Pattern of Cell Proliferation During Budding in the Colonial Ascidian *Diplosoma listerianum*

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Abstract. Many invertebrates reproduce asexually by budding, but morphogenesis and the role of cell proliferation in this diverse and nonconserved regeneration-like process are generally poorly understood and particularly little investigated in didemnid ascidians. We here analyzed cell proliferation patterns and telomerase activity during budding in the colonial didemnid ascidian *Diplosoma listerianum*, with special focus on the thoracic bud where a new brain develops de novo. To help define developmental stages of the thoracic bud, the distribution of acetylated tubulin was also examined. We found extensive cell proliferation in both the thoracic and abdominal buds of *D. listerianum* as well as higher telomerase activity in bud tissue compared to adult tissues. In the parent adult, proliferation was found in various tissues, but was especially intense in the adult esophagus and epicardial structures that protrude into the proliferating and developing buds, confirming these tissues as the primary source of the cells that form the buds. The neural complex in the thoracic bud forms from a hollow tube that appears to separate into the neural gland and the cerebral ganglion. Whereas most of the bud undergoes proliferation, including the hollow tube and the neural gland, the cerebral ganglion shows little or no proliferation. Pulse-chase labeling experiments indicate that the ganglion, as well as the myocardium, in adult zooids are instead composed of postmitotic cells.

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Abbreviations: BrdU, bromodeoxyuridine; TRAP, telomeric repeat amplification protocol.

Introduction

Regeneration involves resumption of morphogenesis in the adult body. Regeneration and repair of the nervous system is limited in vertebrates. However, in tunicates, which are close relatives of vertebrates within the phylum Chordata, regeneration of adult tissues, including the brain, is common. For example, the entire neural complex of the solitary tunicate *Ciona intestinalis* is replaced completely 4 weeks after ablation (Bollner et al., 1995, 1997). Many colonial tunicate taxa routinely generate whole new individuals from adult tissue in a budding process responsible for colony survival and growth (Nakauchi, 1982; Satoh, 1994). Despite widespread and increasing interest in stem cell biology, tissue renewal, and aging, there have been surprisingly few detailed studies of the cellular and molecular mechanisms involved in vegetative propagation by budding in tunicates, the closest relatives to vertebrates that undergo natural adult cloning (Dunn et al., 2008; Nilsson Sköld et al., 2009), including the de novo formation of a new brain. Moreover, while there is great diversity between ascidian families in the detailed modes of budding, the overall bud morphogenesis has underlying similarities in different ascidians (Nakauchi and Kawamura, 1986; Satoh, 1994). Buds normally inherit the parental epidermis, but the remaining bulk of the new tissue derives from stem-cell-like cells that can arise, depending upon the taxon, from several different adult tissues (Nakauchi, 1982). There may also be great plasticity in developmental routes for bud formation even within the same species (Voskoboynik et al., 2007). This diversity is markedly different from larval development, which is highly conserved among all deuterostomes.
Classically, two basic modes of cellular mechanism are distinguished in budding and regeneration events: epimorphosis, involving cell proliferation, and morphallaxis. In morphallaxis, the new tissues are formed by cell migration or direct transformation from existing cells without local cell proliferation. In oligochaetes and sea stars, morphallaxis or direct transformation from existing cells without local morphallaxis, the new tissues are formed by cell migration, involving cell proliferation, and morphallaxis. In distinguished in budding and regeneration events: epimorphosis, involving cell proliferation, and morphallaxis. In hydra, although regeneration after injury is morphallactic, budding instead involves local cell proliferation (Holstein et al., 1991). In the colonial ascidian Botryllus schlosseri, blood-borne stem cells are involved in budding (Laird et al., 2005), and blood cells of the closely related Botryloides leachi and Botryloides violaceus proliferate (Ermak, 1981; Brown et al., 2009). However, cell proliferation levels are not particularly high in the developing buds of botryllines (Rabinowitz and Rinkevich, 2004; Rinkevich et al., 2007; Brown et al., 2009), and a recent conclusion is that most cell proliferation occurs in the blood-borne stem cells before they form the buds (Brown et al., 2009). In Polyandrocarpa misakiensis, which belongs to the same family within the Stolidobranchia, the Styelidae, as the botryllines, budding involves mostly cell migration and transformation of epithelial cells (thus morphallaxis), but also some degree of cell division (Kawamura and Nakauchi, 1986; Kawamura and Fujiwara, 1994; Kawamura et al., 1995). There have so far been no cell proliferation analyses in the more distant and, in this respect, considerably less well studied didemnid ascidians within Aplousobranchia.

Here, we investigate basic developmental processes and cell proliferation patterns during adult budding in the colonial tunicate Diplosoma listerianum (Milne-Edwards, 1841). This species belongs to the Didemnidae, a family in which zooids form two morphologically distinctive buds—a thoracic bud producing the new branchial basket and brain, and an abdominal bud producing the new gastrointestinal tract and heart. Here we especially focus on the thoracic bud for better understanding of how the new brain is formed. Individual asexually propagating clones of D. listerianum have been maintained in our cultures for 19 years, so the cells forming the buds in this species may also have characteristics for long-term self-renewal such as up-regulation of telomerase activity, as described for B. schlosseri (Laird and Weissman, 2004; Laird et al., 2005). We therefore included comparative analysis of telomerase activity in excised buds versus adult zooid tissue. To enhance a classification of the major developmental stages of the thoracic bud, we also documented the expression pattern of acetylated tubulin as a marker for ciliation.

Materials and Method

Study animal and culture process

Experiments were carried out using laboratory cultures of Diplosoma listerianum originally derived from wild United Kingdom populations as described by Ryland and Bishop (1990). Colonies kept in reproductive isolation were grown on glass microscope slides in 800-ml tanks, fed with Isochrysis galbana and Rhinomonas reticulata, and maintained at 16 °C with a regime of 15-h light and 9-h dark. Regular reduction of colonies by cutting and transfer onto clean glass in fresh tanks maintained healthy growth. For details of culture protocols, see Bishop et al. (2001) and Ryland and Bishop (1990).

During budding, a zooid of D. listerianum forms one thoracic bud containing the branchial basket including the ganglion, and an abdominal bud that includes the stomach region (Fig. 1A–D). The two zooids derived from a budding event separate so that the newly budded thorax unites with the old abdomen, and the old thorax with the new. The old abdomen is noticeably more pigmented than the newly budded abdomen (Fig. 1B–D). Budding is not synchronized between the zooids within a colony, so all stages of the process may be present at the same time. When transplanted into a suitable habitat in the field, small colonies from culture are capable of 10-fold increase in the number of zooids in a week (AD Sommerfeldt and JDDB, Marine Biological Association, Plymouth, UK; unpubl.). In laboratory culture, growth is slower, with up to a 4-fold increase per week at 16 °C. Here the budding process typically takes 3 d from the appearance of small buds visible in a live zooid to the separation of the two zooids (3.14 d ± 0.074 s.e., 83 budding events).

Telomerase activity (TRAP) assay

Buds and adult zooidal tissue from six different Diplosoma colonies were separated by manual dissection in RNase-free PBS, quick-frozen in Eppendorf tubes on dry ice and stored at –80 °C. The budding materials were at different budding stages. Telomerase activity using the telomeric repeat amplification protocol (TRAP) was done using the TRAPEze Telomerase Detection kit S7700 (Chemicon International, Inc. Temecula, CA), according to the manufacturer’s protocol, as described by Hernroth et al. (2010). In brief, extracts of buds and adult tissue in each clone were simultaneously homogenized on ice in CHAPS lysis buffer including RNase inhibitor (RNasin: Promega, Madison, WI), incubated on ice for 30 min, and centrifuged for 20 min (12000 g, 4 °C); the supernatant was quick-frozen on dry ice and stored no longer than 20 d at –80 °C before analysis. Protein concentrations were determined using the Micro BCA Protein Assay kit (Pierce Biotechnology, Inc., Rockford, IL). For the TRAP assay, reaction mixtures containing 0.1 and 0.02 μg of protein from extracts of bud and adult tissue were analyzed for each colony. As negative controls, extracts were heat-treated for 15 min at 95 °C, and one reaction mixture was run without any extract. The TSR8 reference template (0.1 amol 1–1) was
used as positive control. The reaction tubes were incubated for 30 min at 30 °C and then amplified by polymerase chain reaction (15 min hot start at 94 °C, cycled 33 times at 94 °C for 30 s, at 59 °C for 30 s, and at 72 °C for 1 min) using HotStarTaq DNA polymerase (Qiagen, Hilden, Germany). The products were separated by 10% polyacrylamide gel electrophoresis, stained with SYBR green (Invitrogen), and visualized under ultraviolet light using a Gel Doc 2000 (Bio-Rad, Hercules, CA). The Wilcoxon signed-ranks test was used as a nonparametric pairwise test to compare values of total band intensity (summary of peak density values from all bands) after background subtraction, in bud versus adult tissue extracts from each colony preparation, as a measure of relative telomerase activity (Sigma Stat, ver. 3.5; Jandel Scientific, San Rafael, CA). The bud and adult material to be compared pairwise were loaded on the same gel.

**BrdU labeling, immunohistochemistry, and microscopy**

Budding colonies were transferred into 5-cm petri dishes for bromodeoxyuridine (BrdU) incorporation using the RPN 202 Cell Proliferation kit (Amersham Biosciences, Uppsala, Sweden). Colonies were initially treated with 25, 100, and 250 mol l⁻¹ BrdU in filtered seawater for 5 h, 1 d, or 3 d at 12 °C. The major difference in labeling was found to depend on the time of BrdU incorporation rather than the administered dose, and 100 mol l⁻¹ BrdU was chosen for standardization. Nonlabeled colonies showed no staining. Pulse-chase experiments were done by post-incubation of BrdU-treated colonies in pure seawater. The colonies were then anesthetized using cold, freshly prepared 0.3% propylene phenoxtol (Clariat GmbH, Sulzbach, Germany) in seawater as described by Bishop and Sommerfeldt (1996).
and fixed overnight in 4% paraformaldehyde in filtered seawater or phosphate-buffered saline (PBS, pH 7.4.) at 4 °C. BrdU incorporation was analyzed by preparing whole-mounts of zooids dissected from treated colonies stored in 70% ethanol, or by processing the colonies for histology. For histology, labeled colonies were rinsed in PBS and dehydrated in an ethanol series (50%, 70%, 90%, 95%, and 100%) for embedding in paraffin wax. After 100% ethanol, the colonies were transferred to Histoclear (National Diagnostics, Hessle, UK), 1:1 Histoclear/melted wax (melting temperature 55–58 °C; from Merck, Darmstadt, Germany), and finally to 100% melted wax for solidification at room temperature. Series of sections of 7–8 μm were transferred onto SuperFrost Plus glass slides. Dried slides were dewaxed in Histoclear and rehydrated in an ethanol series (100%, 95%, 90%, 80%, 70%, and 50%). Endogenous peroxidase activity was quenched by a 10-min treatment in 0.3% H2O2, and the slide washed in PBS and processed for detection BrdU by following the RPN 202 protocol using nickel in the diaminobenzidine solution to produce black/brownish staining. For immunohistochemical analysis of ciliation, a monoclonal acetylated tubulin antibody (clone 6-11B-1 from Sigma-Aldrich, Stockholm, Sweden) and a secondary peroxidise conjugated anti-mouse antibody were instead used. Eosin or Mayer’s hematoxylin, both from Sigma Diagnostics, were used as counterstains. Stained slides were subsequently dehydrated in an ethanol series (50%, 70%, 90%, 95%, and 100%) and finally Histoclear before mounting (Polymount, Polysciences, Inc, War- rington, PA). Slides were analyzed using a Leica microscope adapted for digital imaging with a Canon S40 color camera and Canon imaging software (Leica Microsystems).

**Results**

**Relative telomerase activity**

Analysis of telomerase activity using the TRAP assay showed slightly but significantly higher labeling intensity in bands from tissue extracts of buds compared to the corresponding adult zooid tissue extracts from the same colonial preparation ($P = 0.031$ for both 0.1 and 0.02 μg protein reactions, $n = 6$), indicating higher telomerase activity in buds than in adult tissue (Figs. 1E, F). The only modest increase in telomerase activity in the more concentrated extracts (0.1 μg) compared to in the less concentrated (0.02 μg) suggests polymerase inhibition, saturation, or both, in the former.

**Cell proliferation pattern in adult zooids**

The branchial basket in adult *Diplosoma listerianum* zooids possesses several zones of active cell proliferation (Fig. 2). The endostyle consists of a series of distinct groups of cells in eight major longitudinal zones (Burighel and Clo- ney, 1997). BrdU labeling was detected in the heavily ciliated zones 3 and 5, when animals were incubated in BrdU for 5 h or 1 d (Fig. 2A), whereas 3-d incubations revealed incorporation in all endostyle zones. A regular pattern of positive labeling can be detected in the epithelium surrounding the stigmata (Fig. 2B). After 5 h of labeling, individual cells in the most anterior and posterior parts of the stigmata are stained. After 1 d, labeling has spread outward from the extreme anterior and posterior points, resulting in a chevron pattern of labeling of the stigmata in both adult zooids and late buds. After 3 d, the lateral walls of the stigmata are also labeled. After 1 d, individual cells in the epithelium of the oral siphon are positively labeled, as are cells at the base of the oral tentacles (not shown). In addition, the epithelium of the branchial basket that surrounds the entrance to the esophagus is positively labeled after 5 h in BrdU.

In the abdomen, proliferating cells are situated in the space surrounding the heart (Fig. 2C, arrowhead) and stom- ach (Fig. 3A, arrowhead). These cells are presumably blood cells, given that didemnids have an open circulatory system. The myocardium was unstained after 1 d in BrdU but stained after pulse-chase labeling (Fig. 2C, D), indicative of postmitotic cells in the myocardium.

Analysis of cell proliferation during budding indicates that both the thoracic and abdominal buds that grow out from the abdominal area undergo massive cell proliferation during their development (Fig. 2E, arrows). Epithelial cells around the entrance and exit of the adult stomach also incorporate BrdU (Fig. 2E, arrowheads). Proliferation is, however, comparatively higher at the entrance to the stom- ach after labeling with BrdU for 5 h. The testis is stained after 5 h and more, in agreement with previous research using tritiated thymidine to label *D. listerianum* sperm (Bishop, 1996). A schematic summary of the proliferation pattern in adult zooids after 1 d of BrdU labeling is shown in Figure 2F.

**Cell proliferation pattern in buds and adjacent adult tissues**

In addition to the developing thoracic and abdominal buds, extensive cell proliferation is also seen in the epidermis, esophagus, and epicardium that protrude into the buds (Fig. 3). When incubated for 24 h in BrdU, the epithelium of the esophagus is strongly labeled in the area where the thoracic and abdominal buds are connected to the parental esophagus (Fig. 3A). Most of the epicardium is strongly labeled, with the exception of the more basal cells (Fig. 3B, arrowhead), suggesting presence of more slowly dividing cells in this region of the epicardium. A schematic summary of the tissues involved in thoracic bud development is depicted in Figure 3C.
Cell proliferation pattern in the developing neural complex

The central neural complex in *D. listerianum* is positioned dorsally, close to the oral siphon and opposite to the endostyle (which is ventral) (Fig. 4A–C), as in ascidians in general (Burighel and Cloney, 1997). The ganglion in adult zooids of *D. listerianum* is distinctive and spherical, with an outer cortex one or two cells thick. Just ventral to the ganglion, the discrete and densely ciliated funnel, or dorsal tubercle, connects to the branchial basket at one end and continues at the other into a group of larger globular cells, the neural gland body. A disk-shaped area of pigmented

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**Figure 2.** *Diplosoma listerianum.* (A–E) BrdU incorporation in sections of selected adult tissues. (A) Endostyle (end), cells of zones 3, 5, and 8 show labeling (arrows), while the heavily ciliated zone 1 or the more polarized cells of the other endostyle zones are unlabeled. (B) Branchial basket: a regular pattern of labeling can be detected in the anterior and posterior parts of the epithelium surrounding the stigmata. (C) Abdomen of the adult zooid: BrdU incorporation is evident in blood cells around the heart (arrowhead), but not in the myocardium (arrow). (D) After pulse-chase labeling with 5 h of BrdU labeling followed by 3 days of pure seawater, the myocardium also showed BrdU incorporation (arrow). (E) Section of a whole zooid: intense labeling is seen in both thoracic and abdominal early buds (arrows), epithelial cells around the entrance and exit of the stomach (arrowheads), and stigmata. In all pictures except D, colonies were fixed directly after 1 d of BrdU labeling. (F) Semi-schematic summary of the cell proliferation pattern during budding, where incorporation of BrdU during 1 d of labeling is shown in red. nc = neural complex; es = esophagus. Scale bars in A–E, 100 μm.
cells is located dorsal to the brain. Cell proliferation was seen in the ciliated funnel/neural gland after 5 h to 3 d of incubation with BrdU. The labeling was especially evident in the area of the ciliated funnel/neural gland close to the ganglion (Fig. 4C). The adult ganglion itself showed no proliferating cells. Neither did the neural gland body (sections with 1 d of BrdU incorporation analyzed only).

Analyses of buds at different developmental stages and after different incubation times in BrdU made it possible to examine the development of the thoracic bud and neural

**Figure 3.** *Diplosoma listerianum.* (A, B) Longitudinal sections of developing thoracic bud (insert in A, arrow indicates thoracic bud in section of whole zooid), showing intense BrdU incorporation in areas of the esophagus and in epicardial structures that protrude into the bud, as well as in the outer epithelia that cover the bud (A, arrows). A population of proliferating cells is also situated more distant from the bud in the space surrounding the stomach (A, arrowhead). The epicardial structures show noticeably more BrdU incorporation in the areas adjacent to the bud than in cells more distant from the bud (B, arrowhead shows unlabeled nuclei). (C) Schematic modified from Groepler (1992, *Zoologische Beiträge* 34: 33), presenting the parental tissues involved in development of a thoracic bud—i.e., epithelium, epicardium (ep), and esophagus (es)—based on the results of the BrdU labeling. Cell proliferation is indicated as thicker lines. Scale bar in A, 100 μm.
complex in detail. In early thoracic buds (Phase 2–3), there is a hollow dorsal tube in the area where the nervous system will be located. After 5 h of BrdU incubation, this tube shows evenly distributed proliferation (Fig. 5A, arrow). In Phase 4, when early signs of stigmata can be seen but the endostyle has not yet reached its typical adult form, proliferation is concentrated in the floor of the dorsal tube, leaving an unlabeled dorsal area (Fig. 5B, arrow). Whereas the inner structures of the bud show extensive proliferation, the epidermis is at this stage less labeled (Fig. 5C, arrows). In sections of slightly later buds with developed stigmata (Phase 5), the neural complex now comprises two structures, the neural gland and the ganglion (Fig. 5D, E). In later buds that morphologically have an adult-like endostyle (Phases 6 and 7), the ganglion is spherical and the gland/funnel beneath has the shape seen in the adult. After 3 d of continuous BrdU incubation, the ganglion in later buds shows few or no proliferating cells, while the funnel of the neural gland is highly stained (not shown). However, pulse-chase experiments with 5 h of labeling and 3 d in pure seawater show labeling of the ganglion (Fig. 5F, G). This indicates that the mature ganglion is composed of cells that are no longer dividing—that is, postmitotic. Longitudinal sections reveal an initial BrdU-labeled thickening (Fig. 5H, arrow) in Phase 3 that will form the more distinct hollow tube. At this stage and the next stage (Phase 4) the hollow tube is asymmetrical and leaf-shaped, with the stalk directed posteriorly (Fig. 5I, arrow). BrdU labeling is evident. In the next stage (Phase 5), the neural complex separates into the neural gland and the ganglion, both having elliptical and not yet spherical shapes (Fig. 5J, in square). The formation of the neural complex from the original hollow tube during budding is schematically summarized in Figure 5K, with BrdU labeling in black.

Ciliation pattern in thoracic bud by presence of acetylated tubulin

For further examination and staging of development of the thoracic bud, including the neural complex, we also analyzed timing of the appearance of ciliation in the endostyle and ciliated funnel by the presence of acetylated tubulin (Fig. 6). Adult ciliation is shown in A–C of Figure 6, and the progressive ciliation of the endostyle, stigmata, and ciliated funnel during development of the thoracic bud is shown in D–G.

Discussion

Coloniality is an asexual reproductive strategy that has independently evolved in different families within the ascidians. Of those, budding in the didemnid ascidians is particularly little characterized. Here, we have investigated basic developmental processes during budding in the colonial didemnid ascidian Diplosoma listerianum. Specifically, we analyzed cell proliferation patterns and telomerase...
Figure 5. *Diplosoma listerianum*. Investigation of development of the neural complex by BrdU incorporation during thoracic bud development. (A–E) Frontal/transverse sections of thoracic buds of successive developmental stages in colonies labeled with BrdU for 5 h (A) or 1 d (B–E). (A) Bud phase 2–3: arrow indicates the dorsal hollow tube that will become the neural complex. (B, C) Bud phase 4: arrows indicate unstained areas in the dorsal part of the tube, the developing ganglion, and outer epithelia cells. (D, E) Bud phase 5: arrows in E indicate labeled nuclei of the ganglion (g) and in the dorsal cells of the ciliated funnel (cf). Free unlabeled cells are also seen in proximity to the ganglion (arrowhead in E). (F, G) Fully developed thorax: result of pulse-chase experiment with 5 h of BrdU treatment followed by 3 d of pure seawater, showing labeling of the ganglion (arrows), but not of the ciliated funnel. (H–J) Longitudinal sections of progressive stages of bud development after 1 d of BrdU labeling, showing the neural complex located opposite the developing endostyle (end). The neural complex (nc) originates as a thickening (arrow in H, phase 3), followed by a not yet spherical neural complex (I, phase 4) that subsequently becomes divided into two compartments composed of the elliptical ganglion and the ciliated funnel (J, phase 5, in square). Sections A–G are counterstained with eosin and H–J with hematoxylin. (K) Schematic of the development of the neural complex based on the sections, suggesting formation of the ganglion and the ciliated funnel by separation of the cells of the initial dorsal hollow tube. Scale bars: 50 μm (A–D, F, H–J) and 10 μm (E, G).
activity to gain insight into mitotic capacity and the role of cell proliferation, particularly in thoracic budding including formation of the new brain. We also investigated the distribution of acetylated tubulin as a marker for cilia. Together, our data allow us to present the first picture of the cell proliferation pattern in didemnids, exemplified by *D. listerianum*, and a classification of the developmental stages of the thoracic bud (Table 1). In this classification, we have divided the developmental events into “stages” that are recognizable under stereomicroscopy and more detailed bud or adult “phases” that are recognizable in histological material.

We show highly organized patterns of continuing cell proliferation in mature zooids of *D. listerianum*. From studies based primarily on the unitary stolidobranch *Styela clava*, Ermak (1982) reported renewing cell populations in various epithelia including those of the ciliated funnel, endostyle, stigmata, esophagus, and stomach; these are areas in which proliferation was also noted in *D. listerianum*. This suggests substantial similarity in adult zooid cell and tissue homeostasis between these two distantly related species.

We also show particularly strong and widespread BrdU staining in the buds, indicating that cell proliferation and thus epimorphosis is a major mechanism for budding in *D. listerianum*. Our proliferation data clearly confirm the role of the epithelium, esophagus, and epicardium as sources of cells during didemnid budding, as suggested earlier by histological examination lacking explicit proliferation analysis (Berrill, 1935; Brien and Van den Breede, 1948; Kitazawa, 1967; Groepler, 1992). Thus budding in *D. listerianum* involves multiple tissues. The cells involved could be either differentiated cells, stem cells, or de-differentiated cells, and further work is needed to better understand the relative prevalence of the respective differentiation levels in the cells involved in *D. listerianum* budding.

We found telomerase activity in both adult and bud extracts, but the level of telomerase activity was significantly higher in buds. Our result from *D. listerianum* is therefore similar to data obtained from *Botryllus schlosseri* (Laird and Weissman, 2004). Since budding in both botryllines, and perhaps particularly in *D. listerianum*, involves cell proliferation, elevation of telomerase activity in their developing buds might enable colonial ascidians to maintain the proliferative capacity necessary long-term for asexual reproduction.

Figure 6. *Diplosoma listerianum*. Analysis of ciliation during thoracic bud development by presence of acetylated tubulin. (A) Longitudinal section of an adult zooid undergoing budding, showing intense ciliation in the stigmata of the branchial basket (arrowhead) and in the esophagus, but not in a developing thoracic bud (arrow). (B) Enlargement of the adult neuronal complex showing the ganglion (g) and the ciliated funnel (cf) above the cavity of the ciliated branchial basket. (C) The endostyle of a fully developed zooid, also heavily ciliated. (D–G) Sections of thoracic buds at progressive developmental stages showing absence of ciliation at bud phase 3 (D). In phase 6 (E, F), stigmata rudiments have just started to become ciliated (arrow in F). In a later bud, phase 7 (G), ciliation is established in the ciliated funnel beneath the ganglion, plus the branchial basket, endostyle (end), and stigmata. Scale bars: 10 (A, D–F) and 50 μm (B, C).
propagation. In many mammals, including humans, the drastic decline in overall telomerase activity is associated with cessation of somatic growth, onset of somatic senescence, and loss of cellular renewal (Chang and Harley, 1995; Iwama et al., 1998; Kinugawa et al., 2000; Cawthon et al., 2003; Herbig et al., 2006). However, animals that constantly grow, like some fish, or have extensive regenerative capacity, like sea stars (Elmore et al., 2008; Hernroth et al., 2010), show high telomerase activity in adult tissues, thereby providing proliferative potential.

During asexual budding by colonial tunicates, a new neural complex is formed from pre-existing adult tissue. Work on budding in *B. schlosseri* indicates that a dorsal tube formed by invagination eventually differentiates into the ciliated funnel, neural gland, and dorsal organ; “pioneer cells” delaminate from the dorsal tube, and subsequently from the neural gland, and migrate to form the cerebral ganglion, differentiating into neurons (Burighel et al., 1998; Mackie and Burighel, 2005). The solitary tunicate *C. intestinalis* is similarly able to rebuild a complete neural complex after excision. The cellular source (or sources) for this neural regeneration is not clear, but stem-cell-like hematocytes and dorsal strand tissues have been suggested (Bollner et al., 1997). In a late (Phase 5) bud of *D. listerianum*, some nuclei incorporating BrdU were observed in the ganglion close to the ciliated funnel (Fig. 5E). While it is possible that there is a proliferation zone there, offering some support to the possibility that the ciliated funnel/neural gland gives rise to cells forming the ganglion, the timing of the overall cell proliferation pattern differs widely between the ganglion and the ciliated funnel. From our work, it appears that the ganglion is mainly composed of postmitotic cells, in contrast to the ciliated funnel. The differences in BrdU labeling in the cerebral ganglion compared to the ciliated funnel suggest that in *D. listerianum* both the ganglion and the ciliated funnel derive from cells of the dorsal hollow tube. Cell-tracking experiments would, however, be necessary for a definite understanding of the *de novo* formation of ascidian brains.

Our results demonstrate regionalized cell proliferation in the adult and extensive cell proliferation in the buds. The ganglion, which forms early, and the heart are composed of cells that do not divide in the adult zooid—that is, postmitotic cells. Extensive proliferation was found in the epithelia and in the apparently pluripotent adult esophagus and epidermal structures that protrude into the buds and into the inner structures such as the brachial basket of the new zooid, strongly supporting their contributions in bud formation. We further show that budding tissue has relatively higher telomerase activity than adult tissue, providing elevated mitotic potential that should be important for the capacity and longevity of the epimorphic budding process in *D. listerianum*.

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