Distinct mechanisms underlie oral versus aboral regeneration in the cnidarian *Hydractinia echinata*

Brian Bradshaw[1], Kerry Thompson[2] and Uri Frank[1]*

[1]School of Natural Sciences & Regenerative Medicine Institute

[2] Centre for Microscopy and Imaging, Discipline of Anatomy, School of Medicine

National University of Ireland, Galway

Galway

Ireland

*Corresponding author. Email: uri.frank@nuigalway.ie

Abstract

Cnidarians possess remarkable powers of regeneration, but the cellular and molecular mechanisms underlying this capability are unclear. Studying the hydrozoan *Hydractinia echinata* we show that a burst of stem cell proliferation occurs following decapitation, forming a blastema at the oral pole within 24 hours. This process is necessary for head regeneration. Knocking down *Piwi1, Vasa, Pl10* or *Ncol1* expressed by blastema cells inhibited regeneration but not blastema formation. EdU pulse-chase experiments and *in vivo* tracking of individual transgenic *Piwi*+ stem cells showed that the cellular source for blastema formation is migration of stem cells from a remote area. Surprisingly, no blastema developed at the aboral pole after stolon removal. Instead, polyps transformed into stolons and then budded polyps. Hence, distinct mechanisms act to regenerate different body parts in *Hydractinia*. This model, where stem cell behavior can be monitored *in vivo* at single cell resolution, offers new insights for regenerative biology.

Impact statement

Cnidarians (jellies, corals and their kin) can regenerate any missing body part; we show that stem cell migration underlies head regeneration, but regeneration of other body parts is based on gradual transformation of one tissue type into another.

Funding

Funds were provided by a Principal Investigator Award from Science Foundation Ireland (SFI) to UF (grant number 11-PI-1020), and by the Programme for Research in Third Level Institutions, Co-funded by the Irish Government and the European Union under Ireland's EU Structural Funds Programme 2007 – 2013, to KT.

Introduction

Cnidarians are renowned for their remarkable ability to regenerate any missing body part. Classical work on the freshwater polyp *Hydra* has shown that both head and foot regeneration can occur without a significant contribution from cell proliferation (i.e. through morphallaxis) (Park et al., 1970, Holstein et al., 1991, Marcum and Campbell, 1978b, Marcum and Campbell, 1978a, Dübel and Schaller, 1990, Cummings and Bode, 1984). In planarians, by contrast, proliferation of pluripotent stem cells (called neoblasts) and formation of a mass of undifferentiated cells (called blastema) are required for head, tail and pharynx regeneration (Reddien and Sanchez Alvarado, 2004, Baguna, 2012, Reddien, 2013, Adler et al., 2014). The establishment of a blastema in regeneration has been observed in other taxa including annelid worms (Bely, 2014) and echinoderms (Candia Carnevali, 2006, Kondo and Akasaka, 2010), but the nature of the cells involved is unclear. Urodele amphibians are the only vertebrate tetrapods that can regenerate amputated limbs as adults. They are similar to planarians in their requirement for cell proliferation and blastema formation to complete regeneration, but the cellular source for urodele regeneration is different. In newts, dedifferentiation of cells in the stump provides progenitor cells, but in the axolotl, resident stem cells fulfill the same task (Sandoval-Guzman et al., 2014). Furthermore, amphibian blastema cells are lineage restricted rather than being pluripotent (Kragl et al., 2009).

The ability to regenerate varies greatly among animals (Sanchez Alvarado, 2000, Sanchez Alvarado and Tsonis, 2006, Tanaka and Reddien, 2011, Galliot and Ghila, 2010), with substantial differences sometimes found between closely related taxa: Amphibians, urochordates, planarians and cnidarians all include both groups or

species with excellent regenerative capabilities and their poorly regenerating close relatives (Sanchez Alvarado, 2000, Galliot and Ghila, 2010). One possible explanation for these observations is that the basic genetic toolkit for regeneration is primitive and present in all animals, but that modulation or loss of some components can modify the ability of a given taxon to regenerate. This has recently been shown to be the case in planarians where changes in canonical Wnt signaling underlie differences in regenerative ability between closely related species (Umesono et al., 2013, Liu et al., 2013, Sikes and Newmark, 2013). Hence, studying regeneration in a broad variety of animal models might reveal both regeneration mechanisms that are primitive and widely shared among animals as well as evolutionarily derived ones and could assist in addressing a major question in regenerative medicine, namely why humans are not capable of regenerating many tissues.

A specific difficulty in the study of tissue and organ renewal in higher animals is the fact that, like embryonic development, regeneration is a dynamic process. Therefore, understanding regeneration requires the analysis of individual cells over long time periods covering the duration of the regenerative process. The large size and opaque nature of many animals impede *in vivo* regeneration research at such resolution in most model organisms.

In this study we show that *Hydractinia echinata,* which is a common colony-forming cnidarian in the European North Atlantic (Figure 1), provides a powerful model system to study the cellular and molecular basis of animal regeneration. Indeed, *Hydractinia* is easy to culture in the lab and allows whole mount gene expression analysis, cellular analyses, transgenesis and gene knockdown (Plickert et al., 2012).

The animal reproduces sexually on a daily basis, but also grows clonally by elongation of gastrovascular tubes, called stolons, and asexual budding of new polyps (Figure 1). Finally, they are small and optically translucent, and post-metamorphic animals are sessile and can grow on microscope slides enabling *in vivo* imaging of cellular processes. Like many cnidarians, *Hydractinia* displays a remarkable regenerative ability and growth plasticity, but the molecular and cellular mechanisms underlying these capabilities are not well understood. We have studied both oral (i.e. head) and aboral (i.e. stolon) regeneration in *Hydractinia* and show that stem cell migration and proliferation underlie head regeneration in this animal. Surprisingly, stolon regeneration is achieved through a fundamentally different cellular and morphogenetic process, demonstrating that a single species can apply different mechanisms to regenerate different tissues or body parts.

Results

Head regeneration in *Hydractinia*

Our first aim was to characterize *Hydractinia* head regeneration. For this, polyps were isolated from their colony by a transverse cut close to the polyp-stolon boundary and decapitated (n>300; Figure 2A). The remaining cylinder-like body column was then followed until a new head developed (Figure 2B) and the animals regained the ability to feed. Lesion closure by stretching out of epidermal epithelial cells occurred at both cut ends within six hours following decapitation in all cases. No further development occurred at the aboral end where the stolons were removed (see section below). About 24 hours later, a dome-like tissue appeared at the oral pole. This was followed by the development of a new mouth and tentacles 48-96 hours post decapitation (in over 95% of cases; Figure 2B). Anti-acetylated tubulin antibody staining confirmed that

the nervous system had completely regenerated with both neurons and nematocytes (cnidarian-specific mechanosensory/effector cells) appearing normal as in control animals (Figure 2C-F). The polyps regained the ability to catch prey about two to three days post decapitation when a new head fully formed, but tentacle elongation sometimes continued for an additional few days. The time course of head regeneration (n>300) was variable among polyps, lasting between one and four days, depending on age (young post metamorphosis animals may regenerate faster), genetic background (we use a polymorphic wild type laboratory population), and general state of health (malnourished or otherwise unhealthy animals may display delayed regeneration). No indications for stolon regeneration were observed within the time course of head regeneration. Regeneration of decapitated polyps that remained attached to their colonies was indistinguishable from isolated polyps within the natural variability stated above.

Cell proliferation during regeneration

Head regeneration after decapitation in the freshwater cnidarian, *Hydra*, can occur in the absence of cell proliferation (Park et al., 1970, Holstein et al., 1991, Marcum and Campbell, 1978b, Marcum and Campbell, 1978a, Dübel and Schaller, 1990). We therefore decided to analyze cell proliferation in *Hydractinia* head regeneration. In intact *Hydractinia* polyps, cell proliferation was almost exclusively restricted to a band at the lower part of the polyp body column, with little or no proliferating cells outside of this band. This was shown with both EdU incorporation as well as anti-phospho-Histone 3 (pH3) antibody labeling to visualize mitotic cells (Figure 3A). We then decapitated polyps and allowed them to regenerate. During the course of regeneration we incubated the polyps in EdU for 40 minutes at different times

following decapitation. Animals were immediately fixed and processed for EdU visualization or anti pH3 staining. These experiments showed, first, that wound closure was not associated with enhanced cell proliferation (*Figure 3-figure supplement 1*). However, a striking shift in the spatial distribution of cycling cells was evident 24-48 hpd (hours post-decapitation). In contrast to the lower body column band-fashion distribution of cycling cells in intact polyps, the post decapitation distribution of cycling cells concentrated at the oral pole where the new head was about to form (n=100; Figure 3A). Intact polyps that were labeled by EdU while still connected to their stolonal network showed the same pattern of S-phase cell distribution as polyps with intact heads isolated from their colonies (n=20; *Figure 3-figure supplement 1*). Hence, head but not stolon amputation in *Hydractinia* is followed by the formation of a blastema with highly proliferative cells.

Proliferative cells during regeneration

We then set to address the question of which cell types proliferated during regeneration. For this, we decapitated animals and 24 hours later macerated them as previously described (David, 1973). We also macerated intact animals as control. Cells were spread on glass slides and stained with anti pH3 antibodies. Cnidarians have relatively few cell types including epidermal and gastrodermal myoepithelial cells, several types of neurons, stinging cells (nematocytes), gland cells and stem cells (*Figure 3-figure supplement 2*). Hydrozoan stem cells are called interstitial cells, or i-cells for short. In *Hydractinia*, i-cells reside in interstitial spaces between epithelial cells (mostly epidermal), and at a population level are thought to be pluripotent life long, giving rise to all somatic lineages as well as germ cells (Müller et al., 2004). This is very different from *Hydra* where i-cells do not contribute to the two self-

renewing epithelial lineages (Bode, 1996). Most cell types are easily distinguishable morphologically (*Figure 3-figure supplement 2*; 3B). We have counted mitotic cells in intact versus regenerating animals 24 hpd in three separate experiments and found that on average epithelial cells, as identified by morphology, form less than 4% of the total mitotic cell complement, whereas the vast majority of S-phase cells are i-cells (Figure 3B, C). There was no significant difference in the relative proportion of mitotic epithelial cells in intact versus regenerating animals. Hence, i-cells form the major proliferative cellular component in *Hydractinia* head regeneration, but the contribution of epithelial cells to this process through proliferation and/or transdifferentiation can not be ruled out based on these data.

Cell proliferation is required for regeneration

To address the requirement for cell proliferation for head regeneration we exposed *Hydractinia* polyps to gamma irradiation. We first established that absorbed doses of up to 300 Gy did not completely abolish cell proliferation in decapitated polyps (n=30); *Figure 3-figure supplement 3*). At 500 Gy, S-phase cells were no longer detectable (n=30); *Figure 3-figure supplement 3*) but the animals remained responsive to mechanical stimulation.

To analyze the direct effect of gamma irradiation and assess cell death by apoptosis we performed TUNEL staining on intact and decapitated animals that had or had not been irradiated. We found small numbers of apoptotic cells in non-irradiated animals between 6-24 hpd (n=30). This is consistent with previous work on other animals, where apoptotic cells were shown to play a role in the regenerative process (Hwang et al., 2004, Tseng et al., 2007, Chera et al., 2009, DuBuc et al., 2014). In irradiated

194 animals, the distribution of apoptotic cells was similar to the distribution of
195 proliferating i-cells in non-irradiated animals (n=30; *Figure 3-figure supplement 4*;
196 compare with Figure 3A). We concluded that cycling i-cells are sensitive to gamma
197 irradiation.
198
199 We then irradiated animals at 500 Gy followed by decapitation, and returned them to
200 their culture tanks. None of the animals regenerated at 48 hours following this
201 treatment (n=30; Figure 3D, E; *Figure 3-figure supplement 3*). Animals irradiated at
202 300 Gy or lower did regenerate, but at a markedly slower pace (n=30; *Figure 3-figure*
203 *supplement 3*). Importantly, the lack of EdU incorporation showed that no
204 proliferative blastema developed at the oral pole of animals irradiated at 500 Gy
205 (Figure 3D). Treatment with mitomycin C, a cytostatic drug that was shown to kill i-
206 cells in *Hydractinia* (Müller et al., 2004), had similar effects, with animals failing to
207 regenerate following treatment (*Figure 3-figure supplement 5*). Therefore, cell
208 proliferation and blastema formation are essential for *Hydractinia* head regeneration.
209 This is markedly different from *Hydra* head regeneration, which can occur through
210 morphallaxis in the complete absence of cycling cells.
211
212 **Genes acting during head regeneration**
213 Our next step was to study gene expression during regeneration. We focused on
214 *Piwi1, Vasa, Myc2* and *Pl10*, all standard stem cell markers in cnidarians and other
215 metazoans (Rebscher et al., 2008, Reddien et al., 2005, Alie et al., 2011, Voskoboynik
216 et al., 2008, Juliano et al., 2014, Collins et al., 2013). In addition, we studied *Ncol1*,
217 an early nematocyte differentiation marker in cnidarians (David et al., 2008, Millane
218 et al., 2011). We first established the normal expression pattern in intact polyps using

in situ hybridization. As shown for *Vasa* previously (Rebscher et al., 2008), all i-cell marker genes were expressed in a band fashion at the lower part of the polyp body column, co-localizing with S-phase and mitotic cells (Figure 4A, B; compare with Figure 3A; n=40).

We then decapitated polyps and fixed them at different time points during head regeneration and performed *in situ* hybridization with cRNA probes for i-cell genes. We found that decapitation had a major effect on the distribution of i-cells. Instead of being restricted to the band area, we now saw i-cells at more oral positions. Most strikingly, about 24 hpd, coinciding with the major proliferative peak in the blastema, there was strong expression of i-cell markers and *Ncol1* in the blastema (n=40; Figure 4C, D). Hence, the blastema that forms during head regeneration at the oral pole contains large numbers of i-cells and early nematoblasts in contrast to the absence of these cells in oral areas in intact animals.

Irradiation of animals at 500 Gy resulted in a marked reduction of i-cells and nematoblast marker expression at 24 hours post decapitation (n=10; Figure 4E). This further shows that i-cells are sensitive to irradiation and are required for head regeneration.

Downregulation of i-cell genes inhibits regeneration

To study the role of stem cell genes in head regeneration we used RNAi to knockdown *Piwi1, Vasa, Pl10* and *Ncol1*. RNAi was performed as previously described (Duffy et al., 2010, Duffy et al., 2011, Millane et al., 2011). Briefly, polyps were removed from their colony and were then decapitated. Following decapitation

244 the cylindrical body columns were incubated in seawater containing 30-50 ng/µl
245 double stranded RNA (dsRNA) corresponding to 200 bp coding sequence of *Piwi1, Vasa, Pl10* and *Ncol1*. Control experiments were performed with dsRNA
247 corresponding to the backbone sequence of the pBlueScript cloning vector, which is
248 not encoded by the *Hydractinia* genome. qPCR analysis of *Piwi1* RNAi treated
249 regenerating animals revealed a significant reduction in *Piwi1* mRNA levels
250 comparing to control RNAi (*Figure4-figure supplement 1*).
251
252 Regeneration in animals in which *Piwi1, Vasa, Pl10* or *Ncol1* were knocked down
253 was compromised, with the frequency of regenerating RNAi animals significantly
254 different from the control RNAi (chi-square test, p=0.00001; Figure 4F). Animals
255 treated with control (i.e. non-coding) dsRNA regenerated normally. The experiments
256 were repeated four times with 10 animals for each treatment. To address the role of
257 these genes in i-cell proliferation we treated regenerating animals with dsRNA as
258 described above. Twenty-four hours after decapitation we incubated them in EdU for
259 20 minutes followed by fixation and EdU visualization. We found that knockdown of
260 *Piwi1, Vasa, Pl10,* or *Ncol1* had no visible effect on cell proliferation (Figure 4G). In
261 these RNAi animals the head blastema formed normally yet regeneration was
262 significantly affected, suggesting that these genes are required for differentiation. The
263 specificity of the RNAi treatment was confirmed by knockdown of histones H2A or
264 H4 which strongly reduced EdU incorporation in regenerating animals (*Figure 4-*
265 *figure supplement 1*). Hence, *Piwi1, Pl10,* and *Vasa* expression is not required for i-
266 cell proliferation and for the formation of the head blastema. *Nco11*, which is a
267 nematogenesis marker, is not expressed in cycling i-cells and its downregulation is
268 therefore not expected to affect S-phase cells.

The cellular source of head blastema is migration of stem cells

The experiments described above show that decapitation results in head blastema formation that includes numerous proliferating i-cells. In fully grown, intact animals, i-cells are more or less restricted to the band area in the lower polyp body column, and are nearly absent from the head, which also does not include significant numbers of proliferating cells (n=75/83). We therefore reasoned that in the near absence of resident stem cells in the intact head (Figure 4A), the source of stem cells in the head blastema could either be migration, i.e. i-cells moving from the band in the lower polyp body column, or dedifferentiation of local differentiated cells in the stump. To discriminate between these two options, we performed two types of experiments. First, we pulse-labeled intact polyps with EdU for 60 minutes, followed by decapitation. The animals were intensively washed to remove EdU and left to initiate head regeneration. They were fixed and processed for EdU visualization at 6 and 24 hpd. We found that animals fixed 6 hpd had a strong signal in the band at the lower body column and only a few EdU+ cells were scattered at more oral positions (Figure 5A). However, those animals fixed 24 hpd had also a strong EdU signal in the developed blastema (Figure 5B; *Figure 5-figure supplement 1*). Because no EdU could be incorporated after washing the animals, the EdU+ cells in the blastema must have been the very same cells that were in S-phase in the lower band 24 hours earlier and had migrated to the stump following decapitation. Analysis of pH3 immunoreactivity in EdU pulse chased animals revealed double positive cells in the blastema, indicating that S-phase cells in the band continued to proliferate even after reaching the stump and contributed new cells to the blastema through mitosis (*Figure 5-figure supplement 1*).

Second, to gain a more dynamic view on i-cell migration during regeneration we established transgenic animals expressing GFP under the *Hydractinia* endogenous *Piwi1* genomic control elements. For this, we cloned 2.5 kb upstream and 1.1 kb downstream of the *Piwi1* genomic coding sequence locus and inserted the *GFP* coding sequence instead of *Piwi1* (*Figure 5-figure supplement 2*). This construct was microinjected to one-cell stage embryos as described previously (Künzel et al., 2010). Genomic integration and stable GFP expression occurs in *Hydractinia* within 24 hours (Künzel et al., 2010). GFP expression in transgenic polyps (Figure 5C, D) was consistent with the endogenous expression of *Piwi1* as assessed by *in situ* hybridization (Figure 4A) and immunohistochemistry (*Figure 5-figure supplement 3*). Because genomic integration does not occur in all cells, the animals were mosaics and not all *Piwi1*+ i-cells expressed GFP. This feature was useful because the density of GFP+ cells was not as high as would be expected in a fully transgenic animal, facilitating tracking of single cells *in vivo* (Figure 5D).

Transgenic polyps were isolated from their colonies by a transverse cut close to the polyp-stolon boundary. Polyps were decapitated and then viewed while the head blastema was developing over several hours using a fluorescence stereomicroscope (Figure 5E), time-lapse DeltaVision deconvolution microscope (Figure 5F; Video 1), or Andor spinning disk confocal microscope (Figure 5G; Video 2). *Piwi1*+ cells were observed migrating into the prospective head area (Figure 5E-G; Videos 1 and 2) and no evidence for dedifferentiation was evident as all viewed recruited GFP+ cells at the blastema were migratory. Some migrating cells underwent mitosis before reaching the blastema; they stopped migrating, completed mitosis, and the two daughter cells

resumed migration (Figure 5G; Video 2). Based on these experiments and the EdU pulse-chasing, we conclude that the primary cellular source for establishing the head blastema and, subsequently, head regeneration is migration of i-cells from the band in the polyp lower body column to the prospective head area. A possible contribution of existing epithelial cells to the regeneration process through mitosis and/or transdifferentiation (body column epithelial cells to tentacle epithelial cells) cannot be ruled out.

Stolon regeneration involves different mechanisms than head regeneration

Hydractinia polyps are not able to regenerate stolons directly from their aboral ends (Müller et al., 1986). Polyps, or even fragments of them, can, instead, transform into stolons but this phenomenon is not well understood (Putnam Hazen, 1902, Müller et al., 1986, Lange, 1991). To study aboral, i.e. stolon, regeneration we removed polyps from their colony by a transverse cut at the lower third of the polyp body column to exclude any stolonal tissue. Isolated polyps healed the cut surface within hours (Figure 6A). We then followed the polyps and photographed them every 24 hours for up to 25 weeks. The polyps appeared normal for days and sometimes weeks, responded to mechanical stimuli by contraction, and were able to catch, kill and ingest brine shrimp nauplii, resembling a solitary *Hydra* polyp. No blastema developed at the aboral pole after stolon removal, and the general distribution of EdU+ cells was largely similar to polyps that were labeled on their colony. (***Figure 3-figure supplement 1***). Over the next weeks, however, the polyps started resorbing their tentacles and thereby lost the ability to feed (Figure 6A). Next, the entire head structure disappeared and each polyp's cylindrical body column started to elongate, thereby decreasing its diameter (Figure 6A). New branches appeared at irregular

intervals and some developed into polyps with fully functional heads and tentacles, thereby regaining the ability to feed (n=200; Figure 6B). Chitin secretion (which is stolon specific in *Hydractinia* (Lange, 1991) commenced (Figure 6C)) and eventually, sexual polyps developed and produced fertile gametes (Figure 6D). Based on chitin secretion and ability to generate polyps, it appeared that the polyp body column had transformed into stolons rather than regenerated new stolons from the aboral stump.

To characterize the molecular events associated with the transformation of polyps to stolons we first studied the expression of *Wnt3* during this process. *Wnt3* is an established oral marker in *Hydractinia* (Müller et al., 2007, Plickert et al., 2006, Duffy et al., 2010), but is also expressed weakly in the i-cell band of polyps (Plickert et al., 2006). We found that polyp heads that were losing their tentacles had also lost *Wnt3* expression (Figure 6E). By contrast, *Wnt3* mRNA reappeared in the oral tip of new polyps budding from this newly transformed tissue (Figure 6F). The transformed polyp expressed the gene in a more ubiquitous fashion (see below). Hence, loss of oral *Wnt3* expression preceded, or accompanied, the loss of head characteristics such as tentacles and mouth.

To further analyze the polarity of polyps that transformed to stolons we performed *in situ* hybridization using i-cell marker cRNA probes. In normal, non-regenerating polyps, i-cells were largely restricted to the band area at the polyp lower body column (Figure 4A). In stolons, by contrast, i-cells were equally distributed in the epidermal interstices along the stolon flanks (*Figure 6-figure supplement 1*). Hence, we hypothesized that a polyp to stolon transformation should be reflected by a spatial change in i-cell marker expression from restricted band-like to ubiquitous. Indeed, *in*

situ hybridization of *Piwi1, Vasa, Pl10, Myc2* and *Ncol1* on isolated polyps that lost head structures showed a stolon-like expression pattern of these genes (Figure 6G; *Figure 6-figure supplement 2*).

To summarize this point, *Hydractinia* aboral regeneration is not direct and proceeds through three stages: First, loss of anterior-posterior polarity; second, full transformation of the polyp into a stolon; third budding new polyps and regaining oral-aboral polarity. Hence, *Hydractinia* polyps can regenerate a head through i-cell migration and blastema formation, but they cannot directly regenerate stolons; they can transform into stolonal tissue instead.

Discussion

We have studied both head and stolon regeneration in the cnidarian *Hydractinia.* Decapitation was followed by rapid wound healing that primarily involved stretching of epithelial cells without the requirement for cell proliferation (*Figure 3-figure supplement 1*). Thereafter, we monitored the migration of stem cells (i-cells) from their normal position in the band area at the lower polyp body column to the prospective head, and their proliferation to form a head blastema. Of note, our data show that not all i-cells migrate to the stump, consistent with migratory vs non-migratory i-cell sub-populations. New head structures developed within 2-3 days, after which most i-cells disappeared from the head area and resumed their normal position in the band. Gamma irradiation abolished blastema formation and regeneration altogether. By contrast, individual knock down of each one of the i-cell genes *Piwi1, Vasa* and *Pl10,* and the early nematogenesis marker *Ncol1* did not prevent blastema formation, but did inhibit regeneration. Hence, the role of these

394 genes might be related to the ability of i-cells to differentiate rather than to keeping
395 them undifferentiated. Similar results were obtained with *Smedwi2* (a *Piwi*
396 homologue) knockdown in planarians (Reddien et al., 2005), but knocking out a
397 different stem cell gene, *Sox2*, in axolotl inhibits proliferation of neural progenitors
398 (Fei et al., 2014).
399
400 A common view in the literature has been that cnidarians can regenerate through
401 morphallaxis, i.e. without contribution from cell proliferation (Park et al., 1970,
402 Holstein et al., 1991, Marcum and Campbell, 1978b, Marcum and Campbell, 1978a).
403 More recent studies conducted on *Hydra* and on the sea anemone *Nematostella*
404 *vectensis*, however, have shown that cell proliferation accompanies the regeneration
405 of cnidarian heads under normal circumstances, but the necessity of proliferation was
406 only demonstrated in *Nematostella* decapitation and *Hydra* bisection (Miljkovic-
407 Licina et al., 2007, Chera et al., 2009, DuBuc et al., 2014, Passamaneck and
408 Martindale, 2012). Our results support the new emerging view on head regeneration
409 in the Cnidaria and are consistent with Passamaneck & Martindale's hypothesis
410 (Passamaneck and Martindale, 2012) that *Hydra's* ability to regenerate a head in the
411 absence of cell proliferation is evolutionarily derived within this phylum. This
412 scenario suggests that blastema formation is an evolutionarily primitive hallmark of
413 distal regeneration in animals.
414
415 Isolated polyps were unable to directly regenerate stolons from their aboral end like
416 they do following decapitation from the oral end, consistent with previous studies
417 (Putnam Hazen, 1902, Müller et al., 1986, Duffy et al., 2010), and no blastema
418 formed at the aboral stump following removal of the stolons (*Figure 3-figure*

419 *supplement 1*). Instead of regenerating stolons, isolated polyps lost oral-aboral
420 polarity, and polyp identity altogether, and transformed into stolons. This process
421 lasted for many weeks, thereby demonstrating their remarkable growth plasticity.
422 Polyp to stolon transformation was preceded by loss of oral *Wnt3* expression and oral-
423 aboral polarity, and acquisition of a ubiquitous, stolon-like distribution of i-cells, as
424 opposed to the band like distribution typical of polyps. The newly transformed stolons
425 budded new polyps and became fully functional, sexually mature colonies. These
426 data, therefore, show that *Hydractinia* polyps possess tissue pluripotency. For now,
427 however, we cannot discriminate between the scenarios of pluripotent i-cells versus
428 several self-renewing, but lineage restricted, progenitors. So why do polyps not
429 directly regenerate stolons? We suggest that tissue polarity along the oral-aboral axis
430 prevents direct stolon regeneration. In a previous study, it has been shown that Wnt
431 signaling promotes oral structures in *Hydractinia*, but represses stolons (Duffy et al.,
432 2010). Downregulation of *Wnt3* or *Tcf* in decapitated polyps induces phenotypes
433 reminiscent of polyp to stolon transformation in the present study, but require shorter
434 time to develop (Duffy et al., 2010). We show that in the absence of experimental
435 manipulation, *Wnt3* expression and oral-aboral polarity are lost spontaneously in
436 isolated polyps, enabling the transformation of the polyp into stolonal tissue that can
437 bud new polyps. Possibly, *Wnt3* not only maintains oral- but also polyp- identity, and
438 stolons develop by default in its absence. A summary of the two distinct regenerative
439 processes in *Hydractinia* is schematically illustrated on Figure 7.
440
441 In conclusion, our results, and results published over the past few years by others
442 (Chera et al., 2009, Kragl et al., 2009, Sandoval-Guzman et al., 2014, DuBuc et al.,
443 2014), show that the mechanisms governing animal regeneration can be not only

species-specific, but also tissue-specific within a single species. Some regeneration mechanisms, like blastema formation, are conserved in animals, and their modulation over evolutionary times may have affected the regenerative ability of different species. An exciting development in the study of regeneration is provided by the ability to track individual, transgenic cells *in vivo* using *Hydractinia* as an animal model. *In vivo* cell migration assays have been performed in *Hydra* (Khalturin et al., 2007), but the sessile nature of adult *Hydractinia* facilitates long-term studies at single cell resolution.

Materials and Methods

Animal culture

Colonies of *Hydractinia echinata* were cultured in artificial seawater at 18ºC under 14/10 light/dark regime. They were fed brine shrimp nauplii four times a week and ground fish once a week.

Animal maceration

Animals were anesthetized for 30 minutes in 4% MgCl in seawater. They were then placed in a Glycerin/Acetic acid/Seawater (1:1:13) solution for 10 minutes, followed by incubation in Glycerin/Acetic acid/Distilled Water (1:1:13) for two hours. They were then pipetted up and down to complete maceration and fixed in 8% formaldehyde for 30 minutes. Cells were spread on a glass slide and dried overnight. Cell counting of macerated cells was performed by eye using a FV1000 Olympus confocal scanning laser microscope.

EdU labeling

EdU incorporation was performed for 20-60 minutes at a concentration of 150 μM. For visualization, animals were fixed and processed using the Click-iT® EdU Alexa Fluor® 488 Imaging Kit (Life Technologies) according to the manufacturer's protocol.

Gamma irradiation

Gamma-irradiation was carried out using a ^{137}Cs source at a dose-rate of 12 Gy/min.

TUNEL

TUNEL staining was complete as per the manufacture's protocol (Life Technologies). Click-iT® TUNEL Alexa Fluor® 488 Imaging Assay, Cat: 10245

Immunohistochemistry

Animals were fixed in 4% paraformaldehyde in PBS for 60 minutes and then washed three times in PBS 0.3% Triton (PBST) and blocked for 30 minutes in 2% BSA/PBST. Primary antibodies (anti-Hiwi (a kind gift from Dr Celina Juliano, Yale University), anti acetylated tubulin (T7451, Sigma), anti-phospho H3 (ab5176 Abcam)) were diluted 1:500-1:2000 in BSA/PBST and incubated overnight at 4ºC, followed by three washes with PBST then blocked for 30 minutes in 5% serum in BSA/PBST. Secondary antibodies (Alexa Fluor 488 goat anti-rabbit IgG (A-11008, Invitrogen), Alexa Fluor® 546 Phalloidin LifeTec (A22283)) were diluted 1:500 in BSA/PBST/serum and incubated for 1 hour at room temperature. Animals were washed three times with PBST, incubated in 1:2000 Hoescht (20 mg/ml), DAPI (1:5000) or phalloidin (1:2000) in PBST and washed a further three times in PBST. Animals were mounted in mounting medium (F4680, Sigma).

494

495 ***In situ* hybridization**

496 *In situ* hybridization was performed as previously described (Gajewski et al., 1996).
497 Templates for DIG-labeled RNA probes (Roche) were generated by PCR. RNA
498 synthesis was performed by SP6 and T7 RNA polymerases according to the
499 manufacturer's protocol (Fermentas). The sequence of the oligonucleotides is given in
500 Table 1. *In situ* hybridization was performed at 55ºC.

501

502 ***In vivo* microscopy**

503 Animals were embedded in Ibidi μ-dish (#81156) using 1% low-melt agarose in
504 seawater. They were observed using either a fluorescence stereomicroscope,
505 DeltaVision deconvolution microscope, or Andor spinning disc confocal microscope.
506 For time-lapse movies, images or stacks were taken every five minutes.

507

508 **RNAi**

509 RNAi was performed as previously described (Duffy et al., 2010, Millane et al., 2011,
510 Duffy, 2012). Templates for RNA synthesis were generated by PCR (see
511 oligonucleotide list on Table 1). Sense and antisense RNA strands were generated as
512 for *in situ* hybridization but were annealed by heating them together to 70ºC and
513 allowing them to cool down at room temperature. Animals were incubated in seawater
514 to which dsRNA at 20-40 μg/ml was added directly after decapitation. The
515 experiments run until the control animals had regenerated (usually between 3-5 days).
516 dsRNA solution was replaced every 24 hours.

517

518 **Quantitative, real-time PCR**

mRNA was extracted using standard Trizol/chloroform extraction technique and cleaned over RNeasy minikit (QIAGEN 74104) according to the manufacturer's protocol. RNA was reverse transcribed using Omniscript RT kit (QIAGEN 205110). qPCR was run on a StepOne Plus (Life Technologies) using TaqMan chemistry. Experiments were performed on three colonies using three technical replicates for each.

Generating transgenic animals

We cloned the genomic regions 2.5 kb upstream and 1.1 kb downstream of the *Hydractinia Piwi1* coding sequence into a modified pBluescript backbone (Künzel et al., 2010) and replaced the coding sequence by *GFP*. (*Figure 5-figure supplement 2*). One-cell stage embryos were microinjected with 200 pl volume of the plasmid at a concentration of 4–5 µg/µl as previously described (Künzel et al., 2010, Millane et al., 2011, Duffy, 2012, Kanska and Frank, 2013).

ACKNOWLEDGEMENTS

We thank members of our lab for assistance and discussions. Anti-hydra Piwi (Hywi) antibodies were a kind gift from Celina Juliano, Yale University. Elaine Dunleavy (Centre for Chromosome Biology, NUIG) is kindly acknowledged for assistance with DeltaVision microscopy.

References

ADLER, C. E., SEIDEL, C. W., MCKINNEY, S. A. & SANCHEZ ALVARADO, A. 2014. Selective amputation of the pharynx identifies a FoxA-dependent regeneration program in planaria. *eLife,* 3**,** e02238-e02238.

ALIE, A., LECLERE, L., JAGER, M., DAYRAUD, C., CHANG, P., LE GUYADER, H., QUEINNEC, E. & MANUEL, M. 2011. Somatic stem cells express Piwi and Vasa genes in an adult ctenophore: ancient association of "germline genes" with stemness. *Developmental Biology,* 350**,** 183-97.

BAGUNA, J. 2012. The planarian neoblast: the rambling history of its origin and some current black boxes. *Int J Dev Biol,* 56**,** 19-37.

BELY, A. E. 2014. Early Events in Annelid Regeneration: A Cellular Perspective. *Integr Comp Biol*.

BODE, H. R. 1996. The interstitial cell lineage of hydra: a stem cell system that arose early in evolution. *J Cell Sci,* 109 (Pt 6)**,** 1155-64.

CANDIA CARNEVALI, M. D. 2006. Regeneration in Echinoderms: repair, regrowth, cloning. *Invert. Surv. J,* 3**,** 64-76.

CHERA, S., GHILA, L., DOBRETZ, K., WENGER, Y., BAUER, C., BUZGARIU, W., MARTINOU, J.-C. & GALLIOT, B. 2009. Apoptotic Cells Provide an Unexpected Source of Wnt3 Signaling to Drive *Hydra* Head Regeneration. *Dev Cell,* 17**,** 279-289.

COLLINS, J. J., 3RD, WANG, B., LAMBRUS, B. G., THARP, M. E., IYER, H. & NEWMARK, P. A. 2013. Adult somatic stem cells in the human parasite Schistosoma mansoni. *Nature,* 494**,** 476-9.

CUMMINGS, S. & BODE, H. 1984. Head regeneration and polarity reversal in *Hydra* attenuata can occur in the absence of DNA synthesis. *Wilhelm Roux's archives of developmental biology,* 194**,** 79-86.

DAVID, C. N. 1973. A quantitative method for maceration of hydra tissue. *Development Genes and Evolution,* 171**,** 259-268.

DAVID, C. N., OZBEK, S., ADAMCZYK, P., MEIER, S., PAULY, B., CHAPMAN, J., HWANG, J. S., GOJOBORI, T. & HOLSTEIN, T. W. 2008. Evolution of complex structures: minicollagens shape the cnidarian nematocyst. *Trends Genet,* 24**,** 431-8.

DÜBEL, S. & SCHALLER, H. C. 1990. Terminal differentiation of ectodermal epithelial stem cells of Hydra can occur in G2 without requiring mitosis or S phase. *The Journal of Cell Biology,* 110**,** 939-945.

DUBUC, T. Q., TRAYLOR-KNOWLES, N. & MARTINDALE, M. Q. 2014. Initiating a regenerative response; cellular and molecular features of wound healing in the cnidarian Nematostella vectensis. *BMC Biol,* 12**,** 24.

DUFFY, D. J. 2012. Instructive reconstruction: A new role for apoptosis in pattern formation: Instructive apoptotic patterning establishes de novo tissue generation via the apoptosis linked production of morphogenic signals. *BioEssays : news and reviews in molecular, cellular and developmental biology*.

DUFFY, D. J., MILLANE, R. C. & FRANK, U. 2011. A heat shock protein and Wnt signaling crosstalk during axial patterning and stem cell proliferation. *Developmental Biology,* 362**,** 271-281.

DUFFY, D. J., PLICKERT, G., KÜNZEL, T., TILMANN, W. & FRANK, U. 2010. Wnt signaling promotes oral but suppresses aboral structures in *Hydractinia* metamorphosis and regeneration. *Development,* 137**,** 3057-3066.

FEI, J. F., SCHUEZ, M., TAZAKI, A., TANIGUCHI, Y., ROENSCH, K. & TANAKA, E. M. 2014. CRISPR-Mediated Genomic Deletion of Sox2 in the Axolotl Shows a Requirement in Spinal Cord Neural Stem Cell Amplification during Tail Regeneration. *Stem Cell Reports,* 3**,** 444-59.

GAJEWSKI, M., LEITZ, T., SCHLOSSHERR, J. & PLICKERT, G. 1996. LWamides from Cnidaria constitute a novel family of neuropeptide with morphogenetic ativity. *Roux's Arch Dev Biol,* 205**,** 232-242.

GALLIOT, B. & GHILA, L. 2010. Cell plasticity in homeostasis and regeneration. *Mol Reprod Dev,* 77**,** 837-55.

HOLSTEIN, T. W., HOBMAYER, E. & DAVID, C. N. 1991. Pattern of epithelial cell cycling in hydra. *Dev Biol,* 148**,** 602-11.

HWANG, J. S., KOBAYASHI, C., AGATA, K., IKEO, K. & GOJOBORI, T. 2004. Detection of apoptosis during planarian regeneration by the expression of apoptosis-related genes and TUNEL assay. *Gene,* 333**,** 15-25.

JULIANO, C. E., REICH, A., LIU, N., GOTZFRIED, J., ZHONG, M., UMAN, S., REENAN, R. A., WESSEL, G. M., STEELE, R. E. & LIN, H. 2014. PIWI proteins and PIWI-interacting RNAs function in Hydra somatic stem cells. *Proc Natl Acad Sci U S A,* 111**,** 337-42.

KANSKA, J. & FRANK, U. 2013. New roles for Nanos in neural cell fate determination revealed by studies in a cnidarian. *J Cell Sci,* 126**,** 3192-3203.

KHALTURIN, K., ANTON-ERXLEBEN, F., MILDE, S., PLOTZ, C., WITTLIEB, J., HEMMRICH, G. & BOSCH, T. C. G. 2007. Transgenic stem cells in Hydra reveal an early evolutionary origin for key elements controlling self-renewal and differentiation. *Dev Biol,* 309**,** 32-44.

KONDO, M. & AKASAKA, K. 2010. Regeneration in crinoids. *Dev Growth Differ,* 52**,** 57-68.

KRAGL, M., KNAPP, D., NACU, E., KHATTAK, S., MADEN, M., EPPERLEIN, H. H. & TANAKA, E. M. 2009. Cells keep a memory of their tissue origin during axolotl limb regeneration. *Nature,* 460**,** 60-5.

KÜNZEL, T., HEIERMANN, R., FRANK, U., MÜLLER, W. A., TILMANN, W., BAUSE, M., NONN, A., HELLING, M., SCHWARZ, R. S. & PLICKERT, G. 2010. Migration and differentiation potential of stem cells in the cnidarian *Hydractinia* analysed in GFP-transgenic animals and chimeras. *Dev Biol,* 348**,** 120-129.

LANGE, R. G., MÜLLER, W. A. 1991. SIF, a novel morphogenetic inducer in Hydrozoa. *Dev. Biol.,* 147**,** 121-132.

LIU, S. Y., SELCK, C., FRIEDRICH, B., LUTZ, R., VILA-FARRE, M., DAHL, A., BRANDL, H., LAKSHMANAPERUMAL, N., HENRY, I. & RINK, J. C. 2013. Reactivating head regrowth in a regeneration-deficient planarian species. *Nature,* 500**,** 81-4.

MARCUM, B. A. & CAMPBELL, R. D. 1978a. Development of Hydra lacking nerve and interstitial cells. *J Cell Sci,* 29**,** 17-33.

MARCUM, B. A. & CAMPBELL, R. D. 1978b. Developmental roles of epithelial and interstitial cell lineages in hydra: analysis of chimeras. *J Cell Sci,* 32**,** 233-47.

MILJKOVIC-LICINA, M., CHERA, S., GHILA, L. & GALLIOT, B. 2007. Head regeneration in wild-type hydra requires de novo neurogenesis. *Development,* 134**,** 1191-1201.

MILLANE, R. C., KANSKA, J., DUFFY, D. J., SEOIGHE, C., CUNNINGHAM, S., PLICKERT, G. & FRANK, U. 2011. Induced stem cell neoplasia in a cnidarian by ectopic expression of a POU domain transcription factor. *Development,* 138**,** 2429-2439.

MÜLLER, W. A., FRANK, U., TEO, R., MOKADY, O., GÜTTE, C. & PLICKERT, G. 2007. Wnt signaling in hydroid development: Ectopic heads and giant buds induced by GSK-3 inhibitors. *Int J Dev Biol,* 51**,** 211-220.

MÜLLER, W. A., PLICKERT, G. & BERKING, S. 1986. Regeneration in Hydrozoa: distal versus proximal transformation in *Hydractinia*. *Roux's Arch. Dev. Biol.,* 195**,** 513-518.

MÜLLER, W. A., TEO, R. & FRANK, U. 2004. Totipotent migratory stem cells in a hydroid. *Dev Biol,* 275**,** 215-224.

PARK, H. D., ORTMEYER, A. B. & BLANKENBAKER, D. P. 1970. Cell division during regeneration in Hydra. *Nature,* 227**,** 617-9.

PASSAMANECK, Y. J. & MARTINDALE, M. Q. 2012. Cell proliferation is necessary for the regeneration of oral structures in the anthozoan cnidarian Nematostella vectensis. *BMC Dev Biol,* 12**,** 34.

PLICKERT, G., FRANK, U. & MULLER, W. A. 2012. Hydractinia, a pioneering model for stem cell biology and reprogramming somatic cells to pluripotency. *Int J Dev Biol,* 56**,** 519-534.

PLICKERT, G., JACOBY, V., FRANK, U., MÜLLER, W. A. & MOKADY, O. 2006. Wnt signaling in hydroid development: Formation of the primary body axis in embryogenesis and its subsequent patterning. *Developmental Biology,* 298**,** 368-378.

PUTNAM HAZEN, A. 1902. Regeneration of *Hydractinia* and *Podocoryne*. *American Naturalist,* 36**,** 193-200.

REBSCHER, N., VOLK, C., TEO, R. & PLICKERT, G. 2008. The germ plasm component Vasa allows tracing of the interstitial stem cells in the cnidarian *Hydractinia echinata*. *Dev Dyn,* 237**,** 1736-1745.

REDDIEN, P. W. 2013. Specialized progenitors and regeneration. *Development,* 140**,** 951-957.

REDDIEN, P. W., OVIEDO, N. J., JENNINGS, J. R., JENKIN, J. C. & SANCHEZ ALVARADO, A. 2005. SMEDWI-2 Is a PIWI-Like Protein That Regulates Planarian Stem Cells. *Science,* 310**,** 1327-1330.

REDDIEN, P. W. & SANCHEZ ALVARADO, A. 2004. Fundamentals of planarian regeneration. *Annu Rev Cell Dev Biol,* 20**,** 725-757.

SANCHEZ ALVARADO, A. 2000. Regeneration in the metazoans: why does it happen? *Bioessays,* 22**,** 578-90.

SANCHEZ ALVARADO, A. & TSONIS, P. A. 2006. Bridging the regeneration gap: genetic insights from diverse animal models. *Nat Rev Genet,* 7**,** 873-84.

SANDOVAL-GUZMAN, T., WANG, H., KHATTAK, S., SCHUEZ, M., ROENSCH, K., NACU, E., TAZAKI, A., JOVEN, A., TANAKA, E. M. & SIMON, A. 2014. Fundamental Differences in Dedifferentiation and Stem Cell Recruitment during Skeletal Muscle Regeneration in Two Salamander Species. *Cell Stem Cell*.

SIKES, J. M. & NEWMARK, P. A. 2013. Restoration of anterior regeneration in a planarian with limited regenerative ability. *Nature,* 500**,** 77-80.

TANAKA, E. M. & REDDIEN, P. W. 2011. The cellular basis for animal regeneration. *Developmental Cell,* 21**,** 172-85.

TSENG, A. S., ADAMS, D. S., QIU, D., KOUSTUBHAN, P. & LEVIN, M. 2007. Apoptosis is required during early stages of tail regeneration in Xenopus laevis. *Dev Biol,* 301**,** 62-9.

UMESONO, Y., TASAKI, J., NISHIMURA, Y., HROUDA, M., KAWAGUCHI, E., YAZAWA, S., NISHIMURA, O., HOSODA, K., INOUE, T. & AGATA, K. 2013. The molecular logic for planarian regeneration along the anterior-posterior axis. *Nature,* 500**,** 73-6.

VOSKOBOYNIK, A., SOEN, Y., RINKEVICH, Y., ROSNER, A., UENO, H., RESHEF, R., ISHIZUKA, K. J., PALMERI, K. J., MOISEEVA, E., RINKEVICH, B. & WEISSMAN, I. L. 2008. Identification of the endostyle as a stem cell niche in a colonial chordate. *Cell Stem Cell,* 3**,** 456-64.

Figure legends

Figure 1. *Hydractinia* life cycle and colony structure. Scale bar 200 µm

Figure 2. Head regeneration in *Hydractinia*. (A) Experimental setup. (B) Live images of regenerating polyp. Scale bar 100 µm. (C-F) Anti acetylated tubulin (green) - phalloidin (red) – DAPI (blue) staining of regenerating polyp. Asterisks are depicted at approximately the same position in each panel. (C) Intact polyp. (D) Four hpd. (E) Twenty four hpd. (F) Seventy two hpd. Nem = nematocyte; Neur = neuron. Scale bars 100 µm.

Figure 3. Cell proliferation during head regeneration. (A) EdU (upper panes) and pH3 (lower panes) labeling of cells. Scale bar 200 µm. (B) Maceration of animals and labeling of cycling cells. Scale bar 10 µm. (C) Percentages of epithelial and i-cells out of the total mitotic cells. (D) Effect of gamma irradiation on cell proliferation and regeneration. (E) Effect of irradiation on nervous system regeneration. Green, anti-acetylated tubulin; red, phalloidin; blue, DAPI.

Figure 3-figure supplement 1. EdU labeling of polyps. (A) S-phase cells during wound closure. (B) S-phase cells in a polyps connected to a colony. (C) S-phase cells in a polyp 24 hours post isolation. Scale bars 200 µm.

Figure 3-figure supplement 2. A selection of dissociated *Hydractinia* cells.

Figure 3-figure supplement 3. Effect of different absorbed doses of gamma irradiation on head regeneration and cell proliferation.

731

732 ***Figure 3-figure supplement 4.*** TUNEL staining of control and irradiated polyps post
733 decapitation.

734

735 ***Figure 3-figure supplement 5.*** Effect of 30 μM mitomycin C on cell proliferation and
736 head regeneration.

737

738 **Figure 4.** Gene expression during head regeneration. (A) Expression of i-cell marker
739 genes in intact polyps. Scale bar 200 μm. (B) Higher magnification of positive cells in
740 the band. Scale bar 10 μm. (C) Expression of marker genes 24 hours post
741 decapitation. (D) Higher magnification of blastema cells expressing i-cell marker
742 genes. (E) Effect of gamma irradiation on i-cell marker gene expression. (F, G)
743 Downregulation of marker genes during regeneration by RNAi. (F) Effect on
744 regeneration and quantitative analysis of knockdown. (G) Effect on cell proliferation.

745

746 ***Figure 4-figure supplement 1.*** Effect of histones H2A and H4 knockdown and
747 quantification of *Piwi1* mRNA following RNAi knockdown. (A) Control RANi. (B)
748 Histone H2A RNAi. (C) Histone H4 RNAi. (D) Relative quantity (RQ) of *Piwi1*
749 expression in control vs *Piwi1* RNAi. Expression levels are normalized to 18S rRNA.
750 Error bars = SD.

751

752 **Figure 5.** The cellular source for head regeneration. (A, B) EdU pulse-chase. (A) Six
753 hpd – most EdU+ cells restricted to the band. Scale bar 50 μm. (B) Twenty four hpd –
754 many EdU+ cells migrated to the blastema. (C-G) Live images of *Piwi1*-GFP+
755 transgenic cells. (C) Transgenic *Piwi1*-GFP+ polyp. Scale bar 200 μm. (D) Higher

magnification of live GFP+ i-cells in vivo. Scale bar 10 μm. (E) Live transgenic polyp pictured at 10 (left) and 24 (right) hpd. (F) Live images of a single, *Piwi1*-GFP+ i-cell migrating to the forming blastema (arrow). (G) Live image of a single, *Piwi1*-GFP+ i-cell dividing during migration (encircled).

Figure 5-figure supplement 1. Proliferation of migrating cells. Animals were EdU pulse labeled for 60 minutes and fixed either intact or at different time points post decapitation. They were then stained with anti-pH3 antibody. (A-D) Intact animals. (E-H) 24 hpd. (I-P) 48 hpd. (A-L) represent projections of multiple confocal stacks; (M-P) are single confocal sections. Scale bars 100 μm (A-L) and 10 μm (M-P).

Figure 5-figure supplement 2. The structure of the construct used to generate transgenic, *Piwi1*-GFP+ animals.

Figure 5-figure supplement 3. Piwi1 immunohistochemistry and co-localization of gene expression and S-phase cells. (A) Co-staining of anti-Piwi1 antibody staining (green) and DAPI (blue). Scale bar 200 μm. (B) Co-staining of anti-Piwi1 antibody staining (green) and EdU (red) Scale bar 20 μm. (C) Co labeling of *Ncol1* (blue) and EdU (green) Scale bar 20 μm.

Video 1. Follow up of individual cells migrating to forming blastema.

Video 2. Follow up on proliferating cell migrating to blastema.

Figure 6. Stolon regeneration. (A) Time course of a single polyp transforming into a

stolon and budding new polyps. Scale bar 200 μm. (B) Sexually mature colonies derived from an isolated polyp. (C) Chitin secretion (arrow) by a polyp that has transformed into a stolon. (D) Early embryos spawned by colony derived from a single polyp. (E) loss of oral *Wnt3* in polyp transforming into a stolon. (F) Oral *Wnt3* expression in newly bud polyps (arrows). (G) Stolon-like expression of i-cell markers in transformed polyps.

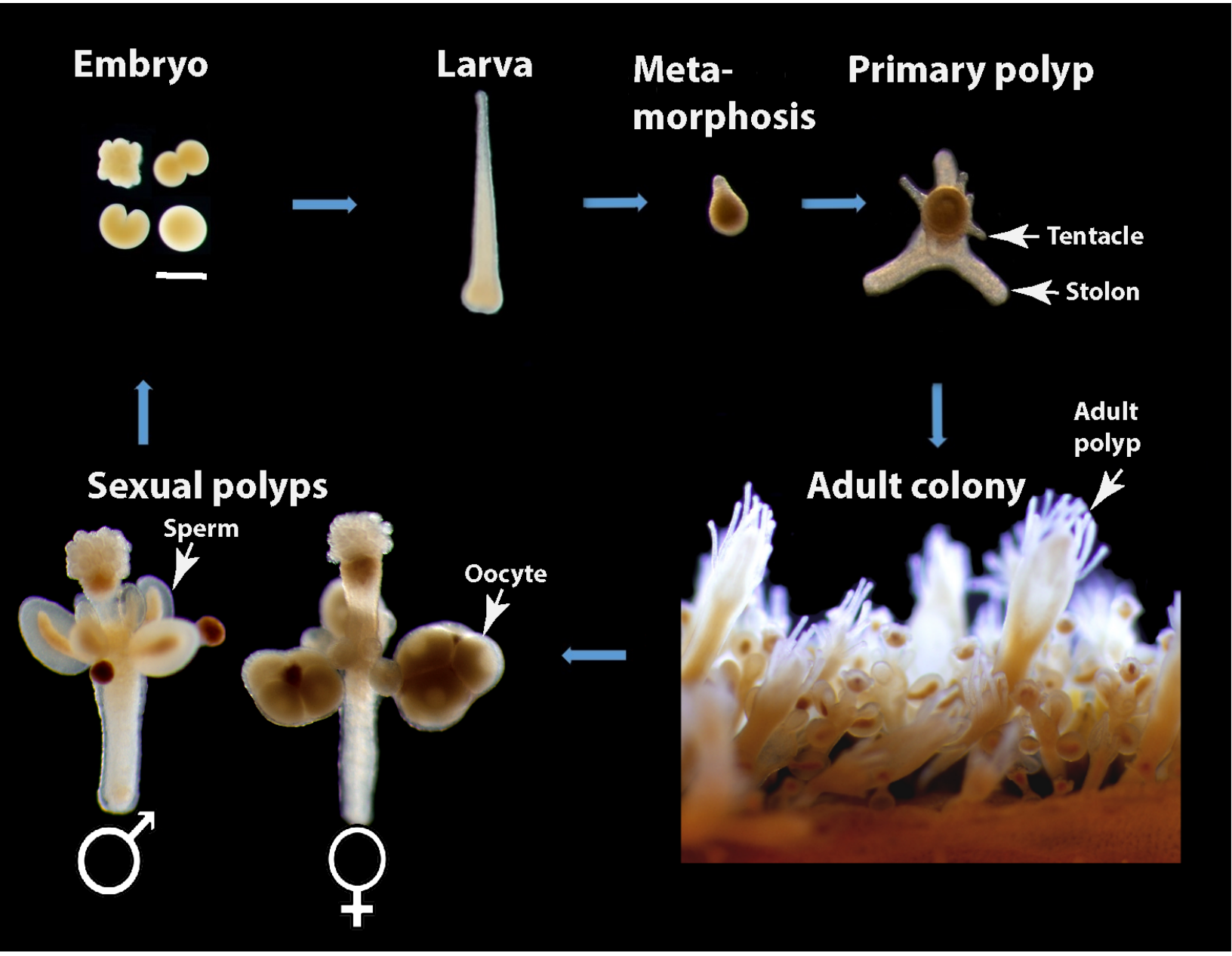
Figure 6-figure supplement 1. Expression of i-cell markers in stolons and *Wnt3* in a primary polyp. Colonies are growing on glass cover slips and images are taken from below, except of *Wnt3* that was taken from above. Scale bar 100 μm.

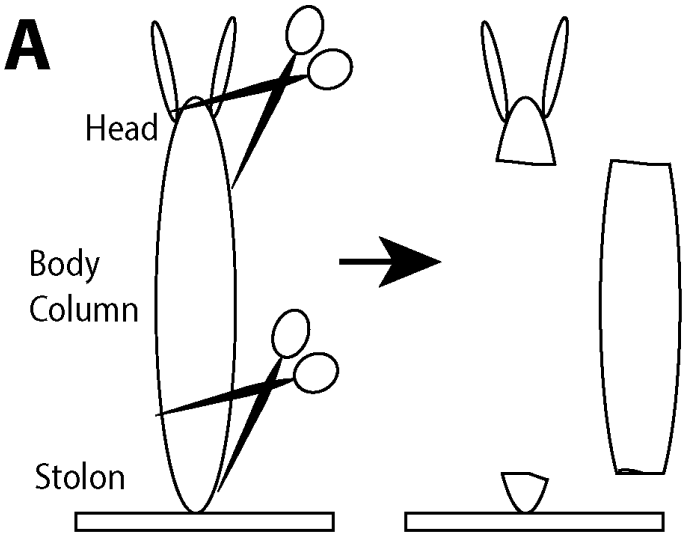
Figure 6-figure supplement 2. Expression of i-cell and nematogenesis markers in polyps that had transformed into stolons.

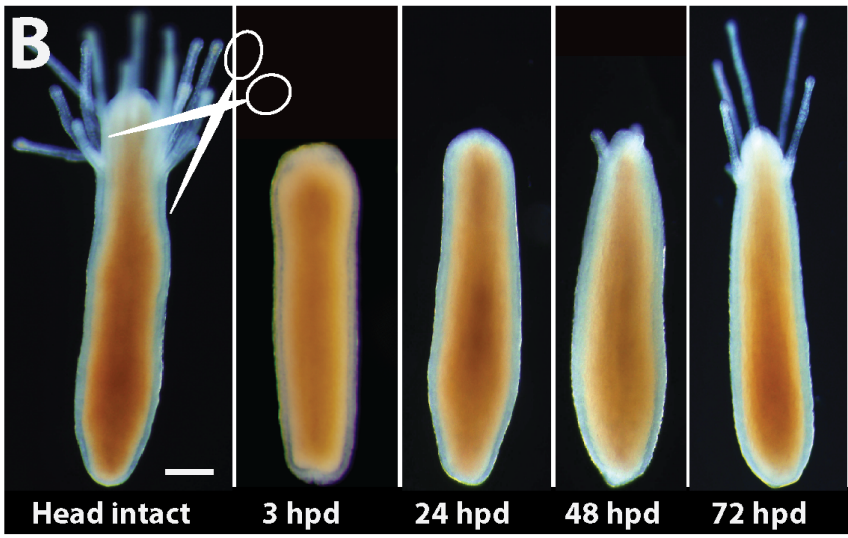
Figure 7. A summary of *Hydractinia* regeneration. Red dots represent proliferating i-cells. (A) A schematic of a normal colony including stolons, feeding and sexual polyps. (B) Head regeneration. Isolation of a polyp from the colony and its decapitation result in migration of i-cells to the head stump but not to the stolon stump. A head blastema, but not stolon blastema, is formed and provides the progenitors for the new head. (C) Transformation of polyps to stolons involves loss of polarity and ubiquitous spread of i-cells.

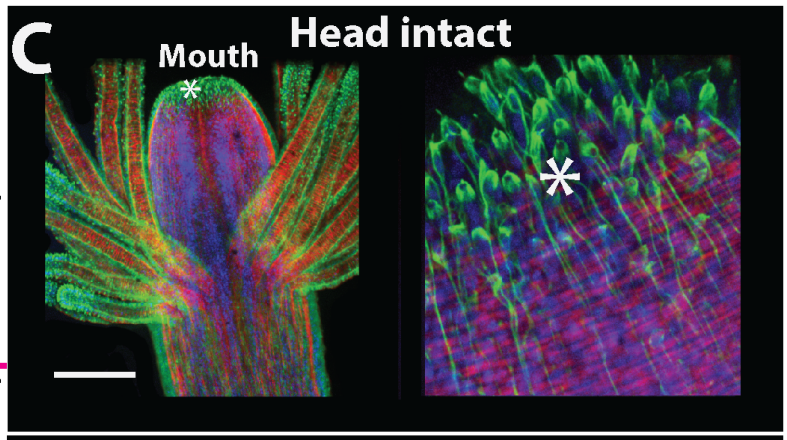
Table 1. Oligonucleotides used in this study

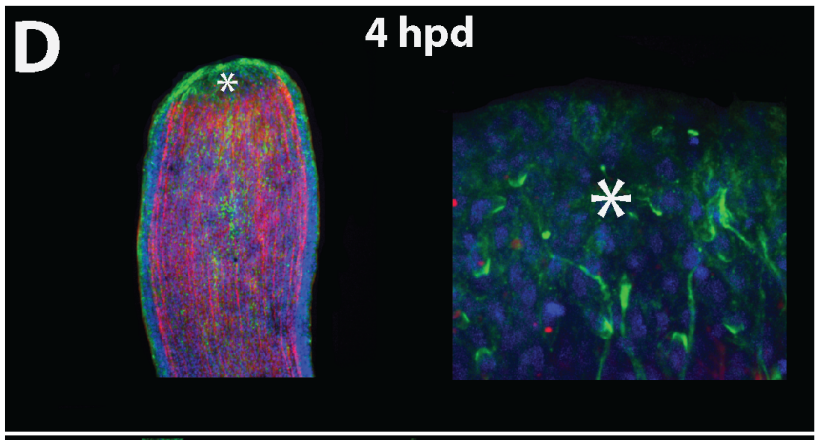
Target	Accession	Primer name	Primer sequence
Piwi1	JG772275.1	CniwiFort7	5′-gatcataatacgactcactatagggagagttgatttcacaatcggttagac-3′
		CniwiRevSp6	5′-agtgcatttaggtgacactatagaagtgtactactactactactggttattt-3′
		PiwiIRNAiT7Fw	5′-gcgtaatacgactcactatagggagagctgtgtggaaagaccagtc-3′
		PiwiIRNAiSP6Rv	5′–tgcatttaggtgacactatagaagtgcgtcaaatccaatcaccatc-3′
		Piwi1qPCRfw	5'-aagtatggcctggcatctca-3'
		Piwi1qPCRrv	5'-cactgtctgctgtcgtaaaacc-3'
		Piwi1qPCRprobe	5'-tgcagtatgaacaagatgtgatgttgtgtgctgatgttcag-3'
Pl10	AB048849.1	Pl10ForT7	5 –gatcataatacgactcactatagggagattctggcaaaacagctgcattt-3'
		Pl10RevSP6	5 -agtgcatttaggtgacactatagaagtgagcttcatccagacaaagaaac-3'
		Pl10rnaiFWT7	5'-gcgtaatacgactcactatagggagagcgtaacacccattttg-3'
		PL10rnaiRVsp6	5'-tgcatttaggtgacactatagaagtgtaaatcacgcgca-3'
Ncol1	JX486117.1	Ncol1-T7fwd	5'–gatcataatacgactcactatagggacgtccaggaccaccaggagta-3'
		Ncol1-Sp6rev	5'–tagcaatttaggtgacactatagaactgggcaacagtattgtggacaaga-3'
Vasa	EF467228.1	HeVASAforT7	5'–taatacgactcactatagggagaaggttcaaagtggttgccattt-3'
		HeVASArevSP6	5'–atttaggtgacactatagaagagtactgccaactttaccaat-3'
		VasaRNAiFWT7	5'–gcgtaatacgactcactatagggagagttgaaatgctgggacaagaagg-3'
		VasaRNAiRVsp6	5'–tgcatttaggtgacactatagaagtggcggtagcgataagaacagtc-3'
Wnt3	AM279678.1	Wnt3ForwardPrimerT7	5'–gatcataatacgactcactataggggagtccgccttcattagtgg-3'
		Wnt3ReversePrimerSP6	5'–tagcaatttaggtgacactatagaatgggcggagtcgtatctatc-3'
cMyc	JF820068.1	cMycInSituFWt7	5'–gatcataatacgactcactatagggcctttaacgcctcccagttct-3'
H2A		H2aRNAiFwd1T7	5'-gatcataatacgactcactatagggatgtctggacgtggaaaagg-3'
		H2aRNAiRv1SP6	5'-tagcaatttaggtgacactatagaaccaatatctcagcagataaatattccaag-3'
		H2aRNAiFwd2T7	5'-gatcataatacgactcactataggggagttggctggtaacgcag-3'
		H2aRNAiRv2SP6	5'-tagcaatttaggtgacactatagaaacttcttctgtcctttgtcgttct-3'
H4		H4RNAiFwd1T7	5'-gatcataatacgactcactatagggatgtctggacgcggaaaag-3'
		H4RNAiRv1SP6	5'-tagcaatttaggtgacactatagaactttagtacacctctggtttcctc-3'
		H4RNAiFwd2T7	5'-gatcataatacgactcactataggggtcaaacgtatctctggccttat-3'
		H4RNAiRv2SP6	5'-tagcaatttaggtgacactatagaaaacctccgaatccgtaaagag-3'
		cMycInsituRVsp6	5'-agtgcatttaggtgacactatagaattgttaacggaaaagggaaaactg-3'
Plasmid		gfpRv-SAC1	5′-aaaaagagctcctatttgtatagttcatccatgccatg-3′
		TerminatorFw-PacI	5′-aaaaattaattaacgtacgggccctttcgtct-3′
Race		NewsplicedleaderFwd	5'-tactcacactatttctaagtccctgagtttaag-3'
		PiwiRV2SP6	5′-tagcaatttaggtgacactatagaaccttagcgccacctgtgc-3′
Cloning		LigDVectorGFP-Fusion	5'-gcggccgctgcagccccggt-3'
		BackbonelactRV1	5'-actggccgtcgttttacaac-3'
		Piwi1ProFw1new	5'-cagatgatccgcagacaatagac-3'
		Piwipromrevin	5'-gttttcttcttataatttttctaaaaactt- 3'
		PiwiRv2SP6	5′-tagcaatttaggtgacactatagaaccttagcgccacctgtgc-3′
		Piwi1TerRV1-PacI	5′-aaaaattaattaagaaggcttacgctagtgtgaattag-3′
		Piwi1TerFw1-SAC1	5′-aaaaagagctcgtagctgcgcgttgtttacg-3′
		GFPSeqFusRev	5'-ttgcatcaccttcaccctctcc- 3'
		PBIGFor	5'-taaaaataggcgtatcacgaggccc-3'

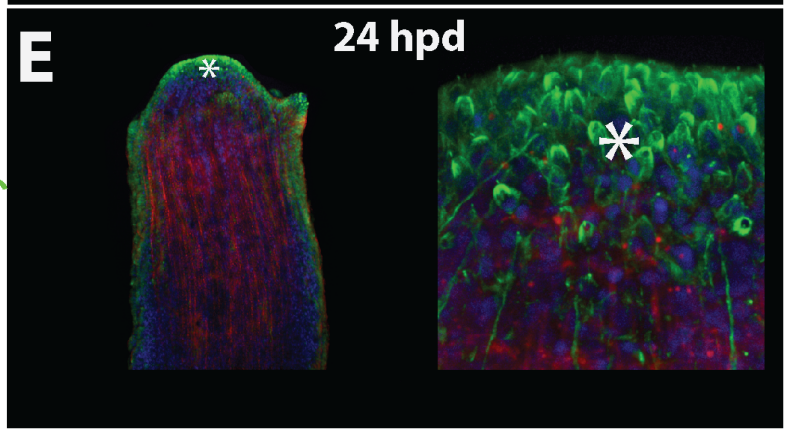

Embryo
Larva
Meta-
morphosis
Primary polyp
Tentacle
Stolon
Adult
polyp
Adult colony
Sexual polyps
Sperm
Oocyte

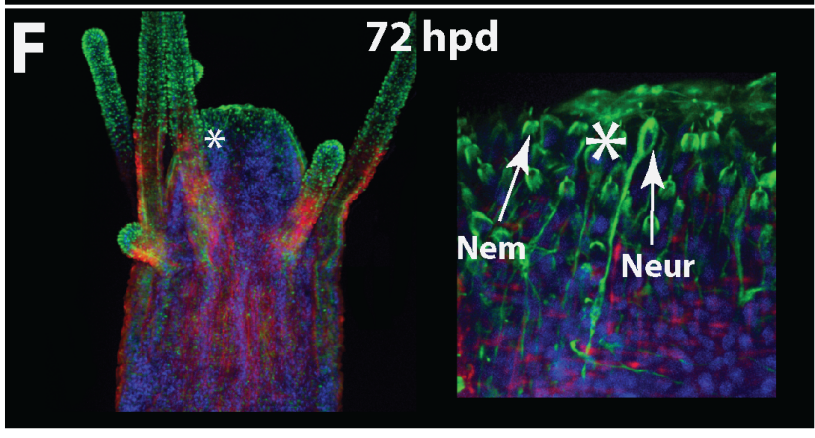




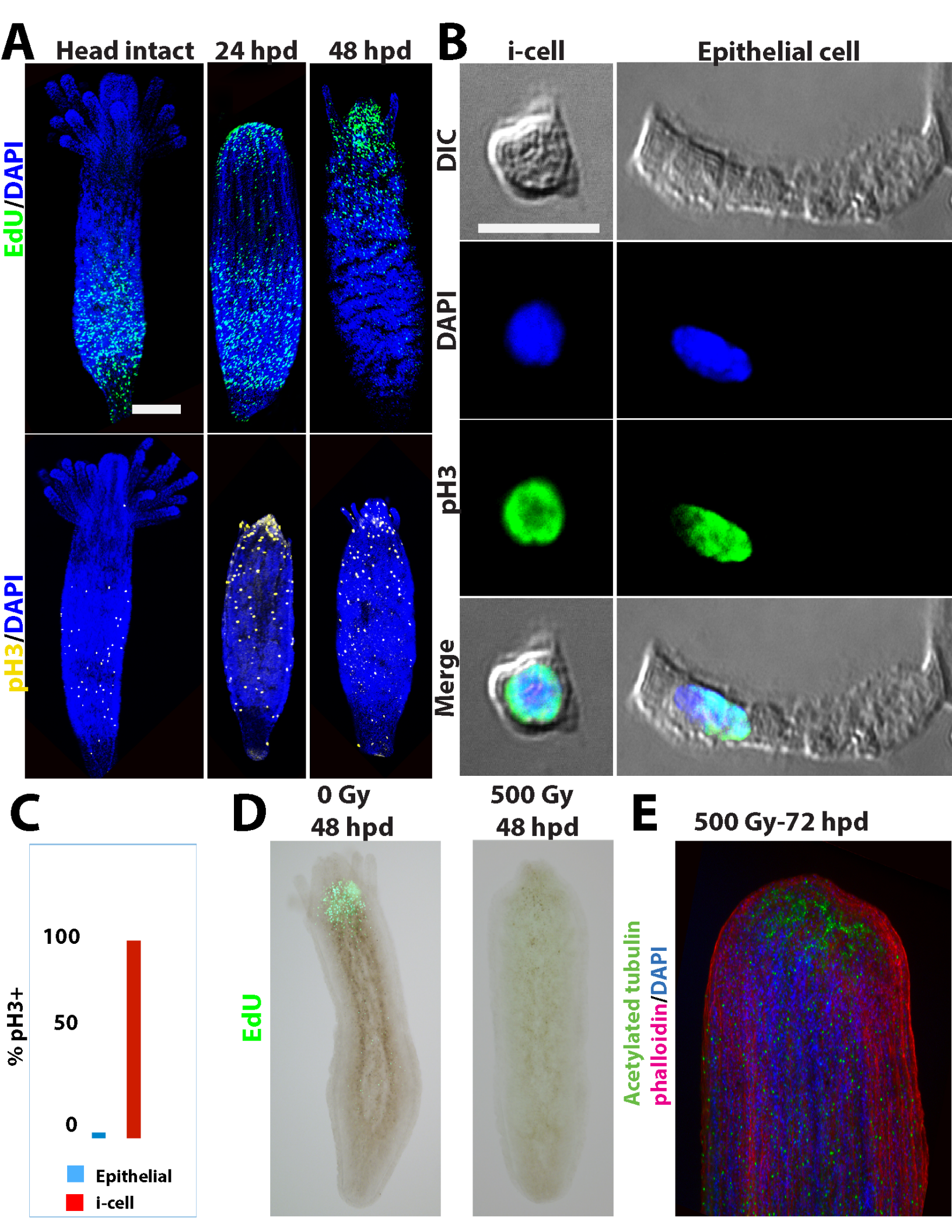


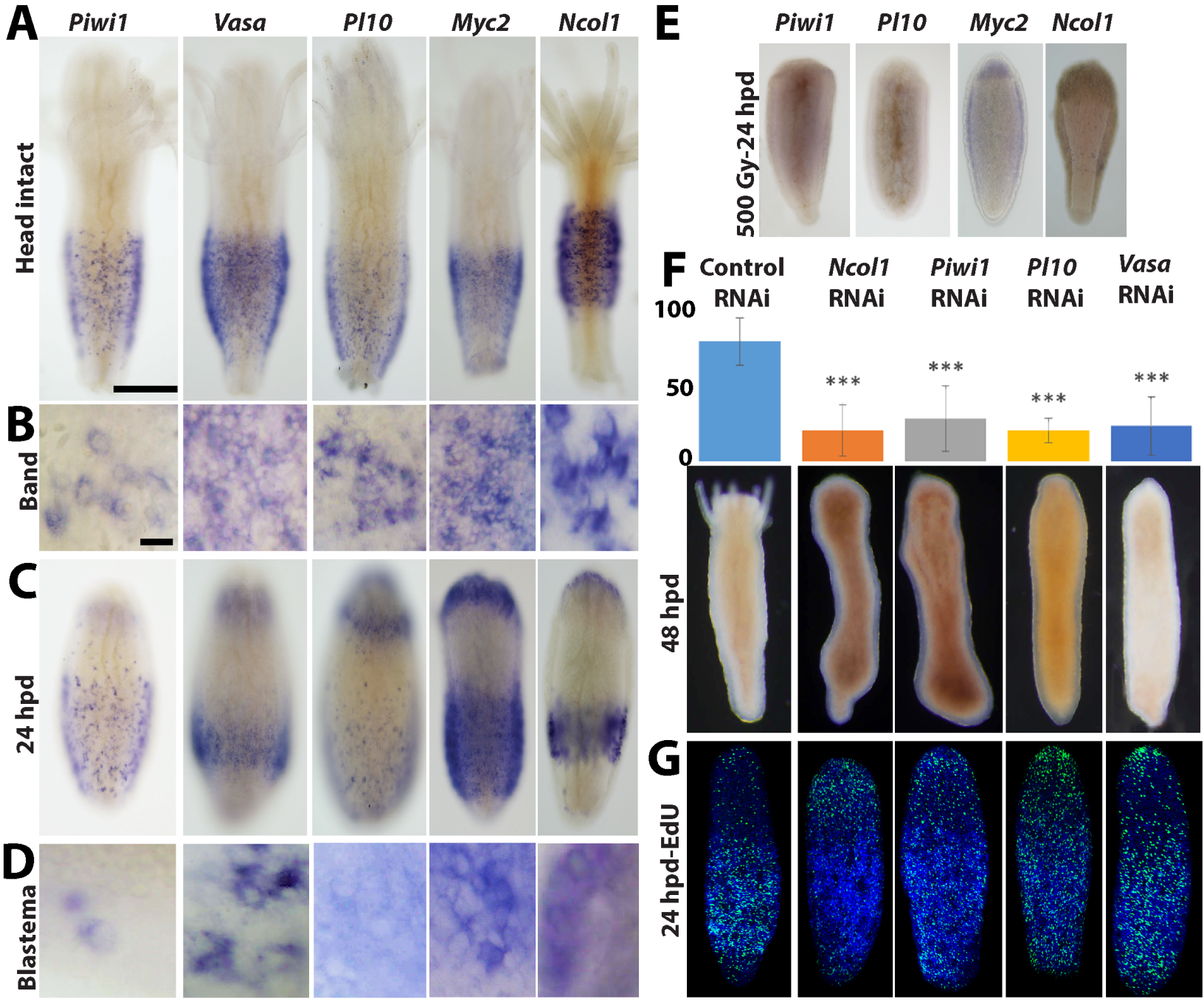


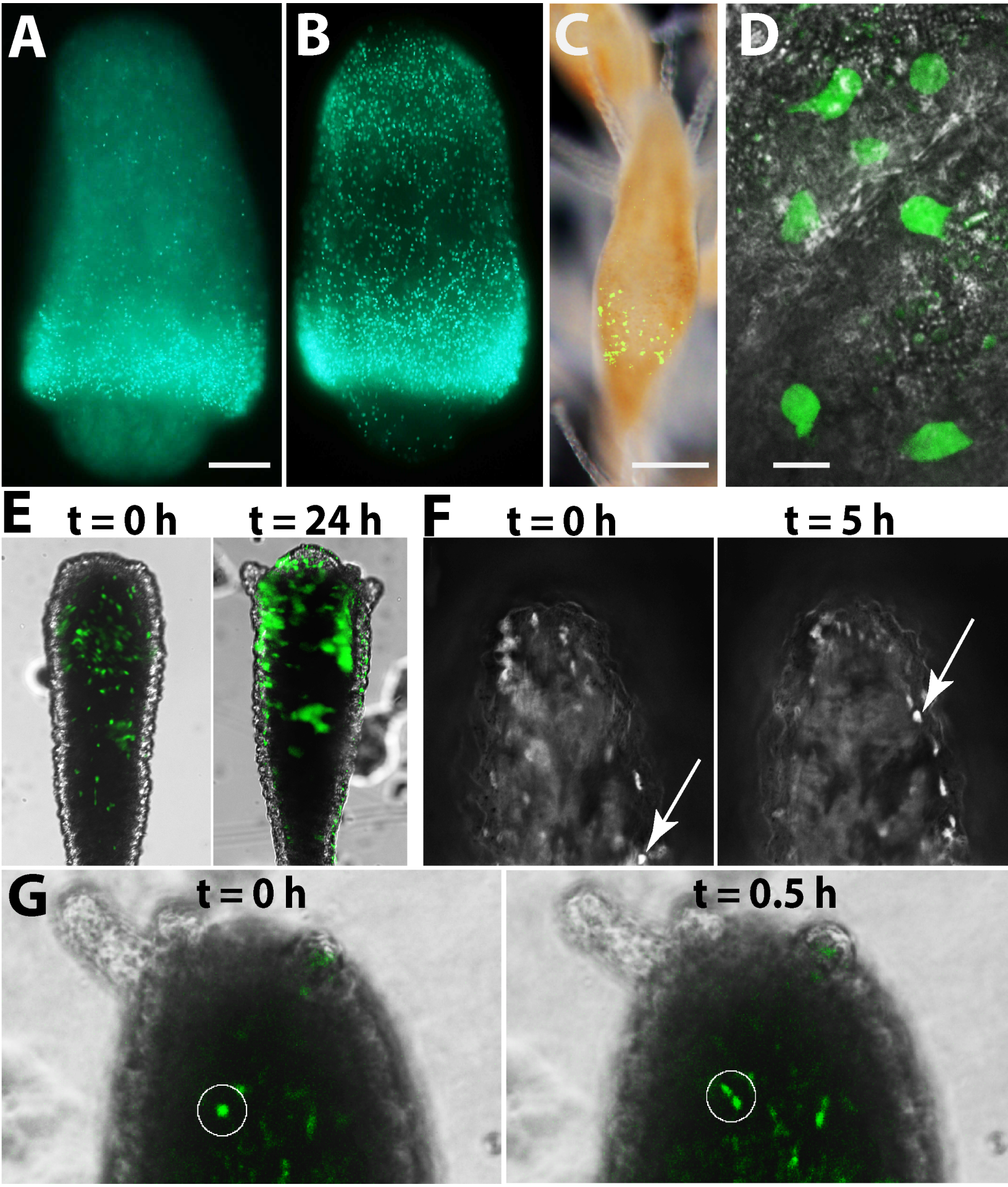




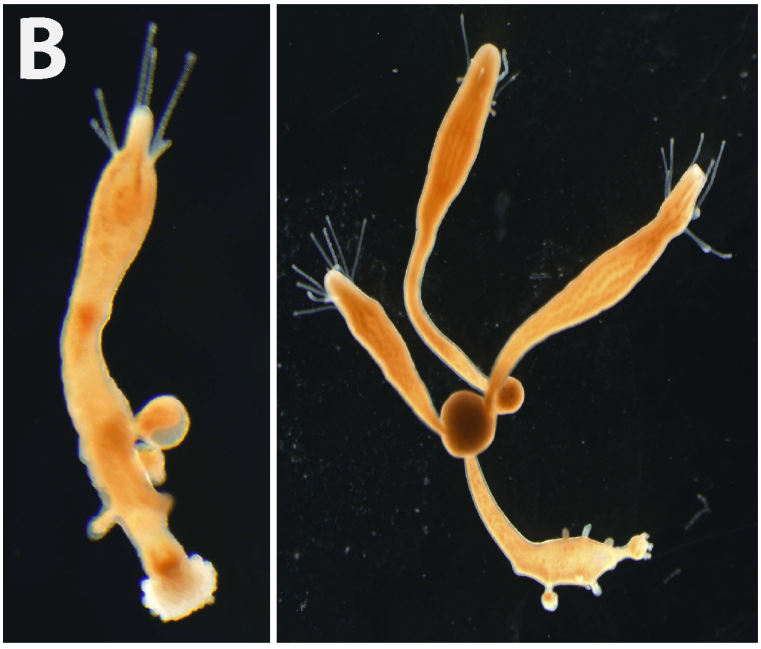
Acetylated tubulin/phalloidin/DAPI

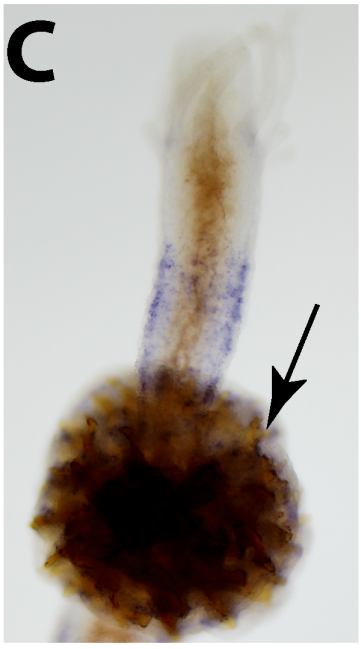

A
Head intact
24 hpd
48 hpd
EdU/DAPI
pH3/DAPI
B
i-cell
Epithelial cell
DIC
DAPI
pH3
Merge
C
% pH3+
100
50
0
Epithelial
i-cell
D
0 Gy
48 hpd
500 Gy
48 hpd
EdU
E
500 Gy-72 hpd
Acetylated tubulin
phalloidin/DAPI

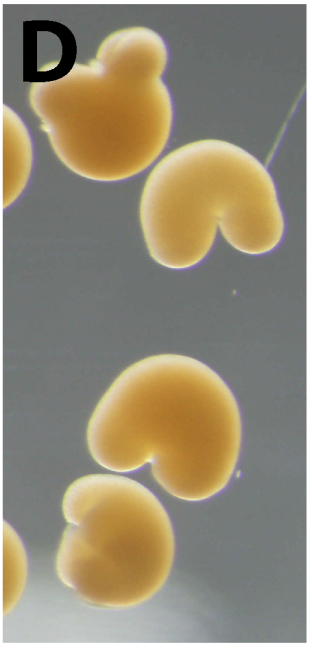

A
Piwi1
Vasa
Pl10
Myc2
Ncol1
Head intact
B
Band
C
24 hpd
D
Blastema
E
Piwi1
Pl10
Myc2
Ncol1
500 Gy-24 hpd
F
Control RNAi
Ncol1 RNAi
Piwi1 RNAi
Pl10 RNAi
Vasa RNAi
100
50
0
***
48 hpd
G
24 hpd-EdU


A
B
C
D
E
t = 0 h
t = 24 h
F
t = 0 h
t = 5 h
G
t = 0 h
t = 0.5 h


A
Day 1
Day 7
Day 10
Day 13
Day 15
Day 18
Day 27


B

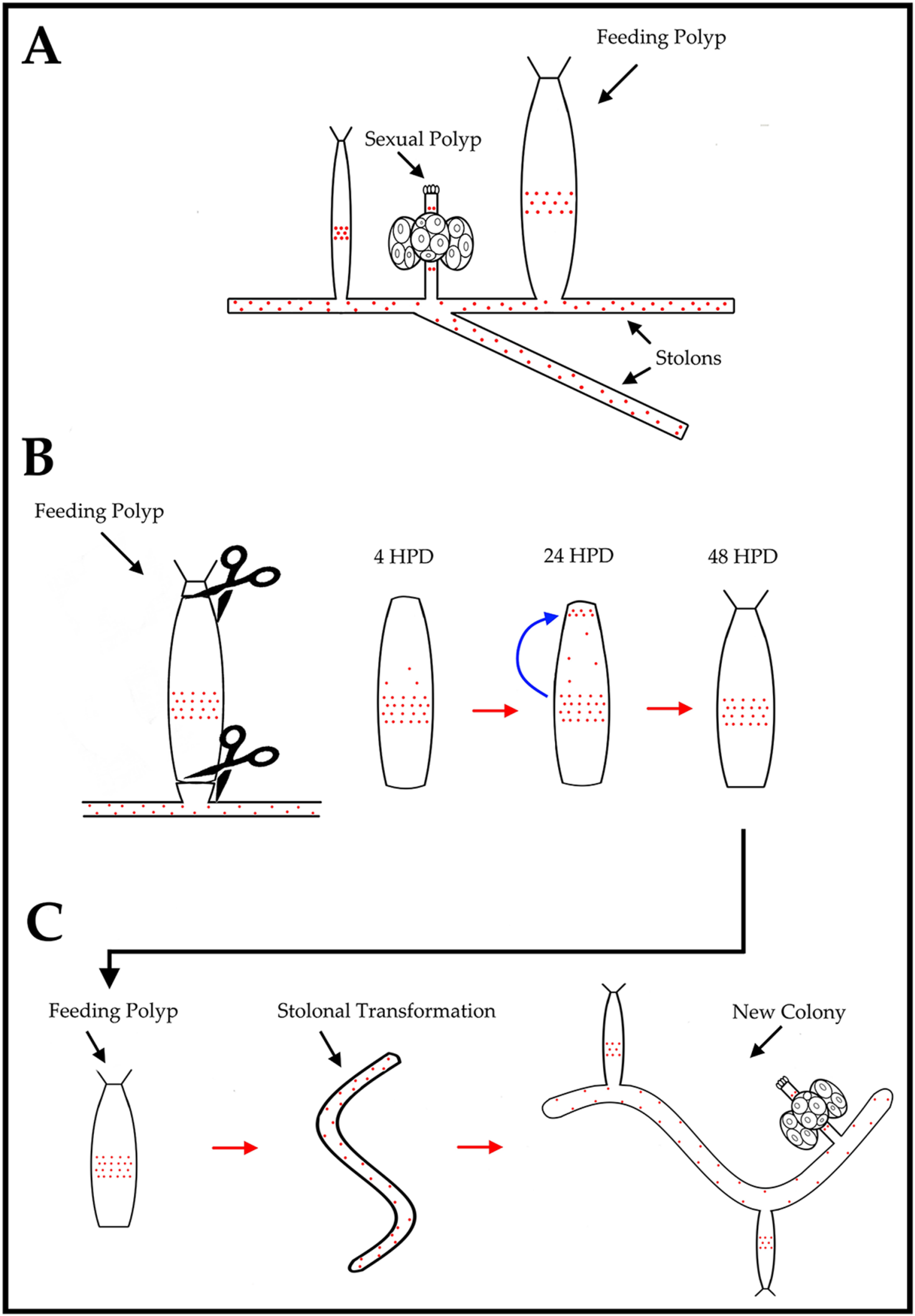

C


D


E
Loss of apical *Wnt3*


F
New apical *Wnt3*


G
Stolon-like distribution of *Ncol1*+ cells


A
Feeding Polyp
Sexual Polyp
Stolons
B
Feeding Polyp
4 HPD
24 HPD
48 HPD
C
Feeding Polyp
Stolonal Transformation
New Colony