Integrin-alpha-6+ Stem Cells (ISCs) are responsible for whole body regeneration in a basal chordate

Susannah H. Kassmer[1], Adam Langenbacher[2], Anthony W. De Tomaso[1]

[1] Molecular, Cellular and Developmental Biology, University of California, Santa Barbara, CA USA. [2] Molecular, Cell, and Developmental Biology, University of California, Los Angeles, CA USA.
Susannah.kassmer@lifesci.ucsb.edu

Abstract:

Colonial ascidians such as *Botrylloides* are the only chordates able to undergo whole body regeneration (WBR), during which entire new bodies can be regenerated from small fragments of blood vessels. Although it has long been suspected that undifferentiated blood borne cells called hemoblasts play an important role, the cellular and molecular mechanisms of this regeneration process are poorly understood. Here, we show that during the early stages of WBR, proliferation occurs only in small, blood borne cells that express *integrin-alpha-6, pou3* and *vasa*. Proliferating cells can be ablated using Mitomycin C (MMC), leading to loss of WBR. However, regeneration can be rescued by injection of blood cells from a healthy individual into MMC treated vessel fragments. Using FACS-based prospective isolation, we found that injection of a single Integrin-alpha-6 (IA6)-positive cell is capable of rescuing WBR. Lineage tracing using EdU-labeling further revealed that IA6+ cells give rise to regenerating tissues. Our data suggest that *ia6+pou3+* cells are proliferating blood borne stem cells that are responsible for whole body regeneration. IA6+ Stem Cells (ISCs) also express *notch* and *frizzled* receptors, and inhibitors of either Notch or canonical Wnt signaling block WBR and reduce proliferation, indicating that these two pathways regulate ISC activation. These results provide important insights into the mechanisms and evolution of chordate regeneration.

Introduction:

The ability to regenerate missing structures is widespread among the metazoan phyla. Some invertebrate species in the groups of Cnidarians, Planarians and Asteroids can regenerate whole bodies from small fragments, while some vertebrates such as amphibians and fish can regenerate distal structures and some organs. In contrast, groups such as insects, birds and mammals have nearly lost the ability to regenerate [1-3]. Just like their general regeneration capacities, the cellular sources for regeneration are highly variable among regenerating species. In the Cnidarian *Hydractinia* and the Planarian *Schmidtea*, pluripotent stem cells are present in the adult and are responsible for regeneration, while in amphibians and zebrafish, lineage-committed progenitors arise from de-differentiation [1, 4]. In many cases, proliferation of undifferentiated cells (whatever their source) will lead to the formation of a blastema in the early stages of regeneration [1].

In many chordates, regeneration is limited to wound healing, for example, amphibians, can regenerate missing limbs and the spinal cord, and fish can regenerate fins and part of the heart. In contrast, basal chordate species, such as the invertebrate chordates of the genus *Botrylloides,* can regenerate whole bodies, including all tissues and organs, from small fragments of vasculature. This process of Whole Body Regeneration (WBR) has been studied in several different *Botrylloides* species, and is initiated when fragments of the extracorporeal vasculature are surgically separated from the colony. During the first 24 hours after separation of the vessel fragment from the colony, the blood vessels undergo regression and remodeling (stage 1, Figure 1B). Blood flow continues and is powered by contractions of the remaining ampullae [5]. During the next several days, the blood vessels continue to remodel and form a dense, contracted network (stage 2, Figure 1B). Within this dense, highly pigmented vascular tissue, an opaque mass of non-pigmented cells becomes apparent, progressing to form a hollow, blastula-like structure. The vascular epithelium wraps itself around this vesicle, leading to the formation of a distinctly visible double vesicle (stage 3 Figure 1B). Folding of this vesicle leads to development of the germ layers, and regeneration begins to resemble the developmental stages of palleal buds (stage 4 Figure 1B). Although multiple double vesicles can initially form, only one will continue to develop into a functional zooid [6]. The heart begins to beat before the development of other organs is complete (stage 5

Figure 1B). After about 10-14 days of regeneration, the fully mature body will begin filter feeding, and over time gives rise to an entire new colony by palleal budding. In summary, WBR is an injury induced regeneration event that must be initiated by either vascular or circulatory cells, or a combination of the two.

We are studying regeneration in the species *Botrylloides diegensis,* a colonial ascidian of the family Styelidae. Ascidians are the sister group of vertebrates, and begin life as a tadpole larvae with a typical chordate body plan. Following a free-swimming stage, the larvae settle and metamorphose into a sessile invertebrate adult. In colonial ascidians such as *Botrylloides*, zooids are connected to each other by a large extracorporeal vasculature and the entire colony is embedded within a gelatinous tunic (Figure 1A). Each zooid undergoes asexual reproduction and gives rise to 1-4 new bodies, which are derived from a region of the zooid called the peribranchial epithelium. This asexual reproduction process is called palleal budding, and leads to expansion of the colony.

In the present study, we aimed to assess whether WBR in *Botrylloides diegensis* is driven by a population of preexisting somatic stem cells. In planarians, a population of proliferating somatic stem cells called neoblasts gives rise to the missing body parts during whole body regeneration [7]. A population of cells with an undifferentiated appearance termed hemoblasts are present in the blood of all colonial ascidians and have been suggested to play roles in WBR [6]. In *Botrylloides violaceus,* 15-20 small hemoblasts that express Piwi protein have been shown to aggregate under the epidermis of a blood vessel during early WBR [8]. In a previous study in *Botrylloides leachii*, Blanchoud et al. showed an increase in the population of hemoblasts very early after injury during WBR [5]. During WBR in *B. violaceus*, circulating hemoblasts that express *piwi* mRNA appear near the early vesicle stage and occasionally within the epithelium of a vesicle [6]. These results had suggested that blood borne stem cells might play a role in WBR in *Botrylloides,* but to date, it has never been directly tested whether such cells give rise to regenerating tissues.

In several species of colonial ascidians, long-lived mobile germline stem cells have been described that are present in the blood of the adult colony and migrate to newly developing buds during repeated cycles of asexual reproduction, where they give rise to eggs and testes [9-15]. The cells express genes associated with germ cells, such as

vasa, *piwi* and *pumilio [12, 13, 16]*. We have previously shown that in *Botryllus schlosseri*, a population of cells enriched for expression of these germline genes can be prospectively isolated based on expression of Integrin-alpha-6 (IA6) [17]. *Vasa* and *piwi* are part of the germline multipotency program [18], and IA6 is a biomarker for various kinds of mammalian stem cells, including embryonic stem cells and primordial stem cells [19]. Therefore we aimed to assess whether a similar population of IA6+ stem cells is present in the blood of *Botrylloides*, and whether this population might play a role in WBR.

Here, we show that during the very early stages of WBR in *Botrylloides*, cell proliferation occurs only in blood borne cells that express *integrin-alpha-6* and the Oct4-like transcription factor *pou3*. We used IA6 as a surface marker to purify a population of blood cells that is enriched for expression of *pou3* and *vasa*, and show that this population is required for regeneration and gives rise to regenerating tissues. We also show that the proliferation of these IA6+ Stem Cells (ISCs) during WBR is regulated by Notch and Wnt signaling.

Results:

The majority of proliferating cells during early stages of WBR express Integrin-alpha-6, pou3 and vasa.

In *Botryllus schlosseri*, we had previously identified a population of germline stem cells (GSCs) that expresses *integrin-alpha-6* and *vasa* [17]. GSCs are found in germline niches in the developing zooids, as well as in the blood. To assess whether Integrin-alpha-6-positive cells might be blood borne stem cells involved in regeneration, we isolated IA6+ cells from the blood of *Botrylloides diegensis* by flow cytometry and assessed for expression of germline pluripotency genes such as *vasa*, *piwi1* and *piwi2* and *pou3.* In mammals, Oct4 (Pou5F1) is a transcription factor that is required for pluripotency [4, 20]. Pou class 5 is a vertebrate specific group of pou genes that likely split from pou3 during vertebrate evolution. Therefore, invertebrate *pou3* is most similar to vertebrate *pou5* [21]. Since vertebrate *pou5* genes play roles in pluripotency, it is likely that *pou5* inherited this function from an ancestral pou paralog, such as *pou3*. In the cnidarian *Hydractinia echinata*, *pou3* plays a role in stem cell pluripotency [22] and we hypothesized that *pou3* might play a similar role in *Botrylloides* stem cells. We cloned

pou3 from *Botrylloides diegensis* and the closely related colonial ascidian *Botryllus schlosseri* and constructed a phylogenetic tree that confirms the close relationship of pou3 to pou5 (Figure S1A). *Pou3* is expressed in germ cells in *Botrylloides diegensis* colonies (Figure S1B), and *vasa* and *pou3* are highly expressed in IA6+ cells isolated from the blood (Figure 1C). Previously, only one *piwi* gene had been reported in *B. leachii [23]*, but we found that, like most animals, *Botrylloides diegensis* has two *piwi* genes ([24, 25], supplemental file). Only *piwi2* is expressed in IA6+ cells, at lower levels than *vasa* (Figure 1C). Using Double FISH, we confirmed that 81% of either *ia6*- or *pou3*-positive cells are double positive, while 9 and 10% express only *pou3* or *ia6,* respectively (Figure 1E). The overlap between *vasa* and *pou3* is 87% (Figure 1D).

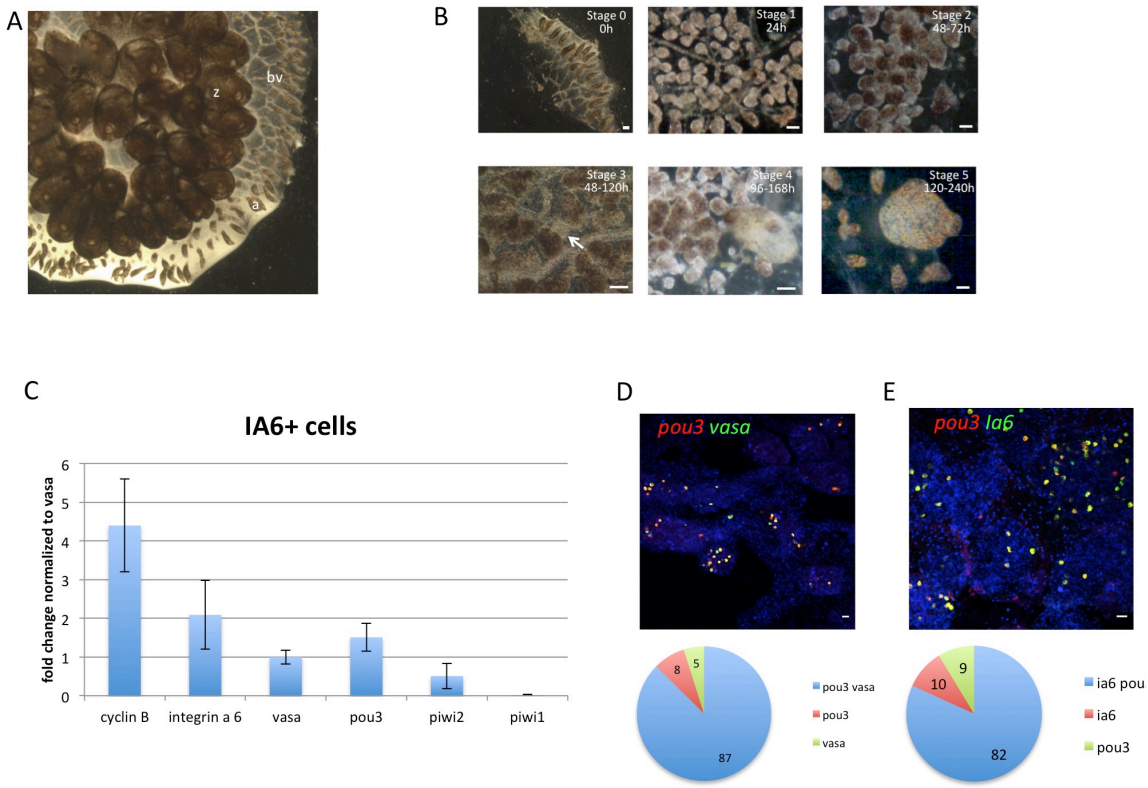


Figure 1: Stages of whole body regeneration, gene expression in IA6+ cells. A) *Botrylloides diegensis*, whole colony brightfield image, dorsal. Zooids (z) are embedded in a common, transparent tunic. Blood vessels (bv) extend throughout the tunic and are shared by the zooids of the colony. At the periphery of the colony, blood vessels form terminal sacs termed "ampullae" (a). Scale bars are 200um. B) Stage of whole body regeneration, brightfield images. Blood vessels are surgically separated from the colony (stage 0). During the first 24h, the blood vessel begin to remodel (stage 2). After 48h, blood vessels become condensed and highly pigmented (stage 2). Regeneration begins when a double vesicle is formed, consisting of two layers of epithelium (stage 3). This

double vesicle undergoes organogenesis (stage 4) and gives rise to a new, filter feeding body (stage 5). C) qPCR analysis showing expression of cyclin B, integrin-alpha-6, vasa, pou3, piwi2 and piwi1 in IA6+ cells isolated by flow cytometry. Data are expressed as averages of fold changes normalized to vasa, n=4, including standard deviation. D) FISH showing co-expression of pou3 (red) and vasa (green) in stage 0. DNA was stained with Hoechst (blue). Single positive and double positive cells were counted using the cell counter feature in FIJI, and for each stage, 4 images from independent samples were counted. Pie graph shows percentages of pou3/vasa-double-positive cells as well as pou3 and vasa single positive cells. E) FISH showing co-expression of pou3 (red) and ia6 (green) in stage 0. DNA was stained with Hoechst (blue). Single positive and double positive cells were counted using the cell counter feature in FIJI, and for each stage, 4 images from independent samples were counted. Pie graph shows the percentages of pou3/ia6-double-positive cells as well as pou3 and ia6 single positive cells. Scale bars 20um.

To assess whether *ia6+ pou3+* cells are involved in responding to injury, we analyzed the expression of these two genes in proliferating cells during the early time points after surgical separation of blood vessels from the colony. S-phase-specific *histone 3* is a marker for proliferation in plants and animals [26-29]. Analyzing expression of *histone 3* together with *integrin-alpha-6* by double fluorescent in situ hybridization (FISH), we found that 90% of all *integrin-alpha-6* + cells proliferate in the blood of a healthy colony (stage 0, Figure 2 A and B). After separation of blood vessels form the colony, the overlap between *histone 3* and *integrin alpha 6* stays high during stages 1 and 2 (86 92%, respectively, Figure 2 A and B), and the total number of proliferating *ia6+h3+* cells increases dramatically during stages 1 and 2 (Figure 2C). Furthermore, integrin-alpha-6-positive cells make up the majority of proliferating cells during the early stages of WBR, as the number of *h3* single positive cells is consistently much lower than that of *ia6+h3+* double positives in stages 0, 1 and 2 (Figure 2C). During stage 2, some *ia6+* cells appear to form small aggregates (arrows), and we hypothesize that these aggregates may be early regeneration niches. When the formation of the tissue layer that gives rise to the double vesicle begins (early stage 3, red arrow in Figure 2A) the number of quiescent *ia6+* single positive cells increases to 23%, and the number of *h3+* single positive proliferating cells increases as cell proliferation occurs in growing double vesicles (arrow, Figure 2A). In all stages, there seems to be a continuum of varying *ia6* and *h3* expression levels with single positive cells at either extreme. The images of separate channels in Figure 2 reveal that some cells that appear single positive in the overlay are actually expressing low levels of the other marker. We speculate that this may represent stages of *ia6+* stem cell differentiation, where *ia6* gets downregulated as

cells divide to give rise to *ia6*- progeny. Cells that keep high levels of *ia6* while dividing may be dividing symmetrically to give rise to more stem cells or may be transit amplifying cells.

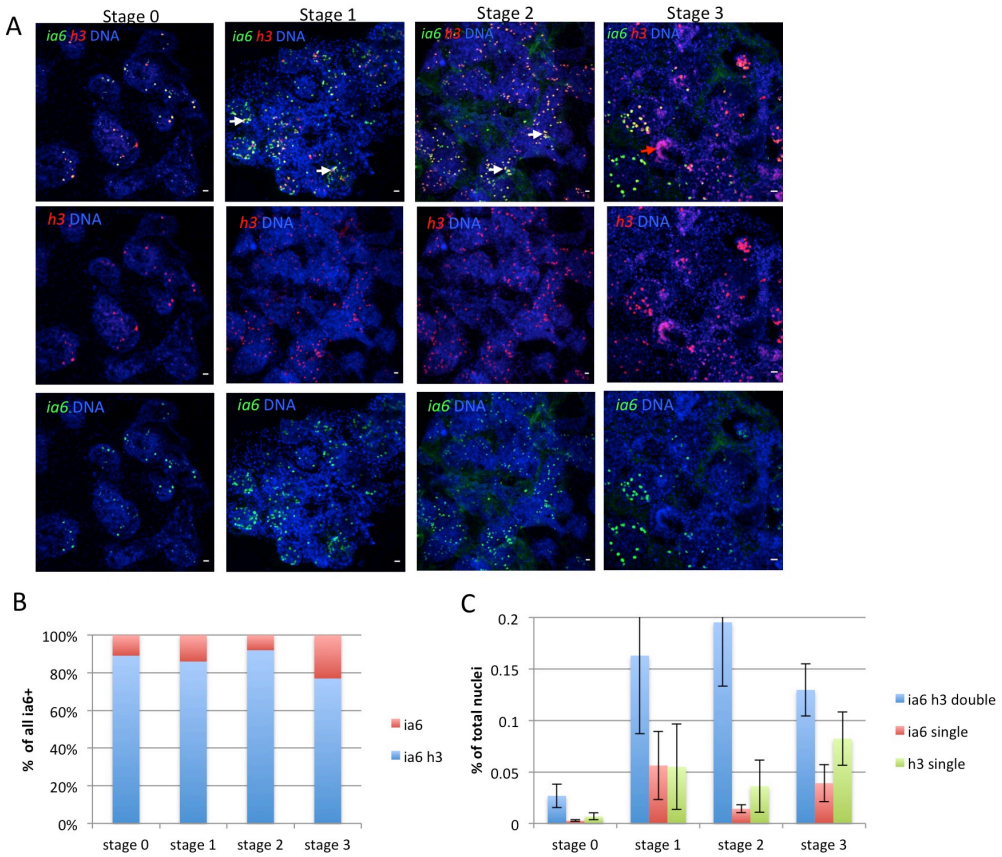


Figure 2: **Whole body regeneration is associated with proliferation of blood borne integrin-alpha-6+ cells.** A: Fluorescent in situ hybridization (FISH) showing expression of *integrin-alpha-6* (*ia6*, green) and *histone 3* (*h3*, red) during stages 0, 1, 2 and 3 of WBR. White arrows indicate clusters of proliferating *ia6*+ cells. Red arrow in stage 3 indicates a developing double vesicle. DNA was stained with Hoechst (blue). Scale bars 20um. B) Single positive and double positive cells were counted using the cell counter feature in FIJI, and for each stage, 4 images from independent samples were counted. Graph shows percentages of ia6/h3 double positive and *ia6*+ cells among all ia6+ cells. C) Single positive and double positive cells were counted using the cell counter feature in FIJI, and for each stage, 4 images from independent samples comprising between 2500 and 3000 cells were counted. Graph shows percentages of ia6/h3 double positive and *ia6*- or *h3*- single positive cells among all Hoechst-positive nuclei. Error bars reflect standard deviation.

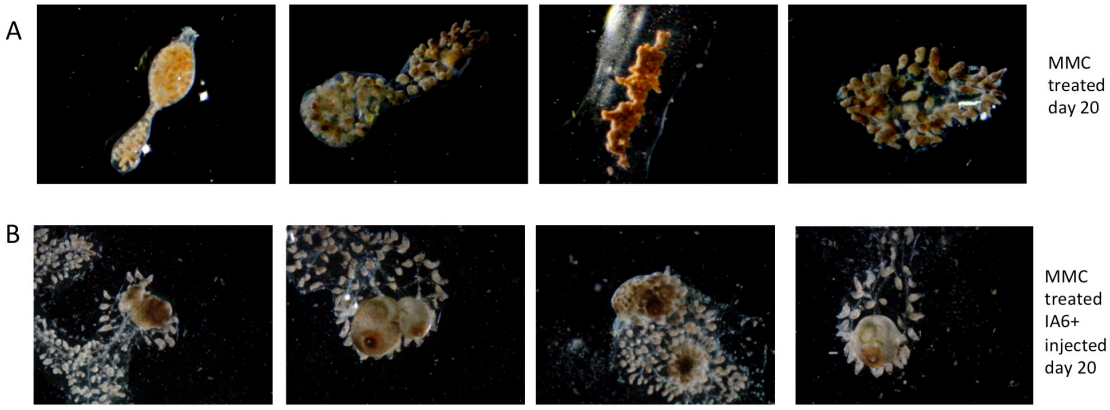
Analyzing expression of S-phase -specific *histone 3* and *pou3* by double FISH, we found that proliferating cells in the blood of *Botrylloides* express *pou3* in uninjured steady state and during the early stages of regeneration (Supplemental Figure 2). 91% of all *pou3*-

positive cells proliferate in steady state, and this percentage stays high during stages 1 and 2. By stage 3, 64% of *pou3*-positive cells become quiescent, and cell proliferation is predominant (71%) in *pou3*-negative regenerating double vesicles (arrows) and blood cells (Supplemental Figure 2). Together, these results show that *ia6+/pou3+* cells proliferate in steady state and make up the majority of proliferating cells during the early stages of WBR. Based on these findings, we hypothesize that *ia6+pou3+* proliferating cells are stem cells responsible for WBR.

<u>IA6+ cells are required for regeneration:</u>

To functionally assess whether proliferating IA6+ cells are involved in whole body regeneration, we ablated proliferating cells using the drug Mitomycin C (MMC). Vascular tissue was soaked in MMC immediately after surgery and treated for 24h until stage 1, when overlap between ia6 and the proliferation marker histone 3 is 93% (Figure 2). In MMC treated vessels at 5 days post treatment, no *pou3*+ or *histone3*+ cells remain (Figure S1D). Elimination of proliferating cells with Mitomycin C at stage 0-1 results in loss of regenerative capacity (Figure 3). Normally, whole body regeneration is complete after 11-14 days (Figure 1), but MMC treated samples do not reach stage 4 or stage 5 even after 20 days (Figure 3A), and most MMC treated blood vessels appear to be permanently arrested in stage 2 (Figure 3A). However, regeneration can be rescued in MMC treated vasculature by injection of blood cells isolated from an untreated, healthy individual, demonstrating that WBR depends on blood borne cells (Figure 3B). To identify the population of cells responsible for this rescue, we injected different populations of cells isolated by flow cytometry. For example, 50 cells that are in G2/M phase of the cell cycle, isolated from the blood of a healthy animal, can rescue regeneration, whereas the same number of cells in G0 phase does not have this ability (Figure 3C). 50 IA6- cells are not able to rescue WBR, and 57% rescue is achieved by injection of 1000 IA6- cells (Figure 3C), indicating that IA6- cells capable of rescuing WBR are very rare (about 1/2000). It is possible that a rare population of IA6- stem cells exists, as almost 20% of pou3+ cells are ia6- (Figure 1E). In contrast, 90% rescue is achieved by injecting 50 IA6+ cells (Figure 3C). We next carried out limit dilution analyses of this population, and found that injection of a single IA6+ cell isolated from the blood of a healthy animal can rescue whole body regeneration in 20% of MMC treated samples (Figure 3C). Using ELDA analysis software [30], we calculated the

estimated frequency of IA6+ cells capable of rescuing WBR to be about 1 in 7.55 (upper estimate 4.63, lower estimate 12.3). These results show that IA6+ cells are required for WBR.



C

Injected cell population	none	IA6- 1000	IA6- 50	G2/M 50	G0 50	IA6+ 50	IA6+ 10	IA6+ 5	IA6+ 3	IA6+ 1	Whole blood
% WBR Stage 5	2%	57%	0%	62%	0%	90%	75%	73%	50%	20%	95%

Figure 3: **IA6+ cells are required for whole body regeneration.** A: Mitomyinc C treatment prevents regeneration. Vessel fragments were treated with MMC for 16h immediately after surgery. MMC was removed and the samples followed for 20 days. Brightfield images of vessel fragments 20 days post MMC treatment. B: Injection of IA6+ cells 24h after MMC treatment rescues WBR. Brightfield images of MMC treated vessel fragments 20 days post injection of IA6+ cells. C: Table showing rates (percentages) of WBR (reaching stage 5) 20 days after injection of different cell populations. For each condition, 16-20 samples were injected and counted. A table of sample numbers is included in Supplement.

<u>IA6+ Stem Cells (ISCs) give rise to regenerating tissues in developing bodies:</u>

Since ISCs appear to be functionally required for WBR, we wanted to assess whether progeny from IA6+ cells is incorporated into regenerating tissues. We used EdU to label IA6+ cells in steady state in an intact animal and subsequently track the presence of their progeny in regenerating tissues by transplanting them into MMC treated vessel fragments. The modified thymidine analogue EdU is incorporated into newly synthesized DNA and detected in fixed tissues using a fluorescent dye. We ensured that EdU could be detected in blood cells at 24h post injection and at stage 3 of WBR (Figure S2A). IA6+ cells were isolated from donor animals that had been injected with EdU the day

before to allow EdU incorporation into the DNA of cycling cells. EdU-labeled IA6+ cells were injected into MMC treated recipients as in the rescue experiments described above. When recipients reached stage 3 or stage 4 of WBR, the regenerating tissues were fixed and stained for EdU. EdU-positive cells were present in early double vesicles in stage 3 (green arrows, Figure 4A). At later stages, when tissues begin to differentiate (stage 4), EdU-positive cells are found in differentiating tissue layers within the developing stage 4 regenerating bud. (Figure 4B, red arrows). In Botryllids, bodies and vessels are lined by a layer of ectodermal epithelial cells, and some of these cells appear to be labeled as well (Figure 4B, green arrows). These results show that IA6+ cells give rise to regenerating tissues in newly developing bodies.

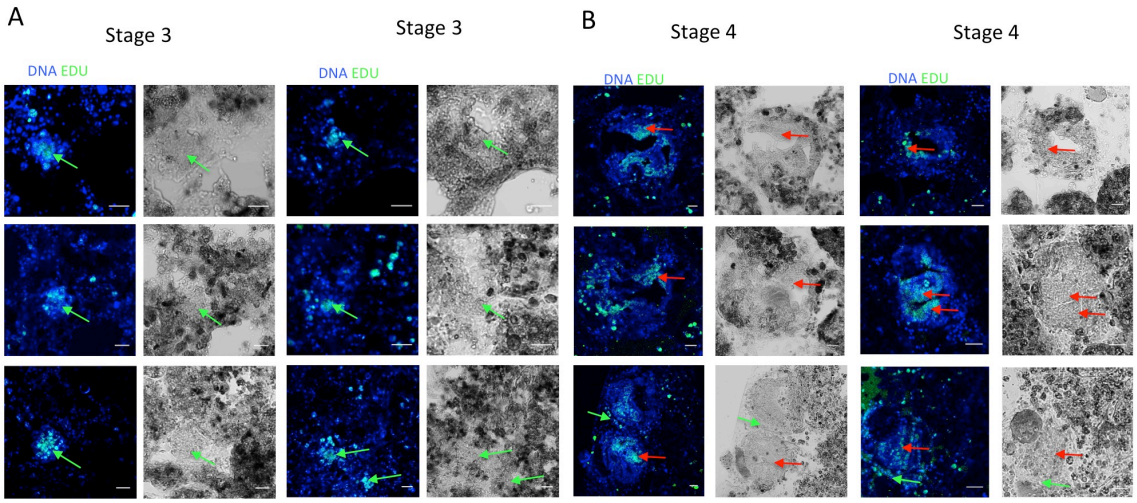


Figure 4: IA6+ cells give rise to regenerating tissues. A) IA6+ cells were isolated from Edu-treated animals and injected into MMC treated vessel fragments. Left panels: Overlay of nuclear staining (Hoechst, blue) and Edu (green) shows Edu-positive cells in the inner cell mass of the double vesicle at stage 3 (green arrows). Right panels are brightfield images of the same tissue. Scale bars 20um. B) Several samples from the same experiment as in A were followed to stage 4 (organogenesis). Edu-positive cells are present in several layers of differentiating tissue (red arrows) and occasionally in the outer epithelial layer (green arrows). Right panels: Overlay of nuclear staining (Hoechst, blue) and Edu (green). Right panels are brightfield images of the same tissue. Scale bars 20um.

Inhibition of Notch or Wnt signaling blocks regeneration and proliferation of ISCs:

We next aimed to analyze the expression of components of the Notch and Wnt signaling pathways in proliferating cells from the blood during the early time points of WBR. These pathways are known to play roles in regulating stem cell activity, proliferation of

blastema cells and cellular differentiation in other organisms [31-38] and are upregulated during WBR in *Botrylloides* [39]. We isolated cycling G2/M cells by flow cytometry from stage 0 as well as at 24h and 48h after injury of vessel fragments. We specifically isolated G2/M cells instead of IA6+ cells in order to be able to analyze gene expression in all proliferating blood cells, even those that may have begun to down-regulate IA6 as they enter into differentiation (Figure 2). As expected, G2/M cells isolated from the blood of healthy, unmanipulated animals (0h), express high levels of *integrin-alpha-6*, mitosis-specific *cyclin b*, and *pou3* (Figure 5A). *Notch 1, notch 2* and the downstream gene *hes1*, as well as the Wnt pathway components *frizzled5/8, disheveled* and *beta catenin* are highly expressed in G2/M cells at 0h, indicating active Notch and Wnt signaling in these cells (Figure 5B). 24h after injury, *notch2, frizzled 5/8* are upregulated in G2/M cycling cells compared to 0h. 48h after injury, *notch2, pou3* and *frizzled 5/8* are even more highly upregulated. *Notch1* is downregulated at 24h and 48h (Figure 5C). *Piwi2* is highly upregulated in cycling G2/M cells at 48h together with *vasa* (Figure S2B). These results show that the pluripotency/stem cell related genes *pou3, ia6, piwi2* and *vasa* are highly expressed in cells that proliferate during the early stages of WBR. Double FISH for *notch1* and *histone 3* shows that *notch1* positive cells proliferate during stage 0 and stage 1, but fewer of these cells are present in stage 2 (Figure 6A). However, the number of *notch2* positive cells increases by stage 2 (Figure 6A).

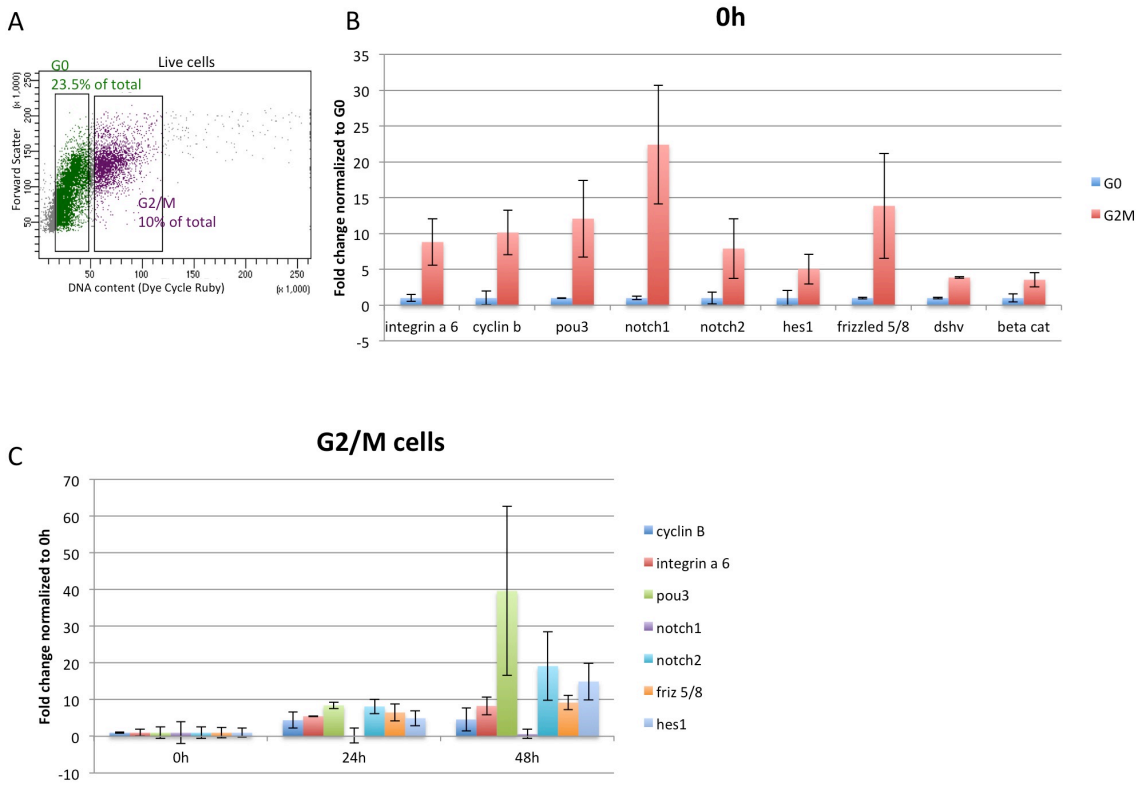


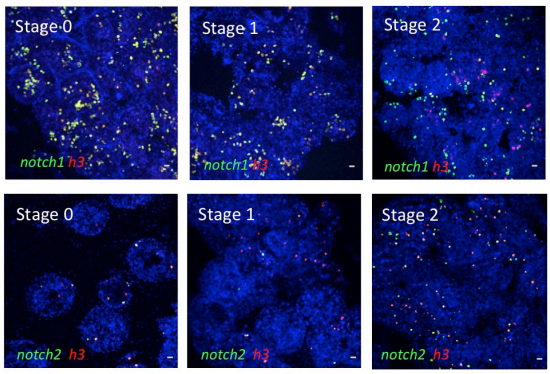
Figure 5: **Gene expression in cycling blood borne cells in steady state and during WBR.** A) Isolation of cycling G2/M cells by flow cytometry. Cells were stained with the live DNA stain Dye Cycle Ruby. Linear analysis of fluorescence intensity plotted against forward scatter (cell size) shows a clear separation of G2/M cells with 2n DNA content and G0/G1 cells with 1n DNA content. G2/M cells comprise 10% of live cells. B) qPCR analysis showing expression of, *integrin-alpha-6, cyclin b, pou3, notch1, notch2, hes1, frizzled 5/8, disheveled (dshv)* and *beta catenin* in G2/M cells in steady state (0h). Data are expressed as averages of fold changes normalized to G0 cells, n=3. C) qPCR analysis showing expression of *cyclin b, integrin-alpha-6, pou3, notch1, notch2,* and *frizzled 5/8* in G2/M cells at 24h and 48h post-injury, Data are expressed as averages of fold changes normalized to 0h (stage 0), n=4. Error bars represent standard deviation.

To assess whether Notch or Wnt signaling are required for WBR, vessel fragments were allowed to regenerate in the presence of inhibitors of either Notch (DAPT) or Wnt signaling (Endo-IWR). Inhibition of either Notch or canonical Wnt signaling blocked regeneration in a dose dependent manner (Figure 6B). In either 2uM of the Notch-signaling inhibitor DAPT or 1uM of the canonical Wnt signaling inhibitor Endo-IWR, the vessel tissue underwent remodeling up to stage 2 in both drugs, but remained in stage 2 while kept in drug (for up to 4 weeks), and appeared otherwise healthy and alive. Upon

removal of the drugs (after 96h of treatment), regeneration progressed normally (14/15 samples reached stage 5 for IWR and 15/16 for DAPT, Figure 6B). Control drugs Exo-IWR (1uM) and the Notch-sparing gamma-secretase modulator E2012 (1uM) did not affect regeneration rates (Figure 6B).

Double FISH for *histone 3* and *pou3* at 72h of treatment showed that only very few proliferating *pou3*-positive cells are present in drug treated vessels compared to controls (Figure 6C). These results show that WBR depends on both Wnt and Notch signaling, and that both signaling pathways control proliferation of ISCs after injury.

A



B

Condition	% stage 4 day 14
Control (no drug)	90%
Notch inhibitor DAPT 2uM	0%
Notch inhibitor DAPT 0.2uM	55%
Wnt inhibitor Endo-IWR 1uM	0%
Wnt inhibitor Endo-IWR 0.1uM	12.5%
Exo-IWR 1uM	83%
E2012 Notch-sparing gamma-secretase modulator 1uM	85%
Remove DAPT 2uM at 96h	89%
Remove IWR at 96h	89%

C

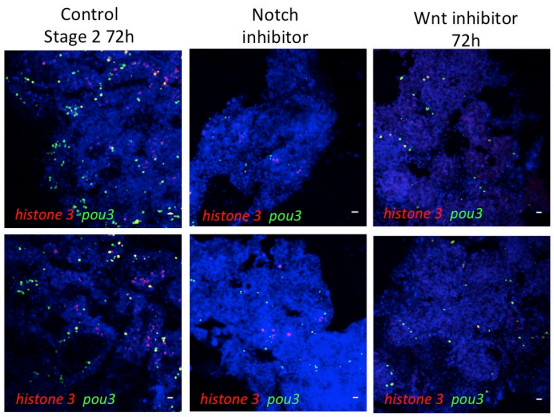


Figure 6: **Inhibition of Notch or Wnt signaling prevents WBR and blocks proliferation of pou3+ cells.** A: top panel: FISH for *notch1* (green) and *h3* (red) during steady state (stage 0) and during early stages of WBR. Bottom panel: FISH for *notch2* (green) and *h3* (red) during steady state (stage 0) and during early stages of WBR. Sacle bar 20um B: table showing the percentages of vessel fragments reaching stage 4 at day 14 post injury during treatment with different doses of inhibitors of canonical wnt signaling (Endo-IWR), Notch signaling (DAPT) or control drugs Exo-IWR (1uM) and the Notch-sparing gamma-secretase modulator E2012 (1uM). The number of colonies that reached stage 4 were counted at 14 days post injury. Percentages represent the number of fragments reaching stage 4, with n= 14-18 fragments for each condition. Controls received vehicle only. C) FISH for *pou3* (green) and *h3* (red) showing a reduction in

proliferating *pou3*+ cells 72h after surgery when treated with an inhibitor of either Notch or Wnt signaling. Sacle bars 20um.

Discussion:

For the first time, we provide definitive functional evidence that blood borne stem cells are responsible for injury induced whole body regeneration (WBR) and give rise to regenerating tissues in an invertebrate chordate. These cells likely belong to the undifferentiated "hemoblast" population that has been described historically in the blood of colonial ascidians [40] [11] and has been implicated in whole body regeneration [6, 8]. But no previous studies have ever directly tested whether hemoblasts or any other blood borne cells play a functional role in WBR.

Here, we show that IA6+ Stem Cells (ISCs) give rise to regenerating tissues during WBR, and that WBR cannot proceed when ISCs are ablated. We show that ISCs are constantly dividing in steady state and express genes associated with pluripotency, such as *pou3*. ISCs also contain germ plasm components such as *vasa* and *piwi,* yet we have shown that they give rise to somatic tissues during WBR. Germ plasm components such as *vasa, nanos* and *piwi* are expressed in stem cells with somatic potential in some invertebrate animals and have therefore been defined as germline multipotency (GMP) genes [18]. Primordial stem cells are defined as evolutionarily conserved stem cells containing germ plasm components that give rise to germ cells and can contribute to somatic tissues. Primordial stem cells are part of the germline and can be involved in asexual reproduction [41]. Examples of germ plasm containing cells that give rise to somatic tissues include small micromeres from sea urchins, neoblasts in planarians, i-cells in hydrozoan cnidarians and archeocytes in sponges. The common stem cell gene repertoire of ancestral metazoan stem cells comprises *cyclin B1* and *vasa* [42]. Both of these genes are expressed in ISCs (Figure 1C). It has been known for a long time that *vasa*-positive germline stem cells are present in the blood of colonial ascidians and persist throughout adult life and give rise to gonads during repeated rounds of asexual reproduction [9, 13, 43]. We hypothesize that in some species of colonial ascidians, the *vasa*+ stem cell population retains somatic potential that is utilized during whole body regeneration, and that a subset of ISCs might be related to primordial stem cells. Future studies will test whether a single ISC is able to give rise to both somatic and germline tissues. ISCs are functionally heterogeneous based on our single cell rescue data. One

out of about 7 IA6+ cells was able to rescue WBR. In future studies, we will use single cell sequencing to assess the heterogeneity within the ISC population and define functional sub-populations.

In *Botryllus schlosseri*, germline stem cells are a population of hemoblasts that give rise to gonads during asexual reproduction (palleal budding), whereas all somatic tissues are derived from the parent zooids peribranchial epithelium and not from blood borne cells [14]. In a previous study, we have shown that IA6 is expressed on *vasa*+ GSC in *Botryllus schlosseri* [44]. In future studies, we will investigate the similarities and differences between IA6+cells from *Botrylloides* and from *B. schlosseri*.

A previous study by Rinkevich et al. suggested that Piwi-positive cells are involved in regeneration in *B. leachii*. Our study revealed several notable differences versus these previous findings. Firstly, in the Rinkevich study, Piwi expression was not seen in blood vessels of steady state colonies and was only detected after 2 days post injury. In addition, Piwi expression was detected only in cells attached to the vascular epithelium, but not in circulating blood borne cells. Following initiation of WBR, Piwi-protein expression was detected in regenerating vessel fragments, and siRNA-mediated knockdown of *piwi* suggested a role of *piwi*+ cells in WBR. [23] However, the nature of these *piwi*+ cells was not explored in detail, and it is not clear which *piwi*-homolog was detected. In contrast, we show *B. diegensis* has two *piwi* homologs, one of which (*piwi2*) is expressed in blood borne ISCs in steady state and is highly upregulated in blood borne G2/M cells at 48h (Supplemental Figure 8B) together with *pou3, ia6* and *vasa*.

In a large-scale gene expression study, Zondag et al. showed that Wnt and Notch signaling components are upregulated during early stages of regeneration in *B. leachii [39]*. Here, we show that these pathways are required for proliferation and possibly maintenance of ISCs. Both of these pathways have been shown to play roles in regulating regeneration and stem cell proliferation in several other regenerating species [2], and Wnt signaling is required for blastema formation [36] and stem cell maintenance [35]. Notch regulates blastema cell proliferation during zebrafish fin regeneration, and it mediates cell fate decisions such as proliferation and differentiation in many stem cell types [33]. Notch also regulates blastema formation during distal regeneration in the solitary ascidian *Ciona* [32]. Future studies will investigate which Notch and Wnt ligands

are expressed by the regeneration niche in *Botrylloides*, and how and when these signaling pathways act on ISCs to regulate proliferation and differentiation.

In summary, we have identified a stem cell population responsible for whole body regeneration in *Botrylloides* and developed the toolsets necessary for future detailed molecular analysis of the stem cells involved in WBR and the pathways that regulate their function.

Methods:

Animals

Botrylloides diegensis colonies used in this study were collected in Santa Barbara, CA and allowed to attach to glass slides. Colonies were maintained in continuously flowing seawater at 19-23°C and cleaned with soft brushes every 14 days.

Whole body regeneration

Under a stereo dissection microscope, blood vessel fragments were surgically separated from the rest of the colony using razor blades. Regenerating fragments were kept attached to slides in flowing filtered seawater at 19-23°C.

Integrin-alpha 6 and cell cycle flow cytometry

Genetically identical, stage matched samples were pooled, and blood was isolated by cutting blood vessels with a razor blade and gentle squeezing with the smooth side of a syringe plunger. Blood was diluted with filtered seawater and passed through 70 µm and 40 µm cell strainers. Anti-Human/Mouse-CD49f–eFluor450 (Ebioscience, San Diego,CA, USA, cloneGoH3) was added at a dilution of 1/50 and incubated on ice for 30 min and washed with filtered seawater. For cell cycle sorting, Vybrant Dye Cycle Ruby Stain (Thermo Fisher Scientific, Waltham, MA, USA) was added at a dilution of 1/100 and incubated for 30 minutes at room temperature. Fluorescence activated cell sorting (FACS) was performed using a FACSAria (BD Biosciences, San Jose, CA, USA) cell sorter. A live cell gate was selected based on forward/side scatter properties, and samples were gated IA6 (CD49f)-positive or –negative based on isotype control staining (RatIgG2A-isotype-control eFluor450, Ebioscience, San Diego,CA, USA). Analysis was

performed using FACSDiva software (BD Biosciences, San Jose, CA, USA). Cells were sorted using high-pressure settings and a 70 µm nozzle and collected into filtered seawater.

Quantitative RT PCR

Sorted cells were pelleted at 700g for 10min, and RNA was extracted using the Nucleospin RNA XS kit (Macherey Nagel, Bethlehem, PA, USA), which included a DNAse treatment step. RNA was reverse transcribed into cDNA using random primers (Life Technologies, Carlsbad, CA, USA) and Superscript IV Reverse Transcriptase (Life Technologies, Carlsbad, CA, USA). Quantitative RT-PCR (Q-PCR) was performed using a LightCycler 480 II (Roche Life Science, Penzberg, Germany) and LightCycler DNA Master SYBR Green I detection (Roche, Penzberg, Germany) according to the manufacturers instructions. The thermocycling profile was 5 min at 95, followed by 40 cycles of 95 °C for 10 sec, 60 °C for 10 sec. The specificity of each primer pair was determined by BLAST analysis (to human, *Ciona* and *Botryllus* genomes), by melting curve analysis and gel electrophoresis of the PCR product. Primer sequences are listed in the Supplemental file. Relative gene expression analysis was performed using the $2^{-\Delta\Delta CT}$ Method. The CT of the target gene was normalized to the CT of the reference gene *actin* : $\Delta C_T = C_{T\,(target)} - C_{T\,(actin)}$. To calculate the normalized expression ratio, the ΔC_T of the test sample (IA6-positive cells) was first normalized to the ΔC_T of the calibrator sample (IA6-negative cells): $\Delta\Delta C_T = \Delta C_{T(IA6\text{-}positive)} - \Delta C_{T(IA6\text{-}negative)}$. Second, the expression ratio was calculated: $2^{-\Delta\Delta CT}$= Normalized expression ratio. The result obtained is the fold increase (or decrease) of the target gene in the test samples relative to IA6-negative cells. Each qPCR was performed at least three times on cells from independent sorting experiments gene was analyzed in duplicate in each run. The ΔC_T between the target gene and *actin* was first calculated for each replicate and then averaged across replicates. The average ΔC_T for each target gene was then used to calculate the $\Delta\Delta C_T$ as described above. Data are expressed as averages of the normalized expression ratio (fold change). Standard deviations were calculated for each average normalized expression ratio (n=6).

Mitomycin C treatment and rescue

Vessel fragments were cut and soaked in 60µM Mitomycin C (Tocris, Bristol, UK) in filtered seawater for 24 hours. MMC was removed and fragments were returned to

flowing seawater for 24 hours before being micro-injected with cells (different numbers of IA6+, IA6-, G0 or G2M, as indicated) isolated from the blood of normal, healthy colonies. Rescue efficiency (number of fragments reaching stage 5) was scored after 20 days.

Fluorescent *in situ* hybridization on cryosections

Vessel fragments at different stages of regeneration were fixed overnight in 4% paraformaldehyde in PBS at room temperature. Samples were washed in PBS and soaked in 15% and 30% sucrose for 30 minutes each before embedding in OCT medium. 20µm sections were cut using a Leica cryostat. Fluorescent *in situ* hybridization was adapted from [45] for cryosections. Briefly, *B. leachii* homologs of genes of interest were identified by tblastn searches of the *B. leachii* EST database (https://www.aniseed.cnrs.fr/aniseed/default/blast_search) using human or Ciona (when available) protein sequences. Primer pairs were designed to amplify a 500-800 bp fragment of each transcript (Primer sequences in Supplemental file). PCR was performed with Hotstar DNA Polymerase (Qiagen Germantown, MD 20874) and products were cloned into the pGEM-T Easy vector (Promega, Madison, WI, A1360). *In vitro* transcription was performed with SP6 or T7 RNA polymerase (Roche, Penzberg, Germany 10810274001, 10881767001) using either digoxigenin, fluorescein or dinitrophenol labeling. Cryosections were air-dried and fixed with 4% PFA for 10 minutes and washed with PBS/1%Triton-X-100. Probes were diluted in hybridization buffer and hybridized at 65 C for 30 minutes. Probes were removed and slides washed with 2xSSC/1%Triton and 0.2xSSC/1%Triton for 15 minutes each at 65C. HRP-conjugated anti-digoxigenin antibody (Roche 1/1000), HRP-conjugated anti-fluorescein antibody (Roche 1/500) or unconjugated anti-DNP antibody (Vector labs 1/100) followed by anti-rabbit-HRP (Abcam, Cambridge, UK, 1/200) were used to detect labeled probes. Fluorophore deposition was performed by incubating slides for 20 minutes at RT in Tyramides (Alexa488-Tyramide, Alexa-555 Tyramide or Alexa-594-Tyramide, all from Thermo Fisher) diluted 1/100 in PBS with 0.001% hydrogen peroxide. Slides were washed twice with PBS and Nuclei were stained with Hoechst 33342 (Life Technologies). Imaging of labeled samples was performed using an Olympus FLV1000S Spectral Laser Scanning Confocal. Image processing and analysis was performed using FIJI.

Tracking of EdU labeled cells

For every 10 zooids, 2µl of 1mM EdU (Thermo Fisher) dissolved in filtered seawater was injected into the blood stream of healthy colonies. After 24 hours, Integrin-alpha-6-positive cells were isolated from EdU-injected colonies by flow cytometry and injected into recipient vessel fragments 24 hours after Mitomycin treatment (see above). At stage 3 (n=10) and stage 4 (n=10) of regeneration, injected samples were fixed and cryosections were prepared as described above. Sections were fixed with 4% PFA for 30 minutes and treated with proteinase K for 7 minutes. Sections were post-fixed with 4%PFA for 20 minutes and blocked with PBS/1% Triton and 3% BSA for 1h. The clickit-reaction cocktail was prepared according to the manufacturer's instructions and the reaction was stopped after 1h at room temperature. Nuclei were stained with Hoechst 33342. Regenerating samples injected with EdU were used as positive control, and uninjected regenerating samples were used as negative controls. Imaging of labeled samples was performed using an Olympus FLV1000S Spectral Laser Scanning Confocal.

Small Molecule Inhibitor Treatment

Vessel fragments were cut and placed in the bottom of 24 well plates. 1ml of filtered seawater containing the Notch-signaling inhibitor DAPT (Tocris, 0.2-2µM), the Wnt-signaling inhibitor endo-IWR1 (Tocris, 0.1-1µM) or control drugs Exo-IWR (1uM) and the Notch-sparing gamma-secretase modulator E2012 (1uM) were added to filtered seawater and replaced every other day. The number of colonies that reached stage 4 were counted at 14 days post injury (n=16 for each condition).

Pou-Phylogenetic analysis

Protein sequences used for phylogenetic analysis were downloaded from the NCBI database or determined in this study. POU protein sequences were aligned using the ClustalW algorithm in the MEGA7 application [46]. Phylogenetic analysis of POU family members was performed with the software RAxML using a maximum likelihood method, the JTT substitution matrix, and empirical frequencies [47]. RAxML software was accessed using the CIPRES Science Gateway (Creating the CIPRES science gateway for inference of large phylogenetic trees. In: Proceedings of the gateway computing environments workshop (GCE), New Orleans, LA, USA; 2010. p. 1–8.) and trees were visualized using the Interactive Tree of Life website (Letunic I and Bork P (2006)

Bioinformatics 23(1):127-8 Interactive Tree Of Life (iTOL): an online tool for phylogenetic tree display and annotation).

Competing interests:

The authors state no competing financial interests.

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References:

1. Li, Q., H. Yang, and T.P. Zhong, *Regeneration across metazoan phylogeny: lessons from model organisms.* J Genet Genomics, 2015. **42**(2): p. 57-70.
2. Sanchez Alvarado, A. and P.A. Tsonis, *Bridging the regeneration gap: genetic insights from diverse animal models.* Nat Rev Genet, 2006. **7**(11): p. 873-84.
3. Sanchez Alvarado, A., *Regeneration and the need for simpler model organisms.* Philos Trans R Soc Lond B Biol Sci, 2004. **359**(1445): p. 759-63.
4. Sanchez Alvarado, A. and S. Yamanaka, *Rethinking differentiation: stem cells, regeneration, and plasticity.* Cell. **157**(1): p. 110-9.
5. Blanchoud, S., et al., *Hematological Analysis of the Ascidian Botrylloides leachii (Savigny, 1816) During Whole-Body Regeneration.* Biol Bull, 2017. **232**(3): p. 143-157.
6. Brown, F.D., et al., *Whole body regeneration in a colonial ascidian, Botrylloides violaceus.* J Exp Zool B Mol Dev Evol, 2009. **312**(8): p. 885-900.
7. Reddien, P.W. and A. Sanchez Alvarado, *Fundamentals of planarian regeneration.* Annu Rev Cell Dev Biol, 2004. **20**: p. 725-57.
8. Oka, H.W.H., *VASCULAR BUDDING IN BOTRYLLOIDES.* The Biological Bulletin, 1959. **117**(2): p. 340-346.
9. Laird, D.J., A.W. De Tomaso, and I.L. Weissman, *Stem cells are units of natural selection in a colonial ascidian.* Cell, 2005. **123**(7): p. 1351-60.
10. Sunanaga, T., H. Inubushi, and K. Kawamura, *Piwi-expressing hemoblasts serve as germline stem cells during postembryonic germ cell specification in colonial ascidian, Botryllus primigenus.* Dev Growth Differ, 2010. **52**(7): p. 603-14.

11. Kawamura, K. and T. Sunanaga, *Hemoblasts in colonial tunicates: are they stem cells or tissue-restricted progenitor cells?* Dev Growth Differ. **52**(1): p. 69-76.
12. Sunanaga, T., Y. Saito, and K. Kawamura, *Postembryonic epigenesis of Vasa-positive germ cells from aggregated hemoblasts in the colonial ascidian, Botryllus primigenus.* Dev Growth Differ, 2006. **48**(2): p. 87-100.
13. Brown, F.D., et al., *Early lineage specification of long-lived germline precursors in the colonial ascidian Botryllus schlosseri.* Development, 2009. **136**(20): p. 3485-94.
14. Carpenter, M.A., et al., *Growth and long-term somatic and germline chimerism following fusion of juvenile Botryllus schlosseri.* Biol Bull, 2011. **220**(1): p. 57-70.
15. Rodriguez, D., S.H. Kassmer, and A.W. De Tomaso, *Gonad Development and Hermaphroditism in the Ascidian Botryllus schlosseri.* Mol Reprod Dev, 2016. **in press**.
16. Langenbacher, A.D. and A.W. De Tomaso, *Temporally and spatially dynamic germ cell niches in Botryllus schlosseri revealed by expression of a TGF-beta family ligand and vasa.* Evodevo, 2016. **7**: p. 9.
17. Kassmer SH, R.D., Langenbacher AD, Bui C, De Tomaso AW, *Migration of germline progenitor cells is directed by sphingosine-1-phosphate signalling in a basal chordate.* Nature Communications, 2015. **6**(565).
18. Juliano, C.E., S.Z. Swartz, and G.M. Wessel, *A conserved germline multipotency program.* Development, 2010. **137**(24): p. 4113-26.
19. Krebsbach, P.H. and L.G. Villa-Diaz, *The Role of Integrin alpha6 (CD49f) in Stem Cells: More than a Conserved Biomarker.* Stem Cells Dev, 2017. **26**(15): p. 1090-1099.
20. Shi, G. and Y. Jin, *Role of Oct4 in maintaining and regaining stem cell pluripotency.* Stem Cell Res Ther, 2010. **1**(5): p. 39.
21. Gold, D.A., R.D. Gates, and D.K. Jacobs, *The early expansion and evolutionary dynamics of POU class genes.* Mol Biol Evol, 2014. **31**(12): p. 3136-47.
22. Millane, R.C., et al., *Induced stem cell neoplasia in a cnidarian by ectopic expression of a POU domain transcription factor.* Development, 2011. **138**(12): p. 2429-39.
23. Rinkevich, Y., et al., *Piwi positive cells that line the vasculature epithelium, underlie whole body regeneration in a basal chordate.* Dev Biol, 2010. **345**(1): p. 94-104.
24. Seto, A.G., R.E. Kingston, and N.C. Lau, *The coming of age for Piwi proteins.* Mol Cell, 2007. **26**(5): p. 603-9.
25. Juliano, C., J. Wang, and H. Lin, *Uniting germline and stem cells: the function of Piwi proteins and the piRNA pathway in diverse organisms.* Annu Rev Genet, 2011. **45**: p. 447-69.
26. Gown, A.M., et al., *Validation of the S-phase specificity of histone (H3) in situ hybridization in normal and malignant cells.* J Histochem Cytochem, 1996. **44**(3): p. 221-6.
27. Osley, M.A., *The regulation of histone synthesis in the cell cycle.* Annu Rev Biochem, 1991. **60**: p. 827-61.

28. Meshi, T., K.I. Taoka, and M. Iwabuchi, *Regulation of histone gene expression during the cell cycle.* Plant Mol Biol, 2000. **43**(5-6): p. 643-57.
29. Langenbacher, A.D., et al., *Whole-mount fluorescent in situ hybridization staining of the colonial tunicate Botryllus schlosseri.* Genesis, 2015. **53**(1): p. 194-201.
30. Hu, Y. and G.K. Smyth, *ELDA: extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays.* J Immunol Methods, 2009. **347**(1-2): p. 70-8.
31. Grotek, B., D. Wehner, and G. Weidinger, *Notch signaling coordinates cellular proliferation with differentiation during zebrafish fin regeneration.* Development, 2013. **140**(7): p. 1412-23.
32. Hamada, M., et al., *Evolution of the chordate regeneration blastema: Differential gene expression and conserved role of notch signaling during siphon regeneration in the ascidian Ciona.* Dev Biol, 2015. **405**(2): p. 304-15.
33. Perdigoto, C.N. and A.J. Bardin, *Sending the right signal: Notch and stem cells.* Biochim Biophys Acta, 2013. **1830**(2): p. 2307-22.
34. Tal, T.L., J.A. Franzosa, and R.L. Tanguay, *Molecular signaling networks that choreograph epimorphic fin regeneration in zebrafish - a mini-review.* Gerontology, 2010. **56**(2): p. 231-40.
35. Reya, T. and H. Clevers, *Wnt signalling in stem cells and cancer.* Nature, 2005. **434**(7035): p. 843-50.
36. Kawakami, Y., et al., *Wnt/beta-catenin signaling regulates vertebrate limb regeneration.* Genes Dev, 2006. **20**(23): p. 3232-7.
37. Vogg, M.C., Y. Wenger, and B. Galliot, *How Somatic Adult Tissues Develop Organizer Activity.* Curr Top Dev Biol, 2016. **116**: p. 391-414.
38. Wehner, D., et al., *Wnt/beta-catenin signaling defines organizing centers that orchestrate growth and differentiation of the regenerating zebrafish caudal fin.* Cell Rep, 2014. **6**(3): p. 467-81.
39. Zondag, L.E., et al., *Uncovering the pathways underlying whole body regeneration in a chordate model, Botrylloides leachi using de novo transcriptome analysis.* BMC Genomics, 2016. **17**: p. 114.
40. Brown, F.D. and B.J. Swalla, *Evolution and development of budding by stem cells: ascidian coloniality as a case study.* Dev Biol, 2012. **369**(2): p. 151-62.
41. Solana, J., *Closing the circle of germline and stem cells: the Primordial Stem Cell hypothesis.* Evodevo, 2013. **4**(1): p. 2.
42. Alie, A., et al., *The ancestral gene repertoire of animal stem cells.* Proc Natl Acad Sci U S A, 2015. **112**(51): p. E7093-100.
43. Carpenter, M.A., et al., *Growth and long-term somatic and germline chimerism following fusion of juvenile Botryllus schlosseri.* Biol Bull. **220**(1): p. 57-70.
44. Kassmer, S.H., et al., *Migration of germline progenitor cells is directed by sphingosine-1-phosphate signalling in a basal chordate.* Nat Commun, 2015. **6**: p. 8565.
45. Langenbacher, A.D., et al., *Whole-mount fluorescent in situ hybridization staining of the colonial tunicate Botryllus schlosseri.* Genesis.
46. Kumar, S., G. Stecher, and K. Tamura, *MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets.* Mol Biol Evol, 2016. **33**(7): p. 1870-4.

47. Stamatakis, A., *RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies.* Bioinformatics, 2014. **30**(9): p. 1312-3.