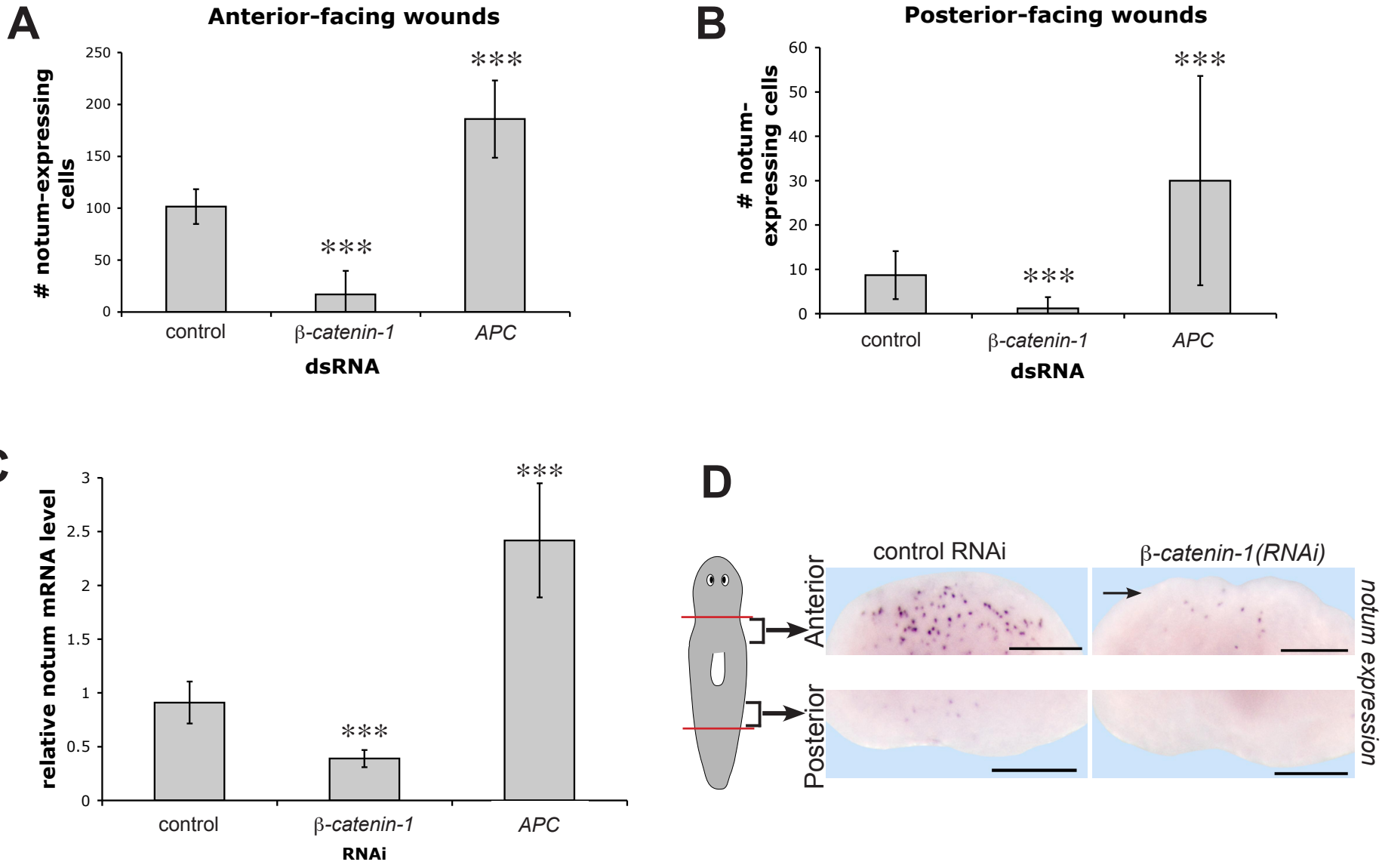

A
Anterior-facing wounds
notum-expressing cells
250
200
150
100
50
0
***
***
control
β-catenin-1
APC
dsRNA
B
Posterior-facing wounds
notum-expressing cells
60
50
40
30
20
10
0
***
***
control
β-catenin-1
APC
dsRNA
C
relative notum mRNA level
3
2.5
2
1.5
1
0.5
0
***
***
control
β-catenin-1
APC
RNAi
D
control RNAi
β-catenin-1(RNAi)
Anterior
Posterior
notum expression

Figure Legends

Figure S1. Alignment of Notum proteins

Alignment of SMED-NOTUM with Notum proteins from other species (Hs, *homo sapiens*; Mm, *Mus musculus*; Tc, *Tribolium castaneum*; Dm, *Drosophila melanogaster*; Nv, *Nematostella vectensis*). Blue dot marks a serine proposed to function in catalysis (*S6*).

Figure S2. *notum* is expressed at anterior-facing wounds. (A-C) *notum in situ* hybridizations in intact animals (A) and in regenerating trunk and tail fragments (B, C) at time points (h) after amputation. Brackets, magnified regions at anterior- or posterior-facing wounds as indicated. Anterior, left (B-C) or top (A). Ventral view (all panels except 72 h), or dorsal view (72 h). Scale bars 200 microns.

Figure S3. Range of defects during anterior regeneration in *notum(RNAi)* animals. Transversely amputated prepharyngeal fragments were injected with 13x3 nl *notum* dsRNA 1 hour and 3 hours after surgery and allowed to regenerate. Animals regenerated with a range of anterior defects: (A) regeneration of two elongated photoreceptors (red arrow) and indented anterior blastema (green arrowhead), (B) regeneration of a single photoreceptor which was located inappropriately (shown, red arrow), (C) regeneration of an anterior blastema that lacked photoreceptors. Diluting the *notum* dsRNA with control dsRNA decreased the penetrance of the PR(absent) phenotype (as shown in C) and increased the penetrance of one or two photoreceptors (as shown in A and B) observed in *notum(RNAi)* animals (*notum(RNAi)*: 82% PR(absent), 18% PR(cyc or abnormal), 0% normal, n=11; compare to *notum(RNAi); control(RNAi)*: 13% PR(absent), 67% PR(cyc or

abnormal), 20% normal, n=15). Therefore, we interpret the PR(absent) phenotype to be the strongest *notum(RNAi)* phenotype. Phenotype abbreviations: cyc, animals with one photoreceptor; elng, photoreceptors elongated; ndnt, indented blastema. Anterior, left. Bars, 500 microns.

Figure S4. Regeneration polarity requires wound-induced expression of *notum*. Freshly amputated pre-pharygeal transverse fragments were injected with control or *notum* dsRNA twice and allowed to regenerate for 15 days. (Upper) Control fragments regenerated normally (100%, n=6) whereas *notum(RNAi)* fragments failed to regenerate two photoreceptors (23%, n=22). (Lower) *In situ* hybridizations of control or *notum(RNAi)* animals probed for *fzd-4* expression. Images are representatives: control RNAi, 5 of 5 animals; *notum* RNAi, 8 of 12 animals. Scale bars, 200 microns.

Figure S5. Quantitation of *notum* mRNA in double RNAi tests with *β-catenin-1* and *wnt1*

Total RNA was prepared from 18-hour regenerating fragments prepared as in Figure 3A and subjected to realtime PCR analysis to quantitate *notum* mRNA versus a *gapdh* control. Values show means of three biological replicates; error bars are standard deviations.

Figure S6. *patched* RNAi results in tail regeneration without affecting *notum* expression.

(A) Animals were administered control or *notum* dsRNA for three days prior to amputation of a prepharyngeal transverse fragment, and either fixed at 18-hours or allowed to regenerate for 8 days. Top, *in situ* hybridizations for *wnt1* expression in control, *notum(RNAi)*, *hedgehog(RNAi)*, and *patched(RNAi)* animals. Bottom left, number of *wnt1*-positive cells (each mean represents an average of the number of *wnt1*-positive cells in at least 7 fragments; error bars, standard deviations). No difference was detected in the average number of *wnt1*-positive cells between control and *notum(RNAi)* animals (2-tailed T-test, $p > 0.7$). *notum(RNAi)* animals from the same cohort failed to form photoreceptors by 8 days (100%, n = 7 animals), indicating effective reduction of *notum* function in this experiment. As previously reported, *hedgehog(RNAi)* regenerating fragments have a reduced number of *wnt1*-positive cells (2-tailed T-test, $p < 0.02$), and *patched(RNAi)* animals have an increased number of *wnt1*-positive cells (2-tailed T-test, $p < 0.0002$). Bottom right, total RNA was prepared from 18-hour regenerating fragments and from three biological replicates of *wnt1* mRNA versus a *gapdh* control. Values show means of three biological replicates normalized such that the control dsRNA treated samples have a *wnt1*-to-*gapdh* abundance ratio of 1; error bars show standard deviations. p-values, two-tailed T-test (*, $p<0.05$; ***, $p<0.001$). (B) Animals were administered control or *patched* dsRNA for three days prior to amputation of heads and tails, and either fixed at 18-hours or allowed to regenerate for 14 days and scored for regeneration defects. Regenerated *patched(RNAi)* animals displayed a range of defects (50% PR(absent), 30% PR(cyc), 10% PR(fsd), n=10) by 12 days after amputation.

Additionally, *patched(RNAi)* animals had excess *wnt1* expression at 18 hours of regeneration (100%, n=7), as reported. However, *notum* expression at 18 hours appeared normal, as it was highly expressed at anterior-facing wounds but weakly expressed or absent at posterior-facing wounds (100%, n=8).

Figure S7. Wnt signaling is necessary and sufficient for wound-induced *notum* expression. (A, B) Quantitation of experiment shown in Figure 4A. Animals were fed bacteria expressing control, *wnt1*, *β-catenin-1,* or *APC* dsRNA for 21 days prior to amputation of heads and tails, fixed 18 hours after surgery, and probed for *notum* expression by *in situ* hybridization. Fixed fragments were scored for their number of *notum*-expressing cells on the ventral surface near the anterior- (A) or posterior-facing (B) wounds. Values show means of ≥ 9 animals examined for each condition, and error bars show standard deviations. (C) Quantitation of *notum* mRNA versus *gapdh* mRNA control by realtime PCR. Values shown are means of total RNA extracted from 8 individual trunk fragments prepared as described in (A,B) above. Error bars are standard deviations. p-values, two-tailed T-test (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

Supplemental References

S1. C.P. Petersen, P.W. Reddien, *Proc Natl Acad Sci U S A* **106**, 17061-6 (2009).
S2. D. Wenemoser, P.W. Reddien, *Dev Biol* **344**, 979-91 (2010).
S3. B.J. Pearson et al., *Dev Dyn* **238**, 443-50 (2009).
S4. P.W. Reddien, N.J. Oviedo, J.R. Jennings, J.C. Jenkin, A. Sánchez Alvarado, *Science* **310**, 1327-1330 (2005).
S5. G.T. Eisenhoffer, H. Kang, A. Sánchez Alvarado, *Cell Stem Cell* **3**, 327-39 (2008).
S6. A.J. Giraldez, R.R. Copley, S.M. Cohen, *Dev Cell* **2**, 667-76 (2002).