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Supplemental Data

Identification of the Endostyle as a Stem Cell Niche in a Colonial Chordate

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Supplemental Results

Calculation of proliferation ratios and fluorescent intensity levels in labeled cells

We quantified the cellular expansion in each observed organ by comparing the number of labeled cells 2 and 10 hours post labeling. Cells from the EN expanded in the zooid by a ratio of 2.3±0.92 (n=5), cells originated from other sites did not expand (expansion factor of 1±0.36, n=11). During this period, the fluorescent intensity of the cells from the EN decreased by about 50% (from 38471±12771n=24 to 16545±4482 n=24, Fig 3). No significant reduction in fluorescence of cells from other sites was observed (37889±18367 n=24 versus 38078±16778 n=24; Fig 3). To assess the contribution of cells from different sites to the budding process, we measured the numbers of labeled cells that reached the primary buds 30 hours post labeling, normalized (divided) by the initial number of labeled cells in the sites of origin. When labeling cells in the EN, we found 5 times more (5.3±0.48 n=8) labeled cells in the primary buds than those originally labeled. In this period (30 hours post labeling), the average fluorescent intensity of these cells decreased by a similar factor (38471±12771 n=24 versus 7213±3677 n=24, 2 and 30 hours post labeling respectively, Fig 3). There was no migration of cells labeled in other sites to developing, regenerating tissues. Ten and 30 hours after labeling, labeled cells were detected in the zooids (mainly at the digestive system and the macrophage islands) and

in the colony vasculature (Svideo 2C; SFig 1; STable 1). Thirty hours post labeling, these cells retained their initial fluorescent intensity (Fig 3).

Supplemental Experimental Procedures

Animals

Colonies of *Botryllus schlosseri* (Pallas) were collected from Monterey marina (Monterey, CA USA). Hatched larvae were settled and maintained as described previously (Boyd et al., 1986). Mature colonies (>3 months old) were chosen for the experiments. Subclones from different genotypes were tested for fusibility by a cut colony assay (Rinkevich and Weissman, 1987). Compatible genotypes, colonies that can fuse upon vasculature contact and form a single chimeric colony, were identified and were used in the relevant experiments.

40 colonies (including 11 chimeras; STable 1) that didn't have a natural background in the Cy5 spectra were chosen for the labeling experiments. Colonies size varied between 3 to 10 zooids (1-2 systems), blastogenic stage on labeling date varied (mainly A or B, few colonies C).

5 pairs of compatible genotypes were used in the transplantation experiments (58 recipients sub clones; STable 2). Most of the colonies that participated in the transplantation experiments were mature reproductive colonies.

Following transplantation, colonies were cleaned and observed at least once a week. Newly settled oozoids, which can potentially serve as a source of contaminating DNA by fusion, were removed upon appearance. Pregnant colonies when identified were transferred to a different aquarium and were observed daily.

At least 3 different colonies were used for the PCNA immunohistochemistry, and Raldh in situ hybridization. 7 colonies were used for preparing frozen sections.

***In vivo* fluorescent cell labeling assay**

We used Vybrant DiD, a carbocyanine lipophilic membrane fluorescent stain (emission-665nm; Molecular Probes, Eugene OR USA) for *in vivo* cell labeling. We have diluted it in a tunicate saline buffer (TS; Negm et al., 1991) and labeled cells in one of the tested sites: endostyle niche (zooid or primary bud), primary bud lateral wall, zooid lateral wall or digestive system, ampulla (STable 1). From the EN, we collected cells that were located on the anterior ventral region of the endostyle epithelium (mainly lymphocyte like cells from the subendostylar sinus). Taking advantage of the transparency of the *Botryllus* body, incorporation of labeled cells into the colony organs was visually confirmed using a 100X magnification Imagexpress fluorescent microscope system (ImageXpress, Molecular devices Corp., Palo Alto, CA). *Botryllus* colonies (ventral side) have high natural fluorescent backgrounds in the 501nm and 565nm emissions spectra (Fitc and Dsred) and a low background in the 665nm emission spectra (Cy5). Therefore we chose the Vybrant DID dye (Cy5) which diffuses laterally to stain the entire cell and fluorescents weakly until incorporated into a membrane. This dye doesn't leak from cell to cell, shows little or no toxicity and is stable for at least 120 hours (Gant et al., 1992). This dye has been successfully applied to mark embryonic neural crest cells and to follow their migration patterns in mouse and chick embryos (Osumi-Yamashita et al., 1996, Serbedzija et al., 1989, 1990). Different dilutions (1:1 – 1:1000; DiD dye:TS) were tested, stock dilutions above 1:100 yielded good labeling results (n=20 colonies preliminary experiments). stock dilutions of 1:100, 1:500 or 1:1000 were used. In all labeling experiments number of labeled cells in the labeling sites decreased over time (few examples: the number of

labeled cells in the EN decreased between X3-X10 within the first 15 hours following labeling; From 42 cells that were labeled in the ampullae only 2 remained labeled 90 hours later). On the other hand, labeled cells could be detected circulating in a colony even 142 hours following labeling. 30 hours following labeling, cells which origin in the endostyle niche lost 80% of their fluorescence intensity, at this time frame, cells from the ampullae did not lose their fluorescence intensity (Fig 3). 188 hours following labeling, labeled cells were rarely. The intensity of bud labeling was correlated to the initial number of labeled cells in the endostyle niche and to the number of endostyle niches that were labeled in a colony. The constant fluorescence and no proliferation and expansion of labeled cells in other sites and tissues negate the possibility of leaking of the lipophilic dye from labeled cells to other cells. We therefore conclude that no intercellular transfer of stain between cells occurred in our experiments and that proliferation of endostyle labeled cells observed is due to- cells proliferation.

Proliferation / migration and fluorescent intensity measurements

Labeled cells in organs (zooid, bud, vasculature) were counted using Image J software, version 1.32j (NIH, USA). Proliferation and migration ratios were calculated on fluorescent images that were taken from the labeling sites, 2 and 10 hours following labeling (Fig 3). “Proliferation / homing” ratios in buds were calculated from initial number of labeled cells and from buds fluorescent images 30 hours post labeling. Fluorescence intensity in labeled cells was measured by the ImageXpress program from fluorescent images taken 2, 10 and 30 hours following labeling (measurement were made per individual cells; Fig 3).

Genotyping

Somatic (a minimum of 5 zooids system per sample) and germ (4-10 male gonads, if arose) tissues were collected every month, 1-6 months following transplantation. Samples were dissected and snap frozen in liquid nitrogen. 259 DNA samples were extracted using a modified version of the Hoss and Pabbo protocol (1993) as described in (De Tomaso et al., 1998). The samples were screened for polymorphism using amplified fragment length polymorphism (AFLP's). AFLP was preformed as described in (Vos et al., 1995; Rinkevich et al., 1998).

DNA samples from transplanted subclones were compared with DNA samples of donor and recipient and of known cut assays (DNA samples which collected from known chimeras following resorption of one partner; Supplemental video 1). To ensure consistency of AFLP fingerprints, some experiments were repeated twice. For each sample, 2 different primer set were used from the following options (ACA, ACG, ACT, AGT, AGC). We concluded that individuals have a "genetic chimerism or parasitism" when the AFLP revealed the presence of genetic fingerprints of the donor.

Reference

Rinkevich, B., and Weissman, I.L. (1987). A long-term study of fused subclones of a compound ascidian. The resorption phenomenon. J. Zool. *213*, 717–733.

Supplemental Tables

Table S1: ***In vivo*** **labeling and tracing of cells expansion and distribution in** ***Botryllus*** **colonies**

In each experiment, 10-40 cells were labeled in one of the following sites: zooid EN, primary bud EN, primary bud lateral wall, zooid lateral wall, zooid digestive system, or the colony vasculature (n=30). In addition, in several experimental groups the vasculature was removed from some colonies (n=12). Two controls were preformed: injection of the dilution solution

into the relevant sites with or without vasculature removal (n=6) and transplantation of labeled cells taken from the vasculature or zooid lateral wall into the EN (n=4). By using time lapse imaging, we tracked the labeled cells and their progeny, and detected their distribution and contribution to the colony tissues, organs and vasculature regeneration (detection sites). n=number of colonies, EN-endostyle niche, vr-vasculature removal, chim-chimera, lw-lateral wall, ds-digestive system, fc-few cells, mac-macrophage, x/y – number of colonies which their labeled cells were detected in the specific organ /number of tested colonies.

Labeled site / Detected Site	Control n=6 (3vr)	Cells from amp or lw inj into EN +vr n=4	EN zooid n=7 (3vr, 3chim)	EN primary bud n=2	Primary bud lw n=8 (2 chim, 4vr)	Zooid lw or ds n=8 (3 chim 2 vr)	Amp n=5 (3 chim)
Secondary bud	0/6	0/4	**5/7**	**2/2**	0/8	0/8	0/5
Primary bud	0/6	1/4 fc	**7/7**	**2/2**	1/8 fc	1/8 fc	1/5
labeled bud only					8/8		
Regenerated vasculature	0/6	0/4	**3/3**	**-**	0/4	0/2	-
Vasculature	0/6	3/4	7/7	2/2	8/8	7/8	5/5
Zooid endostyle labeled zooid	0/6	4/4	7/7	-	0/8	0/8	0/5
Zooid mac. Islands	0/6	3/4	3/7	0/2	7/8	7/8	4/5
Zooid ds	0/6	2/4	3/7	0/2	2/8	5/8	3/5
Zooid near labeling site	0/6	4/4	7/7	-	8/8	8/8	3/5

Table S2**: Induction of chimerism in recipient colonies, following transplantation of few cells.**

Comparison between engraftments of cells origin from the endostyle niche and cells origin from other sites is present in the table. The number of different transplantations and the percentage of those resulting in detectable chimeras are indicated for somatic and germline outcomes. The results which are summarized in this table are based on AFLP analysis of 259 DNA samples. The winner-loser relationship of the genotypes following fusion is presented as winner>loser (between compatible partners, only one genotype can win and

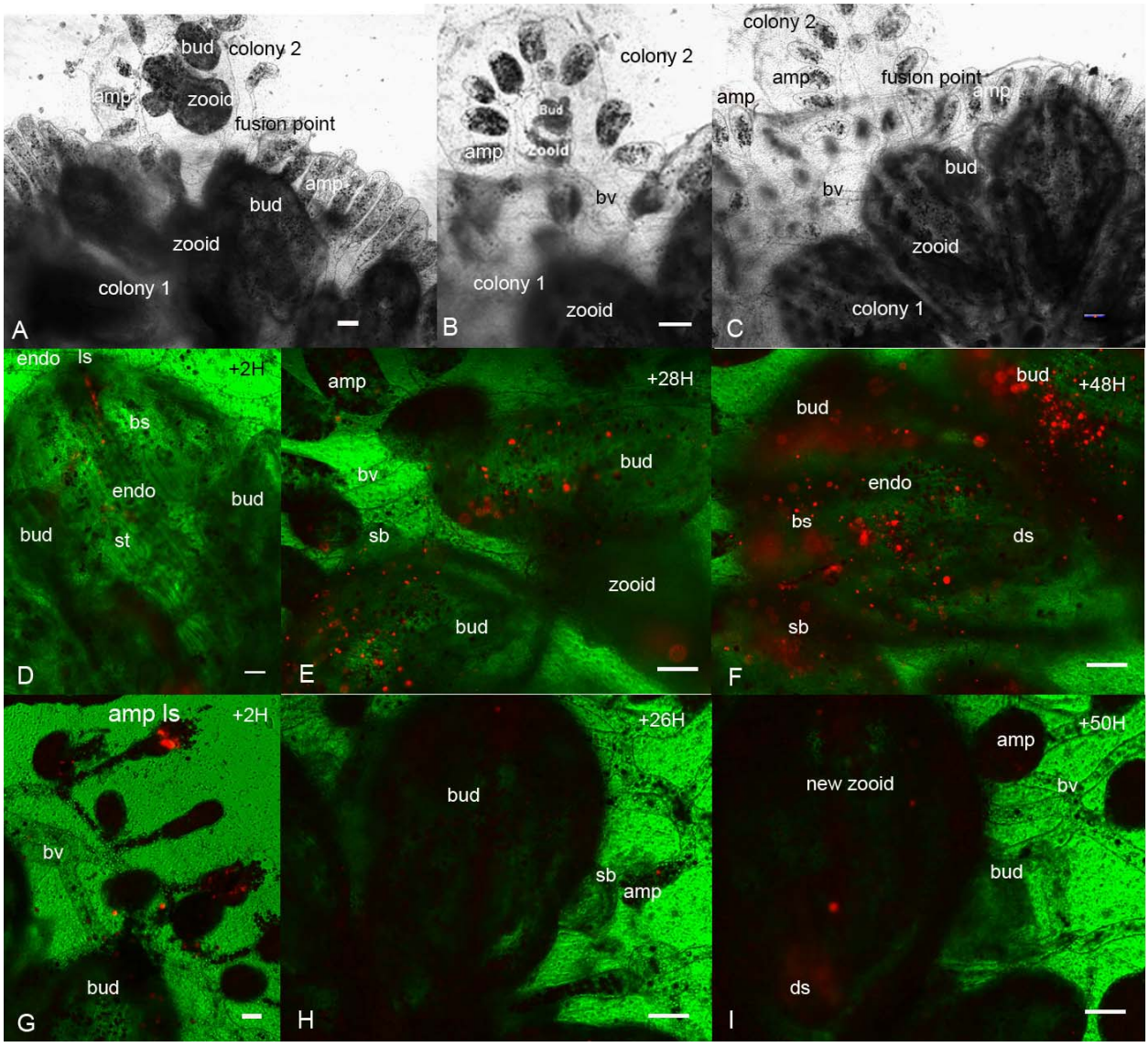
take over its partner genotype). Since germline tissues didn't rise in the tested chimeras, germline hierarchies in our experiments are unknown (marked as'?'). EN-endostyle niche, amp-ampulla, ds-digestive system.

Recipient genotype	**Donor Genotype**	**Fusion winner**	**Transplanted from**	**% of detectable chimeras in the different transplants**		
				n	**%**	**tissue**
2361d	**2362c**	c > d soma	**EN**	**4**	**25**	**soma**
2361d	2362c	c > d soma	amp	4	0	soma
2362c	2361d	c > d soma	EN, amp ds	4	0	soma
1960b	**1960c**	c > b soma germ ?	**EN** EN	**6** 3	**33.3** 0	**soma** germ
1960b	1960c	c > b soma germ ?	amp, ds amp, ds	6 4	0 0	soma germ
1960c	1960b	c > b soma	EN	2	0	soma
2204p	2204t	t > p soma	Amp	2	0	soma
2362c orange	**2362h black**	h > c soma	**EN**	**4**	**75**	**soma**
2362c orange	2362h black	h > c soma	amp, ds	4	0	soma
2362h black	2362c orange	h > c soma	EN, amp	7	0	soma
2190b	**2190c**	c > b soma germ?	**EN** EN	**5** 3	**40** 0	**soma** germ
2190b	2190c	c > b soma germ?	amp, ds amp, ds	4 2	0 0	soma germ
2190c	2190b	c > b soma germ?	EN, amp EN	6 5	0 0	soma germ

Figure S1: ***Botryllus schlosseri*** **chimerism** (A-C) **and the potential of cells to contribute to bud development** (D-I). A. A chimera between colony 1 and its offspring (colony 2), few hours following vascular fusion. B. Same chimera, 57 hours following fusion colony 2, zooid and bud are getting resorbed C. Same chimera 98 hours

following fusion. The zooid and buds of colony 2 were resorbed, their vasculature remained intact.

D. Few labeled cells (red), at the endostyle niche, 2 hours following labeling. E. 28 hours following labeling, labeled cells in the buds, these cells originated from the labeled cells in the endostyle niche. F. 48 hours following labeling, the bud had almost completed its development; labeled cells can be observed in the primary and secondary buds. G. Few labeled cells in 3 ampullae and a bud near the labeling site; these were been tracked 2 hours following labeling. H. Bud near the labeled ampullae, 26 hours following ampullae labeling. Only a few labeled cells were detected (those that were already detected 2 hours following labeling in this bud). I. 50 hours following labeling, the bud from figure 1G became a new zooid. 2-3 labeled cells can be observed in the zooid, the digestive system is labeled too. No labeled cells were tracked in the new zooid's bud. endo-endostyle, sb-secondary bud, amp-ampulla, ds-digestive system, h-heart, bs-branchial sac, st-stigmata, bv-blood vessel, tun-tunic, ls-labeling site, H-hour, scale bar=100µm


bud
colony 2
amp
zooid
fusion point
amp
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zooid
colony 1
A
colony 2
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Zooid
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