Cell Stem Cell, Volume *3*

Supplemental Data

Molecular Analysis of Stem Cells and their Descendants during Cell Turnover and Regeneration in the Planarian *Schmidtea mediterranea*

George T. Eisenhoffer, Hara Kang, and Alejandro Sánchez Alvarado

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

RNA Extraction and Amplification for Microarray Experiments

Total RNA was extracted using Trizol reagent (Invitrogen) from 4 biological replicates of whole wild type asexual planarians and from 4 biological replicates of planarians 24 hours and 7 days after receiving a 100Gy dosage of gamma-radiation (10 worms per biological replicate in all experiments). The total RNA was DNAse I treated (Qiagen) at room temperature for 15min, and cleaned up to remove any remaining organics or contaminants using Qiagen RNAeasy spin columns. RNA quality was assessed using an Agilent Bioanalyzer and the total RNA amplified using the Ambion MessageAmp RNA amplification kit. The quality of the resulting amplified RNA (aRNA) was also examined using the A gilent Bioanalyzer. The aRNA was quantified using a Nanodrop spectrophotometer.

Hybridization and Analyses of Microarrays

The amplified and labeled samples, irradiated (Cy3) and WT (Cy5), were hybridized to a microarray containing 3,435 *Schmidtea mediterranea* ESTs from two asexual cDNA libraries: a neoblast-enriched cell preparation (NB) and animal heads (H) (Sánchez Alvarado et al., 2002) printed in triplicate. The Cy3(irradiated)/Cy5(WT) ratio was calculated using Microsoft Excel, the data was (mean scale) normalized, and then log 2 transformed using Spotfire Decision Site Software for Functional Genomics (TIBCO, v7.0). The transformed data was then analyzed using Significance Analysis of Microarrays (SAM) (Tusher et al., 2001) with a delta 0.85, FDR 0 for WT versus +D1, and delta 1.76, FDR 0 for WT versus +D7 datasets. Spotfire was used for hierarchical clustering analyses and the generation of heat maps.

Whole-mount *in situ* Hybridization

For double fluorescent *in situ* hybridizations, animals were hybridized and washed as previously described (Gurley et al., 2008). The animals were then blocked in MABTB (Maleic Acid Buffer, 0.3% Triton X-100, 1%BSA) and incubated with anti-DIG-POD (1:1000 Roche) overnight at 4°C. Animals were rinsed 4 times every 30 minutes in MABTB, followed by incubation with PBST. Samples were developed with Cy3 tyramide for 20-40 minutes. Tyramide solutions were used according to manufacturer's recommendation (Perkin Elmer 1:50) or synthesized according to (Zhou and Vize, 2004) and used at 1:500-1:1500. Animals were rinsed 5 times over 2 hours, followed by incubation in TNT

(0.1M Tris-HCl pH=7.5, 0.15M NaCl, 0.5% Tween-20). Peroxidase activity of the secondary antibody was inactivated by incubation in 1%H_20_2 in TNT for 45min. Animals were blocked in MABTB and incubated with anti-fluorescein-POD (1:500 Roche) overnight at 4°C. The animals were rinsed in MABTB, incubated in PBST, and developed with fluorescein tyramide. The animals were rinsed with PBS, placed in 50-80% glycerol in PBS and photographed using a Zeiss StereoLumar.V12 equipped with an AxiocamHRc. Some of the resulting specimens were soaked in Hoechst (100µg/mL) to label all the nuclei in the animal, then dehydrated in a series to 100% EtOH, mounted in plastic, and serially sectioned (10µm). Specimens were imaged using a Zeiss LSM-5 Live or Olympus FV1000 confocal microscopes.

Immunostaining

Specimens were fixed as described for *in situ* hybridization and processed as described previously (Newmark and Sánchez Alvarado, 2000; Reddien et al., 2005b). For *in situ* hybridization and BrdU staining, the fluorescent *in situ* hybridization was carried out first, and the animals were then processed for BrdU detection. Specimens were imaged as described for fluorescent *in situ* hybridizations.

Flow Cytometry

Analyses and sorts were performed using either a Becton Dickinson FACSVantage SE High Speed Cell Sorter or a BD Biosciences FACS Aria. Cells

were collected and fixed in cold 70% ethanol, resuspended in PBSTx(0.5), treated with RNase A (5µg/mL), and then propidium iodide (PI) was added at a final concentration of 10µg/mL. For the cell cycle experiments, 1000-5000 cells were analyzed in each independent experiment (n=6) using ModFit *LT* (Verity Software House). For the BrdU time course, a single pulse of BrdU was delivered to ≥30 animals by feeding, and animals were dissociated and processed at different times. At least 50,000 cells from each population were collected and processed as described for the whole animal, with the exception that the secondary antibody anti-rat-HRP (Upstate 1:100) was only incubated for 30 minutes at room temperature. Cells were then rinsed 3 times and incubated with 488 tyramide (Molecular Probes, 1:100) for 30 minutes, rinsed in PBST, treated with RNase A, and then propidium iodide (PI) was added. At least 500 cells were analyzed in each population based on 488 and PI fluorescence in 3-4 independent experiments. *In situ* hybridizations on FACS sorted cells were performed as described (Reddien et al., 2005b).

Quantitative Real Time RT-PCR

Reverse transcription reactions were carried out with 30-150ng of total RNA isolated from FACS sorted cells using the reverse transcription portion of the qRT-PCR kit (Superscript III Platinum Two-Step qRT-PCR; Invitrogen, Carlasbad, CA) to produce cDNA. Gene specific primers were designed for the individual candidate genes using OligoPerfect™ designer (Invitrogen). All quantitative real time RT-PCR was performed in triplicate using an ABI7900HT

Sequence Dectector. The Standard Curve Method (Applied Biosystems, 1997, Updated October 2001) was used to determine the level of gene expression, using the ubiquitously expressed GAPDH (H.8.10b) as a reference.

RNAi

Clones for individual ESTs were cloned in the pR244 vector and then expressed in HT115 to make dsRNA as previously described (Reddien et al., 2005a). A double strength dsRNA/bacteria mix (2X food) was fed to the animals (Gurley et al., 2008). Live animals were photographed using a Zeiss StereoLumar.V12 equipped with an AxiocamHRc.

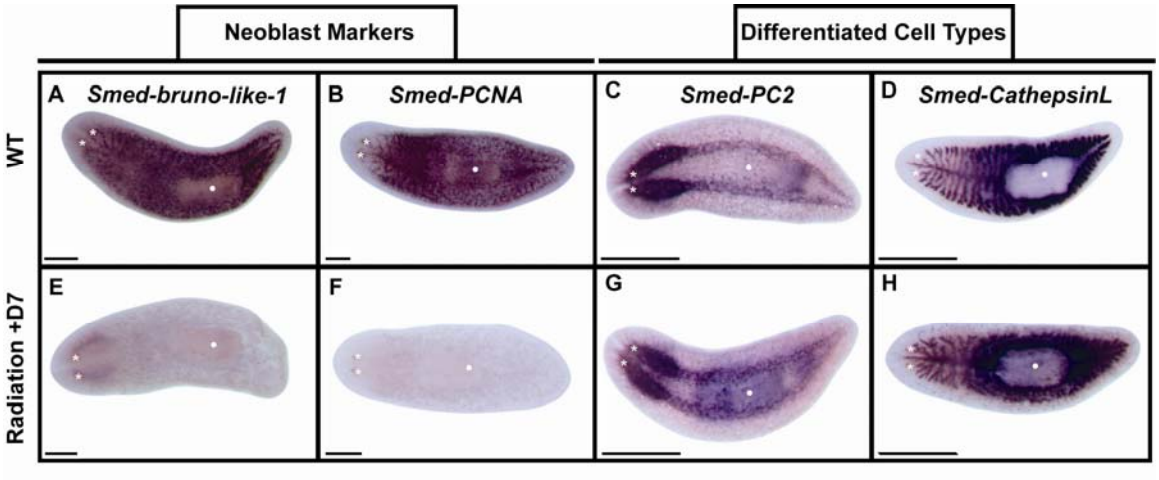

Figure S1. Analysis of neoblasts and differentiated tissues after exposure to irradiation. (A-D) Wild type (WT) and (E-H) irradiated animals hybridized with probes that label the neoblast population and differentiated tissues. Expression of the neoblast markers *Smed-bruno-like-1* (A-E) and *Smed-PCNA* (B-F) are specifically eliminated by irradiation. In contrast, the nervous system marker *Smed-PC2* (C and G) and the gastrovascular system marker *Smed-CathepsinL* (D and H) show no changes in expression 7 days after irradiation. Scale Bars in A-H: 200 μm.

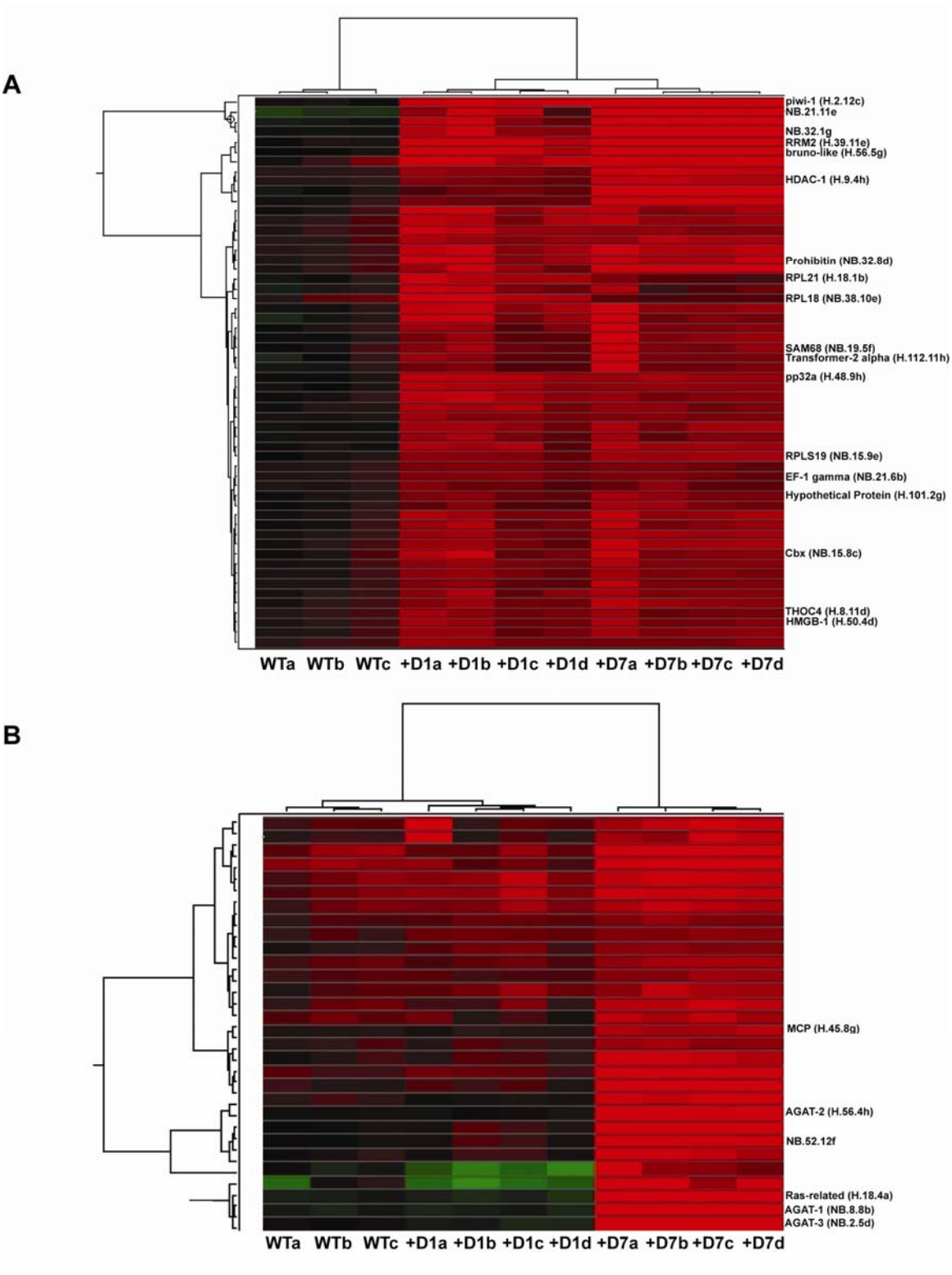
A
piwi-1 (H.2.12c)
NB.21.11e
NB.32.1g
RRM2 (H.39.11e)
bruno-like (H.56.5g)
HDAC-1 (H.9.4h)
Prohibitin (NB.32.8d)
RPL21 (H.18.1b)
RPL18 (NB.38.10e)
SAM68 (NB.19.5f)
Transformer-2 alpha (H.112.11h)
pp32a (H.48.9h)
RPLS19 (NB.15.9e)
EF-1 gamma (NB.21.6b)
Hypothetical Protein (H.101.2g)
Cbx (NB.15.8c)
THOC4 (H.8.11d)
HMGB-1 (H.50.4d)
WTa WTb WTc +D1a +D1b +D1c +D1d +D7a +D7b +D7c +D7d
B
MCP (H.45.8g)
AGAT-2 (H.56.4h)
NB.52.12f
Ras-related (H.18.4a)
AGAT-1 (NB.8.8b)
AGAT-3 (NB.2.5d)
WTa WTb WTc +D1a +D1b +D1c +D1d +D7a +D7b +D7c +D7d

Figure S2. Hierarchical clustering of radiation-sensitive genes. (A) Fifty-six genes with downregulated expression at day 1 (+D1) and day 7 (+D7) after radiation show an expression profile similar to *smedwi-1* and *Smed-bruno-like-1*, known regulators of neoblasts. (B) Thirty genes with severely downregulated expression only at day 7 were identified, many of which are involved in metabolism. The gene name and EST ID are shown for the subset of genes selected for *in situ* validation. The letters a, b, c next to WT, +D1, +D7 refer to individual biological replicates.

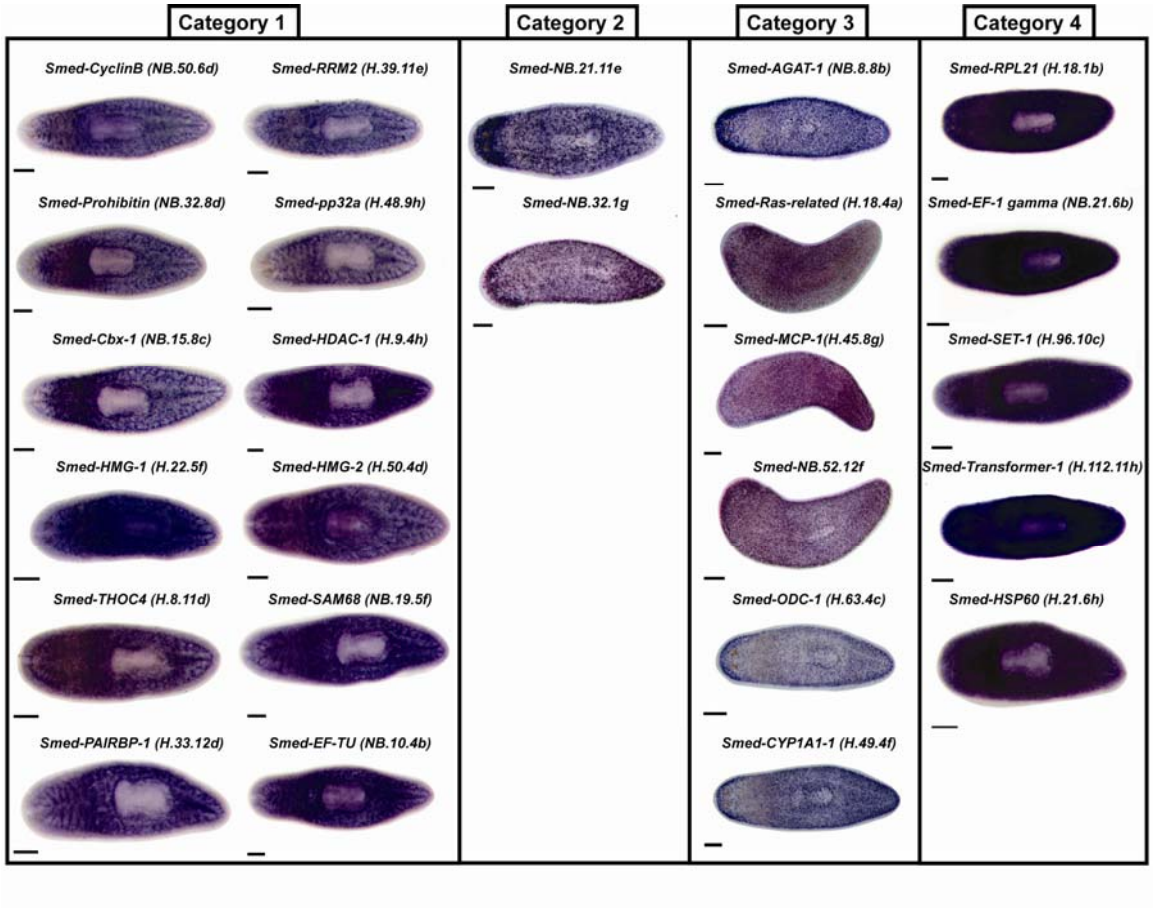

Figure S3. Expression patterns obtained for irradiation-sensitive genes. Genes with downregulated expression levels detected by microarray analyses can be divided into four general categories based on their spatial expression distribution in the whole animal. Category 1 genes yield an expression pattern that is indistinguishable from the known neoblast markers, *smedwi-1*, *Smed-bruno-like-1*, and *Smed-cyclinB*. Genes in Category 2 are expressed in small, discrete cells anterior to the photoreceptors and over the pharynx. Category 3 is defined by genes expressed in cells closer to the animal periphery than those defined by Category 2 (*Smed-AGAT-2* (H.56.4h) and *Smed-AGAT-3* (NB.2.5d) not shown). Genes in Category 4 show wide expression throughout the animal,

except for the pharynx (*Smed-RPL18* (NB.38.10e) and *Smed-Hypothetical Protein* (H.101.2g) not shown). Scale Bars: 200 µm.

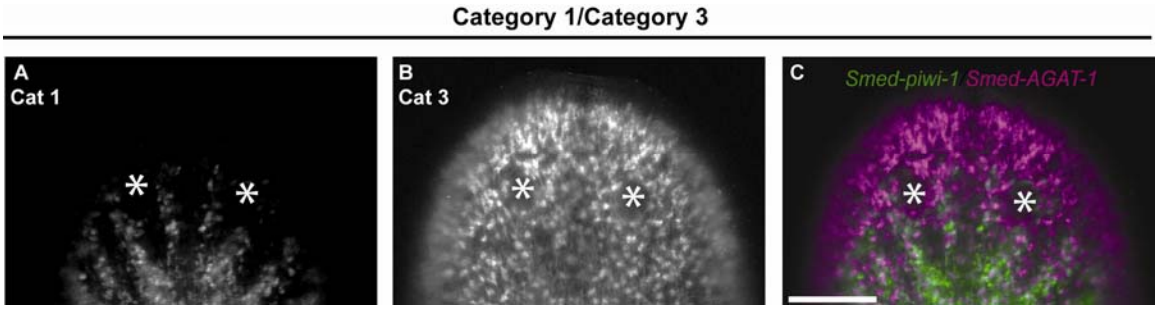

Figure S4. Double fluorescent *in situ* hybridization. (A-C) Single slice confocal images of double fluorescent *in situ* hybridizations for genes from Categories 1 and 3, *smedwi-1* (A) and *Smed-AGAT-1* (B), are not expressed in the same cells (C). Asterisks label the location of the photoreceptors. Scale Bar: 100 µm.

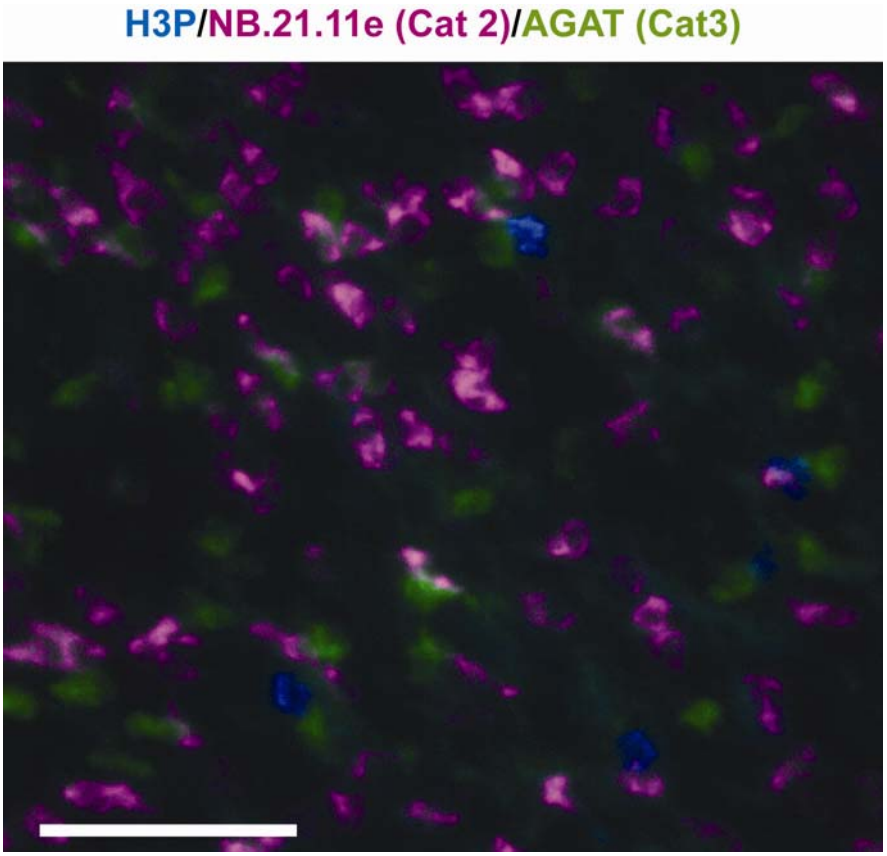

Figure S5. Double fluorescent *in situ* hybridization with H3P staining. Confocal projection of 21 optical sections (1 μm intervals) for a double fluorescent *in situ* hybridization for genes from Categories 2 (magenta) and 3 (green) combined with H3P immunostaining (blue). Scale Bar: 50 μm.

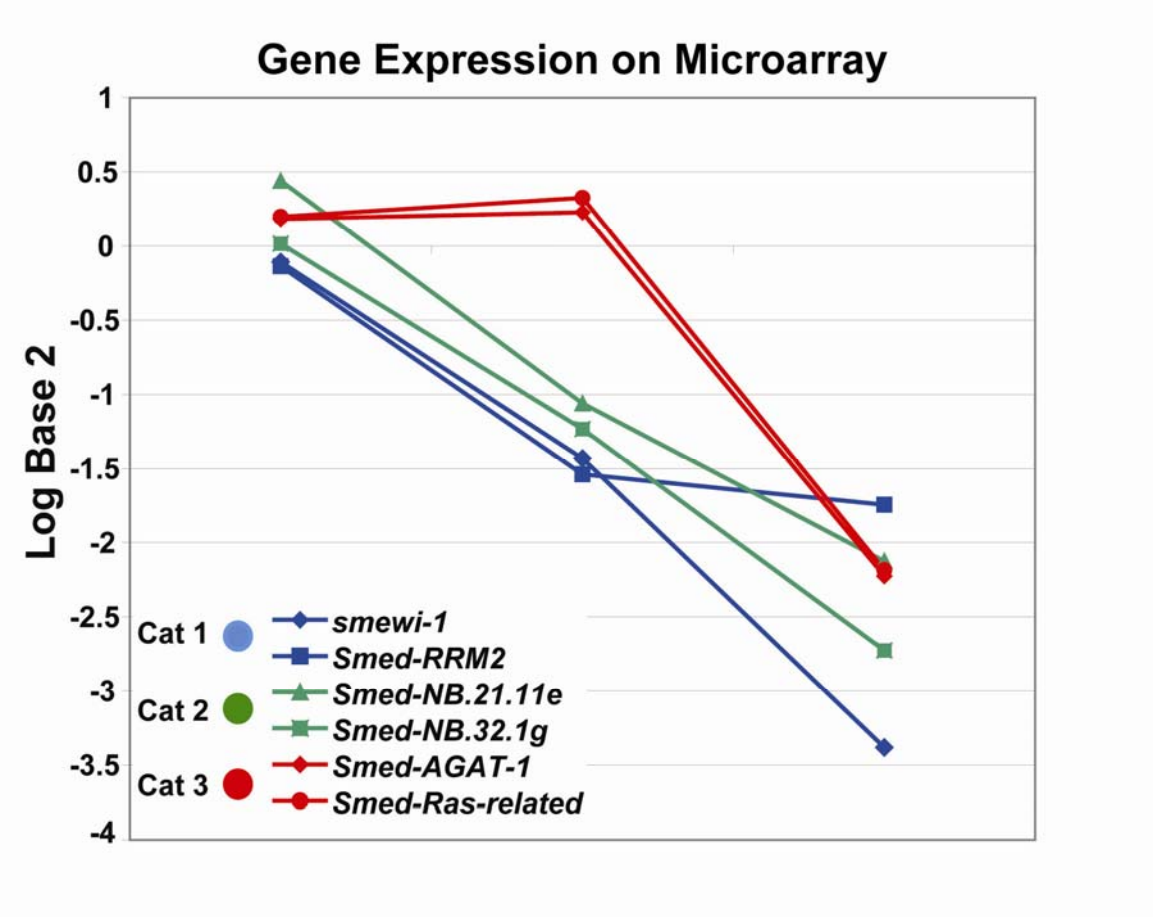

Figure S6. Microarray expression profile data after exposure to irradiation for two genes from each expression category. Category 1 genes are downregulated 24hrs after radiation, but can vary in the severity of downregulation. *Smedwi-1* showed severe downregulation at day 1 (+D1) and decreased further by day 7 (+D7), consistent with expression in dividing and non-cycling ASCs (Figure 3). In contrast, *Smed-RRM2* is expressed in only dividing ASCs and shows downregulated expression at 24hr, but is downregulated at the same level by day 7. Genes in Category 2 disappear with the same kinetics as *smedwi-1*, yet are expressed in post-mitotic cells, suggesting that these cells are the immediate division progeny of neoblasts. *Smed-AGAT-1* and *Smed-Ras-*

related-1 in Category 3 show normal expression at day 1, but are severely downregulated by day 7. WT: wild type.

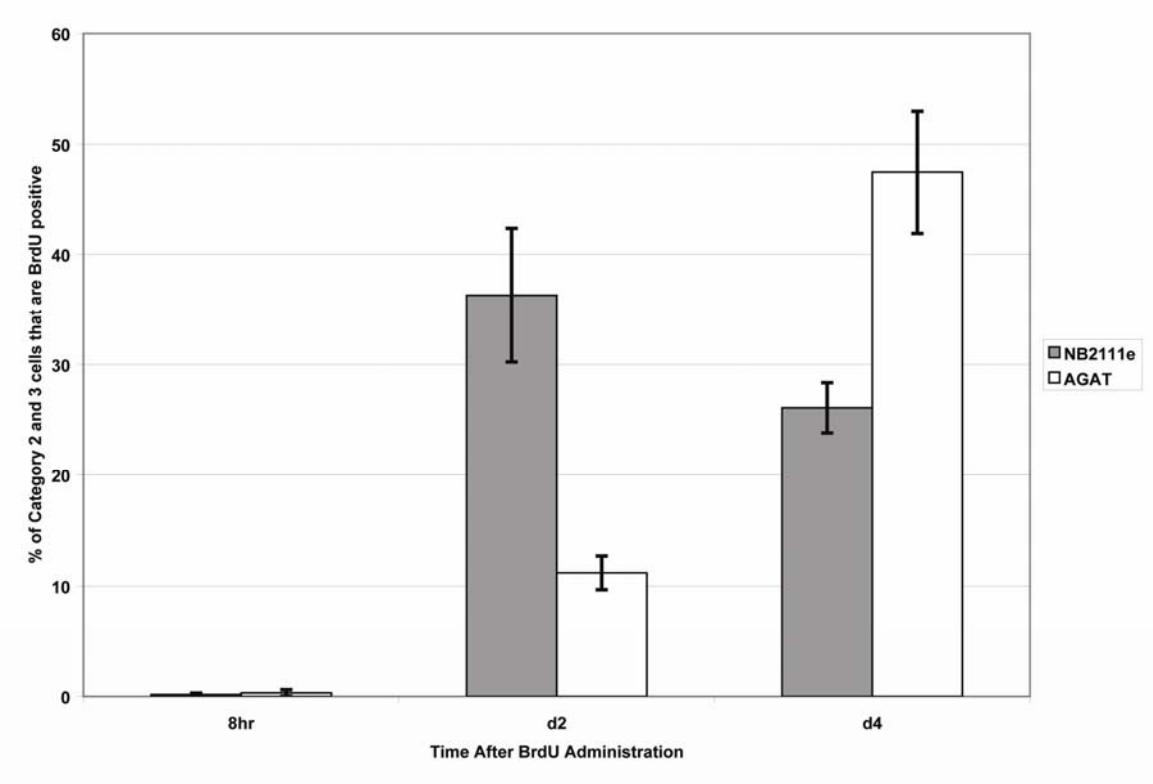

Figure S7. Quantification of BrdU incorporation into different cell types over time during tissue homeostasis. A single pulse of BrdU was delivered by feeding, and *in situ* hybridization of Categories 2 and 3 genes was performed at different times as indicated in Figure 4. The percentage of *in situ* and BrdU positive cells is shown for each time point. Error bars are SEM. 1582-2420 cells were counted from different optical plane confocal images of the anterior region in 4-6 different animals for each time point.

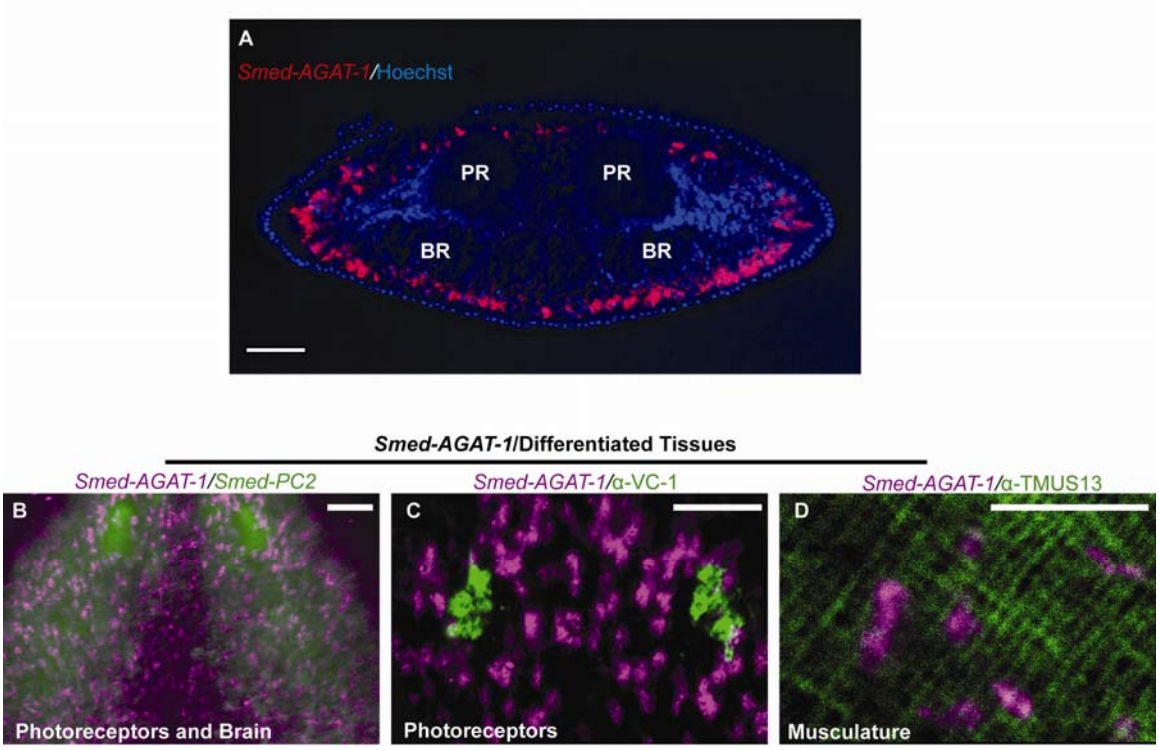

Figure S8. *Smed-AGAT-1* is expressed in committed progeny that are near differentiated tissues. (A) Single slice confocal image of a 10μm transverse section of *Smed-AGAT-1* fluorescent *in situ* animals counterstained with Hoechst. Single slice confocal images of double fluorescent in situ (B) or fluorescent in situ and immunohistochemistry (C, D) illustrating *Smed-AGAT-1* is expressed in committed descendents present just under the basement membrane near many differentiated tissues. PR: photoreceptors, BR: brain. Scale Bars in A-C: 50 μm, D: 25 μm

GEO Series Accession Number: GSE11503

	Cy3 (Experimental)	Description	Cy5 (Reference)
1)	WTSmed-a	pool of 10 WT planarians	WTSmedReference
2)	WTSmed-b	pool of 10 WT planarians	WTSmedReference
3)	WTSmed-c	pool of 10 WT planarians	WTSmedReference
4)	+D1Smed-a	pool of 10 WT planarians 24 hours after exposure to 100Gy	WTSmedReference
5)	+D1Smed-b	pool of 10 WT planarians 24 hours after exposure to 100Gy	WTSmedReference
6)	+D1Smed-c	pool of 10 WT planarians 24 hours after exposure to 100Gy	WTSmedReference
7)	+D1Smed-d	pool of 10 WT planarians 24 hours after exposure to 100Gy	WTSmedReference
8)	+D7Smed-a	pool of 10 WT planarians seven days after exposure to 100Gy	WTSmedReference
9)	+D7Smed-b	pool of 10 WT planarians seven days after exposure to 100Gy	WTSmedReference
10)	+D7Smed-c	pool of 10 WT planarians seven days after exposure to 100Gy	WTSmedReference
11)	+D7Smed-d	pool of 10 WT planarians seven days after exposure to 100Gy	WTSmedReference

Table S1. Microarray sample information and experimental design. WT biological replicates and reference refers to a pool of 10 wild type *Schmidtea mediterranea* that had been starved for one week. Cy3 and Cy5 refer fluorophore used to label the samples for hybridization to the microarray.

Gene	Expression Category	Radiation-Sensitive		Insensitive to Radiation
		X1	X2	Xins
Smed-RRM2	1	31%	0.90%	1.10%
Smed-bruno	1	48%	25%	2%
smedwi-1	1	80%	23.50%	0.30%
Smed-HDAC-1	1	82%	44%	10.55%
Smed-NB.21.11e	2	0.7%	8.5%	1%
Smed-AGAT-1	3	2.4%	12%	2.80%
Smed-AGAT-2	3	2.2%	13.85%	2.40%
Smed-Ras-related	3	4.3%	14.90%	18.50%

Table S2. *In situ* Hybridization of Category 1, 2, and 3 genes in FACS sorted cell populations. FACS defined cell populations were collected, fixed, and *in situ* hybridization was performed using anti-sense riboprobes for genes from the different categories. The percentage of *in situ* positive cells in each population per total number of DAPI positive nuclei is shown.

Gene	Expression Category	RNA mediated interference (RNAi) During Homeostasis and Regeneration	
		Intact	Amputation
Smed-RRM2	1	Hd Regr/Lysis	Little or no new tissue
Smed-HDAC-1	1	Hd Regr/Lysis	Little or no new tissue
Smed-Cbx-1	1	Hd Regr/Lysis	Little or no new tissue
Smed-Prohibitin	1	Hd Regr/Lysis	Little or no new tissue
Smed-THOC4	1	Hd Regr/Lysis	Little or no new tissue
Smed-SLM-1	1	Hd Regr/Lysis	Little or no new tissue
Smed-pp32a	1	OK	OK
Smed-NB.21.11e	2	OK	OK
Smed-NB.32.1g	2	OK	OK
Smed-AGAT-1	3	Lesions	New tissue, but late or no appearance of PR
Smed-Ras-related	3	OK	OK
Smed-NB.52.12f	3	OK	OK

* Previously reported by (Reddien et. al, 2005a)

Table S3. Loss of function Data for Genes from the Different Categories. Summary of RNAi knock-down phenotypes for genes from the different expression categories. Head regression/lysis (Hd Regr/Lysis) defines a neoblast defective phenotype within unamputated animals, and little or new tissue is formed after amputation. *Smed-AGAT-1* produces lesions in intact animals, and while new tissue is formed after injury, the appearance of photoreceptors (PR) is delayed or does not occur.

Smed-RRM2	F- GTTTTGGGTGGAAATGGCTA R- CGGAATCCCTCAAAAGTCAA
smedwi-1	F- GGGCTAATCCAAATCCTGGT R- TGCTGCAATACACTCGGAGA
Smed-HDAC-1	F- GGCTACTGCAGACGACATGA R- TCCCCCAGCTGATAATTGAC
Smed-NB.21.11e	F- GTCTCCCGCCAAATCAAGTA R- TTTCATGCAATCTGCTTTCG
Smed-AGAT-1	F- TCCATCCAGAACCGATTGAT R- CTCCCAAGTCATGGTGGACT
Smed-GAPDH	F- AGCTCCATTGGCGAAAGTTA R- CTTTTGCTGCACCAGTTGAA

Table S4. Primers used in qRT-PCR experiments

Primer sequences (~20 base pairs) for genes from the different expression categories used in quantitative RT-PCR experiments, with expected product sizes from 115-193 base pairs. F=forward, R=reverse.

Smed-PCNA

The *Schmidtea mediterranea* homolog of Proliferating Cell Nuclear Antigen (PCNA) was identified and cloned by Dr. Bret Pearson according to the methods reported in (Gurley et al., 2008), and was kindly provided to us for these studies. *Smed-PCNA* has been given the accession number EU856391.

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