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'-TGAAGCTAGATTTATGTGAAAAAACCA-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

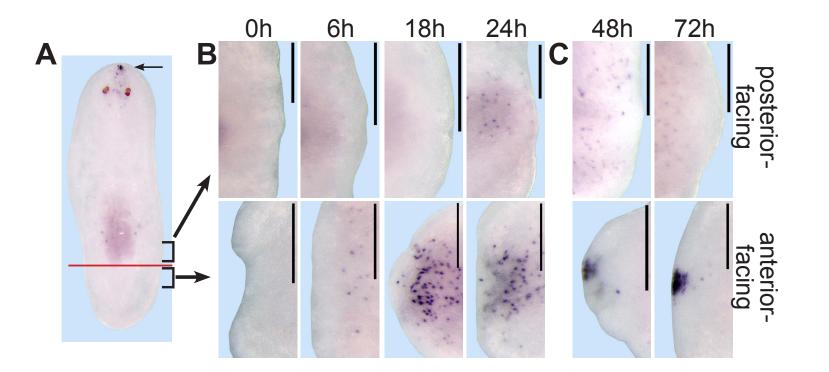
For RNAi by injection, dsRNA was prepared from *in vitro* transcription reactions (Promega) using PCR-generated templates with flanking T7 promoters, purified by phenol extraction and ethanol precipitation, and annealed after resuspension in water. Unless noted otherwise, intact animals were injected 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.

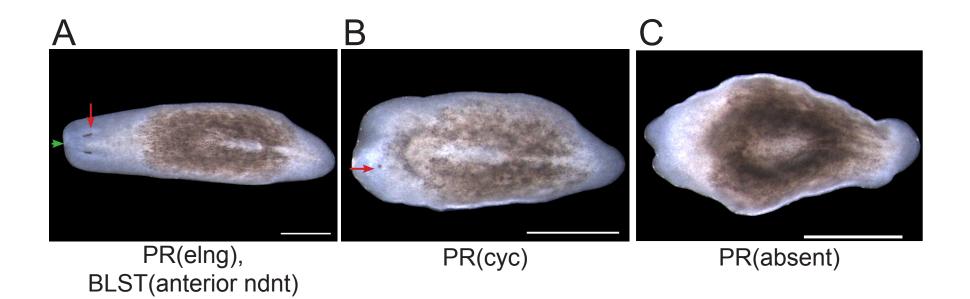
Realtime PCR

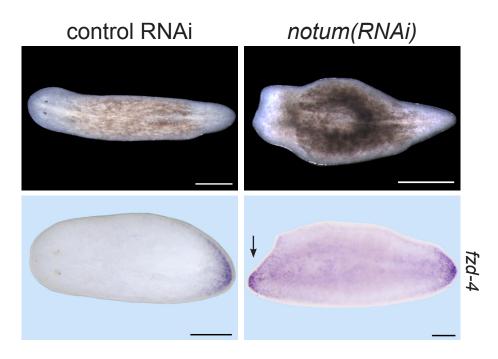
Notum mRNA was detected by realtime PCR using SYBR Green and quantified using the standard curve method. For Figure S5 and S6a, total RNA was isolated from three biological replicates of three regenerating fragments each. For Figure S7C, total RNA was separately isolated from 8 individual prepharyngeal 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'-

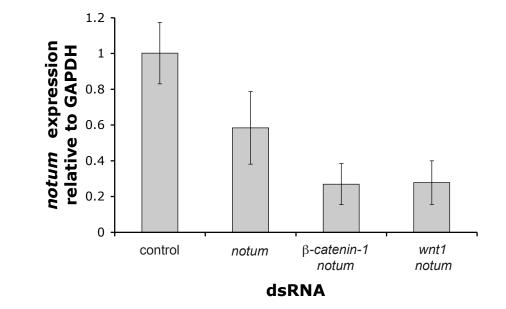
ATCCATTGATCTTCAATAGGCTCA-3', and *gapdh* mRNA was detected with primers described previously (*S5*).

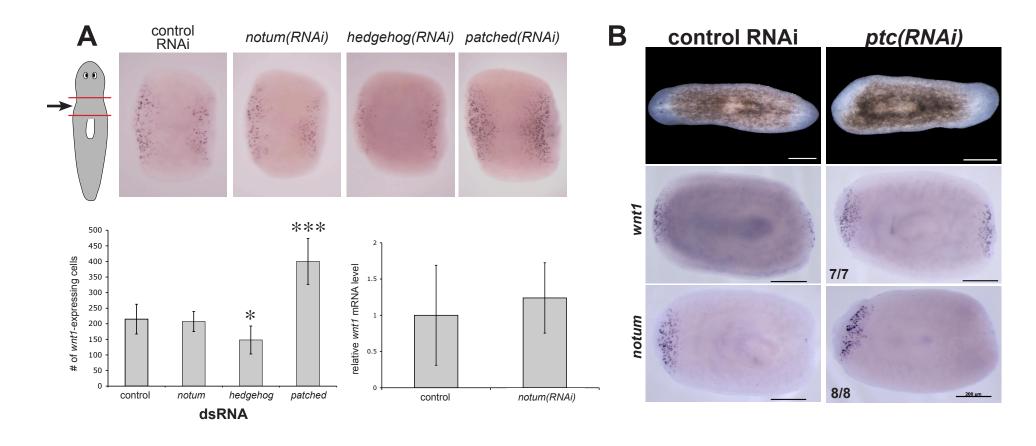
Smed-Notum HsNotum MmNotum TcNotum DmNotum NvNotum	MGGEVRVLLLL	GLLHWVGGSEGRH	(TWRRRGQQPPP (TWRRRGQQPPQP	MKSYLIL PRTEA PPPPPLPQRAEV MRLVVTQL PLLTLLLLLATF	APAAGQPVESFP EPGAGQPVESFP MALWLGLSMAFP SQLPAVCSSSILI	LDFTAVEGNMDSFM LDFTAVEGNMDSFM GTGRRNTHDMDTPI DAASLQEKDPLRDT	FDRINKLNDPQSSN AQVKSLAQSLYPCS AQVKSLAQSLYPCS YTNNIQRFVHR SMNMIQRNYMVMHS	AQQLNEDLRLH AQQLNEDLRLH QENKNEEIRALKR ASGSGDHSRSLKRA	110 KFPNSTNVRCNDGSI HLLLNTSVTCNDGSP HLLLNTSVTCNDGSP VFLSNRSVTCNDGSQ ANLANTSITCNDGSP	AGYYLKESRG AGYYLKESKG AGFYLRKSYT AGFYLRKHPS	SKKWLIFLEGGWYC RRWLLFLEGGWYC RRWLLFLEGGWYC SKKWIIFLEGGWYC SKKWIVLLEGGWHC
Smed-Notum HsNotum MmNotum TcNotum DmNotum NvNotum	FNRENCDSRY-I FNRENCDSRY-S YDHHSCRNRW-I FDVRSCRSRW-N	DTMRRLMSSRDWF STMRRLMSSKDWF .KQRHYMTSTGWF IRLRHLMTSSQWF	SSERQLGGILSNN PRTRTGTGILSSQ PHTRTGTGILSSQ PDARDIGGILSGS PETRDVGGILSPH	PEENPYWWNANM PEENPHWWNANM MEENPFWWNANH PEENPYWHNANH	VFIPYCSSDVWS VFIPYCSSDVWS VFIPYCTSDSWS VLIPYCSSDSWS	GAS – – – SKSEKNE – [–] GAS – – – PKSDKNE – [–] GSK – – – – PHSRSET GTR TEP DTSDRENS	YAFMGALIIQEVVR YAFMGSLIIQEVVR FSFMGSILVQQVVQ NRFMGALILRQVIA	DLTQNQHFKKVH-I ELLGRGLSGAK ELLGKGLSGAK DLLTLGLENSTI ELIPVGLGRVPG	250 2 EVAFVGSSAGGIGVL /LLLAGSSAGGTGVL /LLLAGSSAGGTGVL DLLLTGSSAGGTGVM LLLLAGSSAGGIGVI	LNVDRVAEQLEKI LNVDRVAELLEEI ILNLDPVREFLHDF ILNLDRIRDFLVNE	LGYP-AIQVRGLAD LGYP-SIQVRGLAD KKGLRHIVVKGVTD EKKL-QITVRGVSD
Smed-Notum HsNotum MmNotum TcNotum DmNotum NvNotum	SGWFLDNKQYRI SGWFLDNKQYRI SGWFLDRTPYAI SGWFLDRTPYTI	QSNCTHIYECPPE HTDCVDTITCAPT RSDCIDTINCAPT PTLKPAN PAAVASN	FEAIRRGIRYWNG FDAIRRGIRYWSG /DAIRRGIDLWGG NEAVRQGWKLWQG	VVPERCRRQFQE MVPERCQRQFKE KVPHRCKELY-P LLPEECTKSY-P	GEEWNCFFGYKV GEEWNCFFGYKV DEPWRCYFGYRL TEPWRCYYGYRL	YRHLKNPTFIIQSL YPTLRCPVFVVQWL YPTLRCPVFVVQWL YPTLKTELFVFQWL YPTLKTPLFVFQWL	FDEAQLTVDNVHLT FDEAQLTVDNVHLT FDEAQMDADNVG FDEAQMRVDNVG	GQPVQEGLRLY GQPVQEGQWLY APVTKQQWDY - <u></u> APVTPQQWNY	390 400 QQL GFAAQTL R QNL GRELRHTLK QNL GRELRGTLK HKMGDALRQSFE HEMGGALRSSLD FKI GRKIRESLKRA	QAKGVFAHSCVD DVPASFAPACLS DVQASFAPACLS NVSAVFAPSCIS NVSAVFAPSCIG	EIIIRSHWTDVQV EIIIRSYWTDVQV SVLTKRDWQNVKI GVLFKRDWVNIKI
Smed-Notum HsNotum MmNotum TcNotum DmNotum NvNotum	NNQRLHET NY KGTSLPRALHC KGTSLPRALHC DDISIPEALHC DDISLPSALRC	VDRS VDRS VEQKLHRRRIKKS	KKKVQ SR KRSTEPSTAVSH	NTKMFADQ	PKPLRRQTLN	4QSGGQQRKHNHLS	STLTDEV KEEREERKRLRQEQ	TGKSPCKNLRKPKI LHDSHKASKTPI FHDSHKASKTPI GQKKKRRRKNRKSI RQRRKQRRRQQQQI		.VQASGN	RTSELNLMGR
Smed-Notum HsNotum MmNotum TcNotum DmNotum NvNotum	PQRS1	/LHTQRRK	600 /fleasapqktrs		CP CP HCTI RHRVPRVPEKCG	NRTLSRCA <mark>NAIPL</mark> I VHLVDSCPW FHLVDSCPW HRRLERCSW LRLLERCSW	PHCNPSCPTVRDQF PHCNPSCPTIRDQF PQCNHSCPRLHNPF	TGQEMNVAQFLMH TGQEMNVAQFLMH TGEEMDFIELLKSI TGEEMRFLELLTAI	9 680 YNVRINLIAKSLGIS MGFDMQTVAQPQ MGFDVQTVAQQQ GLDMESVAKALGID GLDIEAVAAALGVD	GLEPSELI GMEPSKLI MQTLNNMDHSELI	_GML SNG S - _GML SNGN - _NLL TQQ SN

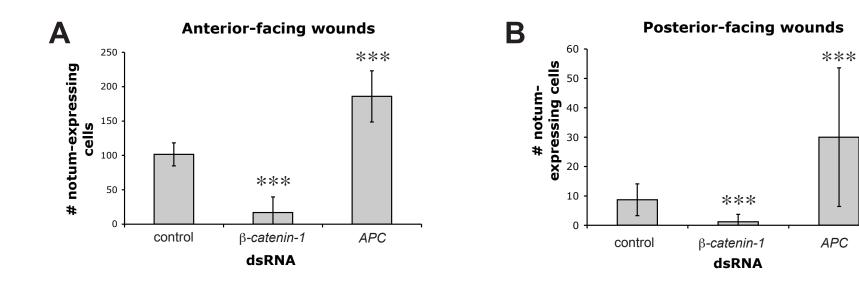












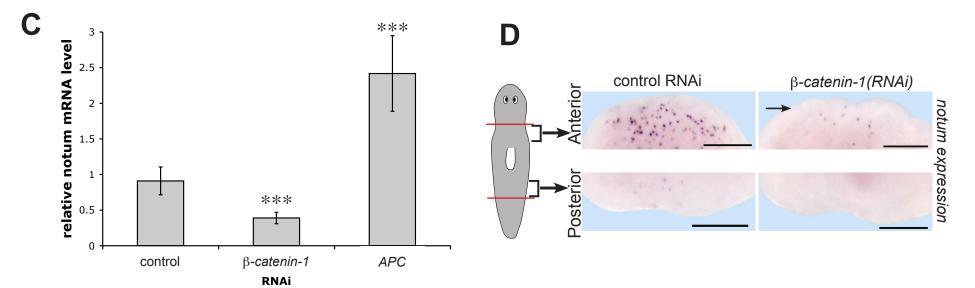


Figure Legends Figure S1. Alignment of Notum proteins

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 posteriorfacing 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 *wnt*1

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 \geq 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).