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1 Tissue homeostasis in sponges: quantitative analysis of cell
2 proliferation and apoptosis

3

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5 Abstract

6 **Background:** Tissues of multicellular animals are maintained due to a tight balance between cell
7 proliferation and programmed cell death. Phylum Porifera is an early branching group of metazoans
8 essential to understand the key mechanisms of tissue homeostasis. This paper is dedicated to the
9 comparative analysis of proliferation and apoptosis in intact tissues of two sponges belonging to
10 distinct Porifera lineages - *Halisarca dujardinii* (class Demospongiae) and *Leucosolenia variabilis*
11 (class Calcarea).

12 **Results:** Labeled nucleotides EdU and anti-phosphorylated histone three antibodies reveal a
13 considerable number of cycling cells in intact tissues of both species. The main type of cycling cells
14 are choanocytes - flagellated cells of the aquiferous system, and the rate of proliferation remains
15 constant in areas containing choanoderm. Cell cycle distribution assessed by the quantitative DNA
16 stain reveals the classic cell cycle distribution curve. During EdU pulse-chase experiment conducted
17 in *H. dujardinii*, the contribution of the choanocytes to the total amount of EdU-positive cells
18 decreases, while contribution of the mesohyl cells increases. It could indicate that the proliferation of
19 the choanocytes is not solely limited to the renewal of the choanoderm, and these cells may
20 participate in general cell turnover through migration. The number of apoptotic cells in intact tissues of
21 both species is insignificant. *In vivo* studies with TMRE and CellEvent Caspase-3/7 indicate that
22 apoptosis might be independent of mitochondrial outer membrane permeabilization in both species.

23 **Conclusions:** A combination of confocal laser scanning microscopy and flow cytometry provides a
24 quantitative description of cell turnover in intact sponge tissues. Intact tissues of *H. dujardinii*
25 (Demospongiae) and *L. variabilis* (Calcarea) are highly proliferative, indicating either high rate of
26 growth or cell turnover. The role of apoptotic processes in intact sponges remains unclear.

27 **Key words:** cell turnover, cell proliferation, apoptosis, Porifera, Calcarea, Demospongiae

1 Introduction

2 Morphogenetic processes underlying the development, growth and regeneration of
3 multicellular animals have long been issues of scientific interest. These phenomena seem to be
4 closely related to a process of physiological regeneration, also known as cell turnover^{1,2}. The cell
5 turnover consists of three key elements: 1) the elimination of old, non-functional cells by programmed
6 cell death; 2) the proliferation of somatic stem cells; 3) the differentiation of newly generated cells
7 along with their integration with the preexisting tissue³. Such tissue renewal through the physiological
8 regeneration is an essential process for multicellular animals, as it allows reparation of minor defects
9 and damages of tissues occurring during their normal functioning^{1,4}. Abnormalities of tissue
10 homeostasis are associated with numerous malfunctions, e.g., oncological and degenerative
11 disorders^{4,5}.

12 Sponges (phylum Porifera) is an early-branching group of metazoans comprising aquatic,
13 mostly filter-feeding sedentary organisms⁶. These animals differ substantially from other metazoans in
14 their body organization: sponges lack tissues and organ systems characteristic of other animals^{7,8}.
15 The central anatomical structure in a sponge body is the aquiferous system, a complex network of
16 canals and chambers through which sponges pump surrounding water for food and oxygen uptake⁹.
17 The rest of the sponge body is composed of the mesohyl, a tissue consisting of an extensive
18 extracellular matrix and numerous wandering cells of different types and functions⁹.

19 Tissues of sponges seem to be structurally and functionally less specialized than tissues of
20 Eumetazoa. The main characteristic of sponge tissues is high plasticity: in particular, sponge tissues
21 are multifunctional, and their cells show broad capabilities of transdifferentiation into other cell
22 types^{10,11}. The tissue plasticity underlays many aspects of sponge biology, participating in constant
23 reorganization of the aquiferous system¹⁰, sexual and asexual reproduction⁸, regeneration¹²⁻¹⁷ and
24 even movement of sponges¹⁸⁻²⁰.

25 At the same time, sponges lack a single category of somatic stem cells. Choanocytes
26 (flagellated cells of the aquiferous system) and some amoeboid cells of mesohyl (e.g., archaeocytes
27 of Demospongiae) are both considered to be the stem cells of sponges^{8,21,22}. As has been shown for a
28 number of species, choanocytes and archaeocytes proliferate in intact tissues, (trans)differentiate into

1 other cell types, take part in regeneration and gametogenesis^{8,14,21,23–25}. They also express some stem
2 cell-specific genes, such as the members of the germline multipotency program^{23,26–28}. Yet,
3 choanocytes are usually interpreted as a minor component of sponge stem cell system – i.e., the
4 stock of archaeocyte progeny that is able to transform back to archaeocytes^{9,23}.

5 For the proper cell turnover, cell proliferation should be balanced by programmed cell death.
6 The most common path of programmed cell death in intact tissues of animals is apoptosis^{3,29–32}.
7 However, immunohistochemical studies reveal few apoptotic cells in intact sponge tissues. Most of
8 them are amoeboid cells located in the mesohyl; caspase-positive choanocytes are extremely rare³³.
9 Meanwhile, some sponges are able to discard certain cell types (e.g., choanocytes, archaeocytes,
10 lophocytes, spherulous and granular cells) into the environment through the aquiferous system^{33–35}.
11 Expelled cells show various signs of degradation, but the nature of this process remains obscure³³.

12 Considering these peculiarities of the tissue organization, sponges can be promising objects
13 for studies regarding the origin and evolution of physiological regeneration mechanisms in metazoans.
14 The aim of this study is the comparative and quantitative analysis of the proliferation and apoptosis in
15 intact tissues of two sponge species, *Halisarca dujardinii* Johnston, 1842 (Demospongiae) and
16 *Leucosolenia variabilis* Haeckel, 1870 (Calcarea). These species belong to different Porifera classes
17 and represent fundamentally different types of the aquiferous system organization^{12,36}. We have
18 combined confocal laser scanning microscopy and flow cytometry to identify proliferating and
19 apoptotic cells, as well as evaluate their number and distribution in the sponge body. Altogether, these
20 data are able to complement the concept of tissue dynamics in these basal metazoans.

21 **Results**

22 ***Cell proliferation in intact sponge tissues: quantitative analysis and*** 23 ***spatial distribution***

24 *Halisarca dujardinii* (class Demospongiae) is a thick globular sponge with 1-2 oscular tubes
25 and a leuconoid aquiferous system (Figure 1A, B). Its body is subdivided into two regions: the
26 ectosome and endosome (Figure 1C). The ectosome represents a superficial region of the body

1 devoid of choanocyte chambers. The external parts of T-shaped exopinacocytes are underlined with a
2 thick layer of diffuse collagen which contains rare spherulous cells (Figure 1C). A dense layer of
3 amoeboid cells (e.g., spherulous and granular cells) and cell bodies of the exopinacocytes are located
4 beneath the diffuse collagen layer (Figure 1C). The internal region of a sponge, the endosome,
5 contains large, tubular choanocyte chambers surrounded by the mesohyl (Figure 1C, D). The mesohyl
6 contains numerous cells (e.g., archaeocytes, lophocytes, spherulous, vacuolar and granular cells) and
7 occupies a significant volume of the endosome (Figure 1C). The structure of the aquiferous system is
8 similar in regions of endosome adjacent to the oscular tube and at some distance (approximately half
9 the body radius) from it (Figure 2A, B). The oscular tube is a principally different region of a sponge,
10 representing a thin-walled outgrowth composed mainly of exo- and endopinacoderm (Figure 2C). A
11 thin layer of mesohyl lies between exo- and endopinacoderm; choanocyte chambers are absent.

12 To evaluate the number and distribution of proliferating cells in intact tissues of *Halisarca*
13 *dujardinii*, we have utilized two markers: 5-ethynyl-2'-deoxyuridine (EdU) and Ser10-phosphorylated
14 histone 3 (pH3) monoclonal antibodies. After 12 hours of treatment with 200 μ M EdU, intact tissues of
15 *H. dujardinii* contain numerous EdU-positive nuclei belonging to a population of cycling cells (Figure
16 2A, B). Fewer cells have nuclei labeled with pH3-antibodies, representing a fraction of cycling cells in
17 the late G2 phase or M phase. Very few pH3-positive cells also contain EdU in the nuclei. The
18 endosome adjacent to the oscular tube and the endosome distant from the oscular tube display
19 similar fraction of proliferating cells: they contain $8.44 \pm 3.08\%$ and $8.10 \pm 1.64\%$ of EdU-positive cells,
20 respectively. Each region also has $0.11 \pm 0.07\%$ of pH3-positive cells (Figure 3A, B; Table 1). Dunn
21 test shows no significant differences between the regions (p -values > 0.9999 for both markers).
22 Furthermore, both regions show a similar distribution of proliferating cells: most of EdU- and pH3-
23 positive nuclei (approximately 90% of all labeled nuclei) are located in the choanocyte chambers and
24 belong to choanocytes, while EdU- and pH3-positive nuclei in the mesohyl are few (Figures 2A, B; 3C,
25 D; Table 1).

26 The oscular tube differs significantly from the regions of endosome. There are only
27 $0.61 \pm 0.64\%$ of EdU-positive cells in the oscular tube, and pH3-positive cells are absent (Figures 2C;
28 3A, B; Table 1). All EdU-positive cells belong to the mesohyl/pinacoderm of the oscular tube, as this
29 region is devoid of choanocyte chambers (Figure 3C). The fraction of EdU-positive cells in the oscular

1 tube is significantly lower than the total fraction of EdU-positive cells in the regions of endosome
2 ($p=0.0157$ and 0.005 compared to the endosome distant from the oscular tube and adjacent to it,
3 respectively) (Figure 3A). However, the fraction of EdU-positive cells in the oscular tube shows no
4 difference with the fraction of EdU-positive mesohyl cells in the regions of endosome (p -
5 values >0.9999 in both cases) (Figure 3C).

6 *Leucosolenia variabilis* (class Calcarea) is an asconoid sponge; its body is a plexus of
7 branching and anastomosing thin-walled hollow tubes (Figure 4A). This plexus - the cormus - gives
8 rise to the oscular tubes and blind outgrowths, the diverticula. The body wall is composed of the
9 exopinacoderm and choanoderm layers separated by thin mesohyl containing few cells, mostly
10 amoebocytes and sclerocytes (Figure 4C-G). The cell bodies with nuclei of T-shaped exopinacocytes
11 and porocytes are located near mesohyl cells (Figure 4E). Still, choanocytes are easily distinguished
12 from other cells as they form a continuous layer of tightly packed pyriform nuclei associated with
13 flagella (Figure 4E-G). Such structure of the body wall is characteristic of most regions of
14 *Leucosolenia* body: diverticula, cormus tubes, and the most part of oscular tubes. Only the oscular
15 rim, the most distal part of the oscular tube, differs in the body wall structure: in this region, the
16 choanocytes are substituted by the flat endopinacocytes (Figure 4C).

17 Intact tissues of *L. variabilis* display a significant number of cycling cells in S and G2/M cells
18 after 6 hours of treatment with $20 \mu\text{M}$ EdU (Figure 5A-G). Few pH3-positive cells display EdU signal in
19 the nuclei (Figure 5H). Regions containing choanocytes exhibit high proliferation rate. Most of these
20 regions (cormus tubes, proximal parts of diverticula, oscular tubes) show similar fractions of
21 proliferating cells (p -values >0.9999 in each pairwise comparison), each containing about 10% of EdU-
22 positive and 0.6% of pH3-positive cells (Figures 5B-G; 6A, B; Table 2). In the distal parts of the
23 diverticula, the proportion of pH3-positive cells remains the same ($0.50 \pm 0.20\%$), while the proportion
24 of EdU-positive cells decreases ($6.53 \pm 2.08\%$) (Figure 6A, B; Table 2). The decrease is significant
25 compared to the distal and middle parts of oscular tubes (p -values = 0.0068 and 0.0022 , respectively);
26 in other pairwise comparisons differences are insignificant, but p -values are slightly higher than 0.05 .
27 The oscular rim has the lowest fraction of proliferating cells compared to other body parts: it contains
28 only $0.40 \pm 0.96\%$ of EdU-positive and $0.17 \pm 0.20\%$ pH3-positive cells (Figure 6A, B; Table 2). In case
29 of EdU, the decrease in the number of labeled cells is significant compared to all body regions, except

1 the distal part of the diverticula; the difference in the number of pH3-positive cells is significant,
2 excluding the distal part of the diverticula and cormus tube.

3 Most EdU- and pH3-positive cells of *L. variabilis* are choanocytes. However, few EdU-positive
4 and pH3-positive cells belong to the mesohyl/pinacoderm layer (Figures 5A-G; 6C, D). The proportion
5 of the choanocytes in the total number of EdU-positive cells remains almost the same (95% on
6 average) in different parts of the oscular tube, cormus and diverticula (Figure 6C; Table 2). In the case
7 of pH3-positive cells, choanocytes usually represent approximately 75% of all late G2/M cells (Figure
8 6D; Table 2). In the distal parts of the diverticula, their proportion decreases to $40.64 \pm 22.29\%$ (Figure
9 6D; Table 2). This decrease is significant in comparison with the cormus, middle and proximal parts of
10 the oscular tube ($p=0.0023$, 0.0003 and 0.0002 , respectively).

11 ***EdU tracking experiment in H. dujardinii***

12 Since labeled nucleotides are transferred to daughter cells after mitosis, EdU allows us to
13 track proliferating cells or their progeny. We treated individuals of *H. dujardinii* with $200 \mu\text{M}$ EdU for 12
14 hours and transported them to the running seawater aquarium. After the transportation, EdU
15 incorporation is ceased, and the distribution of EdU-positive cells in intact tissues of *H. dujardinii*
16 gradually changes: the contribution of the choanocytes to the total amount of EdU-positive cells
17 decreases, while contribution of the mesohyl cells increases (Figure 7A, B). At the beginning of the
18 experiment, mesohyl cells account for only $7.92 \pm 6.11\%$ of labeled cells; 7 days after, their fraction
19 significantly increases, reaching $55.12 \pm 23.15\%$ ($p=0.0026$) (Figure 7C).

20 ***Cell cycle distribution in H. dujardinii and L. variabilis intact tissues***

21 Cell cycle distribution was assessed by the quantitative DNA staining of single-cell
22 suspensions obtained via mechanical dissociation in calcium and magnesium-free seawater
23 (CMFSW-E). The major part of *H. dujardinii* cells ($38.94 \pm 5.90\%$) form a diploid G0/G1 population peak
24 (Figure 8A; Table 3). The tetraploid cells in the G2/M phase form a less distinguishable peak
25 characterized by a 2-fold higher fluorescence intensity than G1/G0 cells (Figure 8A). The G2/M
26 population contains $9.28 \pm 1.78\%$ of all cells (Table 3). Cells in the S phase are located between the
27 G0/G1 and G2/M populations and account for $20.18 \pm 6.14\%$ of the total population (Table 3). The

1 SubG1 population is characterized by lower DNA content than G1/G0 cells (Figure 8A). Scatter plots
2 indicate that the SubG1 population consists of small and highly granulated objects - possibly, dying
3 cells and debris (Figure 8C).

4 CMFSW-E suspensions of *L. variabilis* do not provide reliable data: they contain too many
5 SubG1 and too few G2/M cells to obtain an accurate cell cycle distribution histogram and were
6 excluded from the further analysis. Instead, suspensions made of pre-fixed *L. variabilis* tissues were
7 used. This method provides improved resolution: the suspensions contain fewer SubG1 cells and
8 show well-distinguishable G0/G1 and G2/M peaks. The general appearance of the histogram is
9 similar to *H. dujardinii* (Figure 8B). The proportion of the G0/G1 cells is $53.88 \pm 2.59\%$, S cells -
10 $12.58 \pm 0.76\%$ and G2/M cells - $4.63 \pm 1.79\%$ (Table 3).

11 ***Investigation of apoptotic activity***

12 To study the apoptosis, individuals of *H. dujardinii* and *L. variabilis* were vitally stained with
13 fluorescent substrate for effector caspases – CellEvent – for two hours. Subsequent CLSM studies
14 reveal an insignificant number of apoptotic cells in intact tissues of these sponges (Figure 9A, D).
15 Most of these cells exhibit weak CellEvent fluorescence. Nonetheless, they are easily resolved from
16 autofluorescent cells by nuclear localization of the signal characteristic of CellEvent (Figure 9B-F). In
17 *L. variabilis*, the proportion of CellEvent-positive cells does not exceed 1% in any studied body region
18 (Table 4). There are choanocytes as well as mesohyl cells among these apoptotic cells. The
19 endosome of *H. dujardinii* demonstrates even less apoptotic activity: for 6-9 thousand normal cells,
20 there are usually 1-2 CellEvent-positive ones (Table 4).

21 We also studied suspensions obtained via mechanical dissociation in CMFSW-E. They were
22 stained with CellEvent, fixed, and analyzed via flow cytometer. Cells of *H. dujardinii* form a single
23 peak with low/negative CellEvent fluorescence (Figure 10A, B). The fraction of apoptotic cells is low:
24 CellEvent-positive cells account only for $1 \pm 1.15\%$ of the total population. In contrast, cells of *L.*
25 *variabilis* are clearly divided into two subpopulations by the level of CellEvent fluorescence (Figure
26 10C, D). CellEvent-positive cells with elevated autofluorescence (as evidenced by increased short-
27 wave fluorescence in the PacificBlue channel) comprise $60.8 \pm 13.46\%$ of the total cell population
28 (Figure 10C, D). We consider this result as a sampling artifact (see the “Discussion” section)

1 Suspensions were also studied *in vivo*; during this experiment, non-fixed suspensions were
2 stained for 30 min with CellEvent and TMRE (TMRE indicates the presence of the mitochondrial outer
3 membrane potential - i.e., the viability of the mitochondria). These suspensions were studied both by
4 CLSM and flow cytometry. Four cell types can be distinguished via CLSM (Figure 11A):

5 1) TMRE+ CE- cells possessing functional mitochondria (as indicated by TMRE fluorescence)
6 and no active effector caspases (as clear nuclear CellEvent signal is absent);

7 2) TMRE- CE- cells characterized by the absence of viable mitochondria or active effector
8 caspases (i.e., no CellEvent or TMRE fluorescence observed);

9 3) TMRE+ CE+ cells possessing both functional mitochondria and active effector caspases (bright
10 TMRE and CellEvent signals are present);

11 4) TMRE- CE+ cells distinguished by high activity of effector caspases and the loss of the
12 mitochondrial outer membrane potential; these cells display bright nuclear CellEvent signal
13 but no TMRE fluorescence.

14 The same types of cells have been observed via flow cytometer (Figure 11B-D). Both species
15 demonstrate similar cell distribution. The major part of cells belongs to the TMRE+ CE- population,
16 which resides in quadrant Q1. It accounts for $89.67 \pm 1.06\%$ cells in *H. dujardinii* and $70.55 \pm 1.48\%$
17 cells in *L. variabilis* (Figure 11C, D; Table 5). TMRE+ CE+ population lies in the area of higher
18 CellEvent fluorescence, Q2. This population reaches $7.51 \pm 0.87\%$ in *H. dujardinii* and $8.58 \pm 0.77\%$ in
19 *L. variabilis* (Figure 11C, D; Table 5). TMRE- CE+ cells reside in Q3. They do not exceed 1% of the
20 general population and do not form a distinguishable cluster (Figure 11C, D; Table 5). All CellEvent-
21 positive cells (Q2 and Q3) seem to be small and highly granulated objects, as indicated by light
22 scatter parameters (Figure 11B). TMRE- CE- population is located in Q4 and accounts for
23 $1.67 \pm 0.35\%$ in *H. dujardinii* and $13.70 \pm 3.25\%$ in *L. variabilis* (Figure 11C, D; Table 5). Although these
24 cells are also smaller than TMRE+ CE- cells, they tend to separate from TMRE+ CE+ cells on scatter
25 plots (Figure 11B).

1 Discussion

2 In multicellular animals, the long-term maintenance of tissue homeostasis is achieved by cell
3 turnover^{1,4}. Cell turnover is composed of three complementary processes: cell proliferation, cell
4 differentiation and programmed cell death³. Sponges are basal metazoans well known for their unique
5 tissue dynamics. In this study, we mainly focus on cell proliferation and apoptosis in intact tissues of
6 sponges, providing promising data on the cell turnover in sponges.

7 *Cell proliferation in intact tissues of H. dujardinii and L. variabilis*

8 *Halisarca dujardinii* is a leuconoid demosponge that demonstrates a significant number of
9 cycling cells under the steady-state condition. The presence of EdU- and pH3-positive cells in intact
10 tissues is consistent with flow cytometry data, as the distribution of *H. dujardinii* cells by the DNA
11 content reveals the classic cell cycle distribution curve³⁷. However, the estimates of proliferating cell
12 fractions are slightly different. The proportion of EdU- and pH3-positive cells is about 8% of the total
13 cell number, while S plus G2/M cells together reach 28% - possibly because our CLSM staining is not
14 able to detect G2-phase cells.

15 Different regions of the endosome display active proliferation and do not differ significantly in
16 the proportions of EdU- and pH3-positive cells; the proliferation activity seems to be evenly distributed
17 throughout the sponge's endosome. The oscular tube possesses a different pattern of proliferation
18 and contains few labeled cells. We attribute such difference to the absence of choanocyte chambers
19 and choanocytes in the oscular tube. In other demosponges, choanocytes have already been shown
20 to incorporate labeled nucleotides^{14,33,34,38}; however, they might have retained the BrdU signal as the
21 progeny of other proliferating cells. Here we demonstrate that choanocytes are also labeled with anti-
22 pH3 antibodies. Thus, choanocytes do not only synthesize DNA but also divide, being the main
23 proliferating fraction of cells in *H. dujardinii*.

24 *Leucosolenia variabilis* is a calcareous asconoid sponge. Despite that, the pattern of
25 proliferation is the same as in *H. dujardinii*. A significant number of EdU- and pH3-positive cells and
26 cell cycle distribution curve prove the fact of constant proliferation. As in other calcareous sponges,
27 the major part of DNA synthesizing cells are choanocytes^{12,38}. Most pH3-labeled cells are also

1 choanocytes, although the proportion of choanocytes among all pH3-labelled cells is lower than in *H.*
2 *dujardinii*. Body regions containing choanocytes display a high number of cycling cells and do not
3 differ in the rate of proliferation. The only exception is the distal parts of the diverticula. Fewer EdU-
4 positive cells are found there; interestingly, the proportion of pH3-positive cells remains almost the
5 same. This body region also differs in the proportion of choanocytes among the pH3-positive cells;
6 nearly half of the G2/M cells belong to the mesohyl/pinacoderm layer. It could be evidence of potential
7 growth since new sclerocytes, pinacocytes and porocytes are obviously needed to lengthen the
8 diverticula. In the oscular rim, which is devoid of choanocytes, the intensity of cell proliferation is very
9 low. There are, however, few labeled cells, indicating either slow cell turnover or growth.

10 This study provides the first spatial analysis of cell proliferation in different body regions of
11 sponges representing distinct phylogenetic lines and distinct types of aquiferous system. In general,
12 proliferation activity appears to be evenly distributed throughout sponge tissues (excluding regions
13 that do not contain choanocytes). The proportion of S-phase cells in *H. dujardinii* is lower than in other
14 sponges - in particular, tropical Demosponges, in which this parameter reaches 20-25% on
15 average^{34,38}. Considering calcareous sponges, the proportion of S-phase cells in *L. variabilis* is slightly
16 higher than in *Sycon coactum*³⁸. It should also be noted that during regeneration studies, the
17 proportion of EdU-labeled cells in *L. variabilis* has been rather overestimated compared to current
18 data, possibly because of the counting technique¹². Similar to the previous studies, we detect that
19 DNA synthesis occurs primarily in choanocytes; additionally, we show that the distribution of mitotic
20 cells in adult sponges have similar pattern. Overall, the proportion of proliferating cells in *H. dujardinii*
21 and *L. variabilis* resembles those in animals characterized by fast cell turnover - platyhelminthes,
22 cnidarians, bivalvians³⁹⁻⁴².

23 ***The contribution of choanocytes to homeostasis***

24 The physiological role of proliferation in intact tissues is either growth or renewal³; to
25 understand the general dynamics of sponge tissues, we should figure out the fate of proliferating cells.
26 In intact sponges, the proliferation takes place in the choanoderm. Since the choanoderm is a)
27 metabolically active and b) directly exposed to the environment, we should expect high rate of
28 choanocyte turnover⁴. Similarly to feeding structures of other suspension feeders, constant
29 replacement of old, damaged cells is crucial for the long-term maintenance of the choanoderm⁴²⁻⁴⁵.

1 Despite constantly facing a potentially hostile environment (e.g., toxic and mutagenic factors dissolved
2 in seawater), choanocytes themselves undergo constant proliferation and represent the main source
3 of new cells in the choanoderm. It should be noted here that the proliferation of morphologically
4 differentiated cells is fairly common among metazoans, including vertebrates^{3,46-49}. Particularly
5 interesting is the case of Cnidaria, in which ecto- and endodermal epithelial cells do not only act as
6 barrier and contractile cells but also retain multipotency and the ability to proliferate⁵⁰.

7 In fact, the boundary position of choanocytes might be advantageous since cell division
8 requires significant amount of resources. As the primary food-entrapping sponge cells, choanocytes
9 are provided with sufficient energy for constant proliferation and respond appropriately to
10 environmental changes. Moreover, since choanoderm is obviously enriched in cell-cell interaction and
11 at least partially compartmentalized by extracellular matrix¹⁸, it could maintain the proliferative
12 potential of choanocytes through some signaling system resembling the stem cell niche. In some
13 animals (e.g., *Hydra* and planarians), the concept of niche may be interpreted in a rather broad sense,
14 regarding a whole organ/tissue as stem cell niche^{69,70}. Choanoderm might be just the example of such
15 a "tissue niche".

16 Since the flagellum is resorbed during the cell division, the filtration process becomes
17 impaired; to resolve the conflict between proliferation and food uptake, choanocytes should a)
18 maintain the proliferating fraction at a certain level or b) minimize the period of the absence of the
19 flagellum. The short duration of the M phase is possibly the reason why the proportion of pH3-positive
20 cells does not exceed 1% in any examined area of *H. dujardinii* and *L. variabilis*. Indeed, although the
21 length of cell cycle phases varies significantly among different animals, the M phase is always the
22 shortest (e.g., 3-5% of the total duration of the cell cycle in mammals)⁵¹.

23 It has been shown earlier that choanocytes are the main proliferative fraction of cells in intact
24 tissues of demosponges and calcareous sponges: in most species, the proportion of EdU(BrdU)-
25 positive cells of mesohyl is significantly lower than the proportion of labeled choanocytes^{33,34,38}. Still, it
26 is usually stated that the proliferation of choanocytes is restricted to the self-renewal of the
27 choanoderm, and the mesohyl contains an autonomous population of cycling cells (e.g., archaeocytes
28 of Demospongiae)⁸. According to Funayama, choanocytes and mobile mesohyl cells both represent
29 distinct types of stem cells²¹; however, choanocytes display broad stem cell activity only in calcareous

1 and homoscleromorph sponges⁸. In demosponges and hexactinellids, archaeocytes are thought to
2 be active stem cells; choanocytes are the minor component of the stem cell system, transforming into
3 archaeocytes only under specific circumstances²³.

4 Indeed, we have observed EdU- and pH3-positive cells outside the choanoderm in intact
5 tissues of *H. dujardinii* and *L. variabilis*. However, the fraction of labeled mesohyl cells is too low and
6 variable to assume that the mesohyl contains a large population of cycling cells. Instead, we suppose
7 that at least part of labeled mesohyl cells originates from the choanoderm. Since we have used
8 prolonged incubation in EdU, not all observed EdU-positive cells are actually in the S phase. For
9 instance, few pH3-positive cells also contain the EdU signal, indicating that these cells have finished
10 the S phase and passed through the G2 phase by the moment of fixation. Since DNA synthesis
11 occurs predominantly in the choanoderm, it is tempting to assume that these pH3-positive cells have
12 migrated to the mesohyl from the choanoderm. In such case, some of the EdU-only-positive cells in
13 the mesohyl could be choanoderm-derived cells that have not yet reached the M phase.

14 EdU tracking experiment in *H. dujardinii* provides us with more clues. After the removal of the
15 EdU from the medium, the proportion of labeled mesohyl cells continuously increases. There are
16 several ways to interpret this result. It could well be that the proliferation of mesohyl cells drive the
17 increase in the proportion of labeled mesohyl cells. Comparing to choanocytes, cell cycle of
18 archaeocytes might be way shorter. If so, EdU-labeled progeny of archaeocytes would easily
19 outnumber slowly proliferating choanocytes by the final time point. Apparently, this is not the case with
20 *H. dujardinii*: assuming that the S and M phases of archaeocytes are very short, we would have seen
21 much more EdU-positive cells in mesohyl immediately after the treatment with EdU (i.e., at the initial
22 time point). Thus, there is no evidence that archaeocytes proliferate faster than choanocytes.

23 On the other hand, the proportion of labeled mesohyl cells can increase only due to the loss
24 of EdU-positive choanocytes - e.g., label dilution or cell shedding. The first option assumes that
25 labeled nucleotides are equally distributed between daughter cells; after the division, the intensity of
26 the signal decreases by two. Continuous proliferation may cause the intensity of the fluorescence to
27 drop beyond the registration threshold, making it hard to detect some EdU-positive cells. Since
28 choanocytes are actively cycling, they are more affected by the label dilution than mesohyl cells. On
29 the other hand, the division of an EdU-positive cell should also result in appearance of two EdU-

1 positive daughter cells. This process could at least partially compensate for the decrease in the
2 number of EdU-positive cells through label dilution.

3 The second option considers an extensive shedding of choanocytes described in the previous
4 works; in these studies, choanocytes were found to be massively discarded into the environment.
5 Nonetheless, there is little physiological sense in getting rid of newly produced (i.e., EdU-positive)
6 cells. One should expect that old, non-proliferative choanocytes are predominantly shed. Finally,
7 extensive cell shedding is characteristic of sponges having high microbial abundance in their
8 tissues³⁴; in turn, *H. dujardinii* should be categorized as the low microbial abundance sponge³⁶.

9 In line with similar experiments on planarians, the label flux in *H. dujardinii* can also occur via
10 cell migration⁵². Since choanocytes are the main DNA synthesizing population of cells, the increase in
11 the proportion of EdU-positive mesohyl cells could be a consequence of the migration of choanocytes
12 (or their progeny) into the mesohyl. Of course, the population of archaeocytes might be self-renewing
13 to some extent, but the label flux can be an indirect evidence that choanocytes contribute to the pool
14 of mesohyl cells. Even if archaeocytes take part in the renewal of the choanoderm, the general tissue
15 dynamics leans towards the opposite direction.

16 The same process may occur in intact tissues of *L. variabilis*. We have not performed pulse-
17 chase experiments of this species; however, the spatial heterogeneity of the proliferation might serve
18 as potential evidence of cell migration. For instance, it is doubtful that DNA synthesis occurs in the
19 oscular rim. In this body region, the decrease in the proportion of EdU-positive cells outstrips the
20 decrease of pH3-positive cells. The observed variation in the ratio of EdU/pH3-positive cells could be
21 explained by the alterations in the cell cycle length – e.g., the presence of fastly cycling cells.
22 However, it is unlikely since we would have seen much more EdU-positive cells that retain the label
23 after the S phase. We suppose that pH3-positive cells in the oscular rim actually might have migrated
24 from the more proximal parts of the oscular tube possessing well-developed choanoderm. In the distal
25 part of the diverticula, there are fewer pH3-positive choanocytes than in other parts of the body;
26 however, the proportion of EdU-positive choanocytes and mesohyl cells remains the same. This can
27 be a result of cell migration: choanocytes occasionally leave the choanoderm and contribute to the
28 growth of the diverticula, giving rise to mesohyl/pinacoderm cells.

1 To sum up, choanocytes are apparently the major part of cycling cells not only in calcareous
2 sponge *L. variabilis* but also in demosponge *H. dujardinii*. It seems that the proliferation of
3 choanocytes is not solely limited to the renewal of the choanoderm. It has been previously shown that
4 choanocytes contribute to gametogenesis^{8,53–55}, regeneration^{12,14,16}, and express certain stem cell
5 markers (at least in demosponges and homoscleromorphs)^{26,28,56,57}. Moreover, it seems that
6 choanocytes as the main proliferating fraction of cells may also participate in cell turnover through cell
7 migration⁵⁷. Altogether, these data suggest that the role of choanocytes in the stem cell system of
8 sponges can be underestimated. They may be the major source of new cells (including cells of
9 mesohyl) in both calcareous sponges and demosponges during regular cell turnover.

10 ***Apoptotic activity in intact tissues of sponges***

11 Under the steady-state condition, cell proliferation is counterbalanced by the process of cell
12 elimination. Apoptosis is considered to be the most common way of cell death in animals^{3,32,58}. It is a
13 tightly regulated and complex process, which results in the fragmentation of a dying cell into apoptotic
14 bodies³¹. Once these apoptotic bodies are engulfed by other cells, neat cell death is reached. It
15 should be noted that tissues exposed to the adverse environment usually display high rates of
16 physiological turnover (e.g., mammalian epidermis and intestine, gills, intestine and mantle of
17 mollusks, epithelia of ascidians and cnidarians)^{42,58–62}. Given the fact that choanocytes are highly
18 proliferative, we would expect a significant amount of dying cells.

19 CLSM studies using CellEvent staining indicate that apoptotic cells are almost absent in intact
20 tissues of *H. dujardinii* and *L. variabilis*: less than 1% of cells contain active effector caspases. We
21 consider this result to be the most relevant - in particular, it fits well with other known studies^{16,33,34}.
22 Surprisingly, there are significantly more CellEvent-positive cells in the CMFSW-E suspensions
23 analyzed with the flow cytometer. These cells are small, highly granulated and contain active effector
24 caspases; thus, they demonstrate classic features of apoptosis^{63,64}. Suspensions of *H. dujardinii*
25 contain few apoptotic cells. The case of *L. variabilis*, in which CellEvent-positive cells comprise up to
26 60% of the general population, is more intriguing.

27 SubG1 cells detected by means of DNA content are also to be mentioned here. These small,
28 highly granulated cells characterized by fragmented DNA should be considered apoptotic. Since cell

1 debris and bacteria might also be detected as a SubG1 population, it does not represent an accurate
2 estimation of the number of apoptotic cells. However, SubG1 cells may serve for indirect rough
3 evaluation of apoptotic activity. The presence of SubG1 cells seems to be an inevitable consequence
4 of sample preparation⁶⁵. Yet, the proportion of SubG1 cells differ drastically between species:
5 CMFSW-E dissociation does not significantly affect *H. dujardinii*, but leads to a massive transition of
6 *L. variabilis* cells to the SubG1 population. Obviously, CMFSW-E suspensions of *L. variabilis* indicate
7 massive cell death caused by sample preparation; the apoptotic response develops quickly – within
8 one or two hours. More importantly, this is not the case of suspensions made of pre-fixed tissues. It
9 seems dissociation itself results in the activation of the apoptotic machinery in cells of *L. variabilis*.
10 Thus, the observed number of CellEvent-positive cells and SubG1-cells in the suspensions of *L.*
11 *variabilis* cannot be considered an adequate estimation of the apoptotic activity.

12 The observed interspecific variability of the apoptotic response might be associated with the
13 phylogenetic position and type of body organization. While the major part of *H. dujardinii*
14 (Demospongiae) body is occupied by mesenchymal tissue, *L. variabilis* (Calcarea) is mainly
15 composed of epithelial-like layers of choanoderm and pinacoderm. Overall, it seems that calcareous
16 sponges represent more epithelial-like organization relying on tighter cell-to-cell interactions. For
17 example, the regeneration occurs via blastema formation and mesenchymal morphogeneses in
18 demosponges, while in calcareous sponges, it depends on the morphallactic reorganization of
19 adjacent intact tissues through epithelial morphogeneses^{11,12,66}. The disruption of the natural system
20 of cell interactions during the CMFSW-E dissociation could lead to the massive induction of apoptotic
21 activity in calcareous sponges.

22 Certainly, some degree of apoptosis upregulation should be expected in non-fixed
23 suspensions. Indeed, apoptotic cells account for 7-8% of the general population in both species
24 during *in vivo* studies; however, despite the apoptosis upregulation, *in vivo* experiment sheds some
25 light on the mechanisms of apoptosis. In mammalian cells, double staining with TMRE and CellEvent
26 usually reveals the presence of three populations: 1) intact TMRE+ CE- cells characterized by intact
27 mitochondria; 2) early apoptotic TMRE- CE- cells, in which mitochondrial transmembrane potential
28 has vanished; 3) late apoptotic TMRE- CE+ cells, which contain non-functioning mitochondria and
29 active effector caspases⁶⁷. This situation is typical for mammalian cells since the permeabilization of

1 the inner mitochondrial membrane is shown to be tightly associated with the mitochondrial outer
2 membrane permeabilization (MOMP)⁶³. In turn, MOMP is the critical event during the apoptotic
3 response, resulting in the release of some pro-apoptotic proteins from the mitochondrial
4 intermembrane space into the cytoplasm. The most crucial pro-apoptotic protein is cytochrome c,
5 which is able to bind to cytoplasmic protein APAF1, forming the apoptosome and beginning to recruit
6 effector caspases^{32,68}. In flatworms (*Dugesia dorotocephala* and *Schistosoma mansoni*) and
7 echinoderms (*Strongylocentrotus purpuratus* and *Dendraster excentricus*), the activation of effector
8 caspases is also cytochrome-c dependent⁶⁹. Thus, active effector caspases in their cells should be
9 detected only after the inner membrane permeabilization and MOMP. Some invertebrates, however,
10 do not fit into this scenario⁷⁰. In common model objects, *Caenorhabditis elegans* and *Drosophila*
11 *melanogaster*, the activation of effector caspases is not associated with the MOMP. In such case, the
12 promotion of apoptotic cell death does not depend on the presence of cytochrome c in the cytoplasm;
13 even if the MOMP is present, it is more a consequence than a reason for apoptotic response^{71,72}.

14 Our data indicate that the apoptosis in sponges might resemble the scenario of
15 *Caenorhabditis elegans* and *Drosophila melanogaster*: a significant amount of TMRE+ CE+ cells has
16 been observed in *H. dujardinii* and *L. variabilis* suspensions. TMRE+ CE+ cells contain active effector
17 caspases but possess well-functioning mitochondria. Unstained negative control suspensions indicate
18 that the high level of TMRE fluorescence in these presumably early apoptotic cells is specific.
19 Apparently, *H. dujardinii* and *L. variabilis* cells maintain functional mitochondria during apoptosis.
20 Thus, there is a possibility that the apoptosis in sponge cells is MOMP-independent. Therefore, in
21 contrast to mammalian cells, the TMRE- CE- population may contain not early apoptotic cells but
22 represents a population of damaged or dead cells and cell debris. Rare TMRE- CE+ cells might be
23 interpreted as non-viable, late apoptotic cells with non-functional mitochondria.

24 **Conclusions**

25 Here we provide quantitative and spatial analysis of cell proliferation and apoptosis in two
26 sponges belonging to different classes of Porifera. Intact tissues of *Halisarca dujardinii*
27 (Demospongiae) and *Leucosolenia variabilis* (Calcarea) are highly proliferative, indicating either high
28 rate of growth or cell turnover. In both species, the most proliferating fraction of cells are choanocytes,
29 and the proliferation occurs evenly throughout the areas of the body containing these cells. The

1 proliferation of choanocytes maintains the tissue homeostasis in the choanoderm and, possibly, in the
2 mesohyl/pinacoderm. Further investigations with *in vivo* cell tracking and *in situ* hybridization are
3 needed to prove if the choanocytes or their progeny actively differentiate into other cell types.

4 The number of apoptotic cells in intact tissues of both species is insignificant; however, there
5 is a possibility that a short duration of apoptosis might compensate for the low proportion of apoptotic
6 cells. Regarding that apoptotic response is developed rather quickly under certain conditions (e.g.,
7 dissociation of *L. variabilis*) it could still be involved in the maintenance of the tissue homeostasis. For
8 instance, choanocytes might undergo some kind of anoikis, the detachment-induced form of
9 apoptosis^{73,74}. In tissues exposed to the environment, cell extrusion may serve as an alternative to the
10 engulfment of apoptotic bodies. It is commonly thought that cell extrusion promotes anoikis via loss of
11 survival signaling through cell-cell and cell-matrix interactions^{75,76}, although the activation of caspases
12 may occur prior to cell shedding⁷⁴. There is a possibility that choanocytes actually undergo apoptosis,
13 but the activation of caspases takes part mostly after the detachment.

14 Obtained data will serve as a basis for further detailed researches of tissue homeostasis in *H.*
15 *dujardinii* and *L. variabilis*. While we have an information about spatial distribution and intensity of cell
16 proliferation and apoptosis for a number of sponge species, many other processes and mechanisms
17 contributing to the tissues homeostasis in sponge remains uncharacterized, e.g., the behavior and
18 fate of proliferating cell progeny, temporal characteristics of the proliferation, apoptosis and
19 differentiation, and, finally, the signaling systems maintaining proliferative potential of cells. Altogether,
20 these data would help us to establish a comprehensive model of the tissue dynamics in these basal
21 metazoans.

22 **Experimental Procedures**

23 ***Sampling and laboratory maintenance***

24 *Halisarca dujardinii* (Demospongiae, Chondrilla) and *Leucosolenia variabilis* (Calcarea,
25 Leucosolenida) were collected in the Kandalaksha Bay of the White Sea near the Pertsov White Sea
26 Biological Station of Lomonosov Moscow State University (66°34' N, 33°08' E) during August 2019-

1 2020. Sponges were collected in the upper subtidal zone with the substrate (brown algae *Fucus* and
2 *Ascophyllum*) and cleared of epifauna. Specimens were kept in laboratory aquariums with running
3 natural seawater of ambient temperature. Each experiment was carried out on individuals of the same
4 size collected the same day. Sponges were kept in open plastic Petri dishes filled with 5 ml of 0.22
5 μm -filtered seawater (FSW) during the experiments. Petri dishes were kept at 8-12°C on a shaker for
6 better stirring and aeration of FSW.

7 ***Histological studies of intact tissues***

8 For histological studies, sponge tissues were fixed for 2 hr at 4°C with 2.5% glutaraldehyde
9 (Electron Microscopy Science, 16020) and post-fixed for 1 hr at room temperature (RT) with 1% OsO₄
10 (Electron Microscopy Science, 19100). Fixation and post-fixation were done in modified 0.1M Na-
11 Cacodylate buffer (0.1M Na-Cacodylate, 85.55 mM NaCl, 5 mM CaCl₂, 5 mM MgCl₂; pH 7.0-7.5).
12 After the post-fixation, specimens were dehydrated and embedded in Epon/Araldite epoxy embedding
13 media (Electron Microscopy Science, 13940) according to the standard protocol described earlier
14 (Lavrov et al., 2018; Lavrov, Ereskovsky, 2021).

15 Semi-thin sections (1 μm) were cut using LKB V and Leica UC6 ultramicrotomes. The
16 sections were stained with 1% toluidine blue – 0.2% methylene blue mixture for 1-1.5 min at 60°C and
17 studied with Carl Zeiss Axioplan 2 microscope (Carl Zeiss) equipped with digital camera AxioCam
18 HRm (Carl Zeiss) and software AxioVision 3.1 (Carl Zeiss).

19 ***Study of cell proliferation with CLSM***

20 We used 5-ethynyl-2'-deoxyuridine (EdU) and Ser10-phosphorylated histone 3 (pH3)
21 monoclonal antibodies as the markers of cell proliferation. EdU is a nucleoside analog of thymidine,
22 which incorporates into nuclei of proliferating cells during DNA synthesis marking cells in the S-
23 phase⁷⁷. Antibody against phosphorylated histone 3 labels cells in the late G2-phase and M-phase⁷⁸.
24 To mark choanocytes' flagella, we used antibodies to acetylated- α -tubulin.

25 The EdU (Lumiprobe, 10540) stock solution was prepared in dimethyl sulfoxide (DMSO, MP
26 Biomedicals 196055). Optimal EdU concentration and incubation time were found out during the
27 preliminary studies. *H. dujardinii* individuals were incubated in 5 ml of FSW with 200 μM EdU for 12

1 hours, *L. variabilis* - in 5 ml of FSW with 20 μ M EdU for 6 hours. Sponges kept in 5 ml FSW with 100
2 or 10 μ M DMSO served as negative controls. We examined 8 individuals of *H. dujardinii* and 6
3 individuals of *L. variabilis*.

4 After the EdU incubation, all individuals were fixed with 4% paraformaldehyde in phosphate-
5 buffered saline (4% PFA PBS, Carl Roth 0335.2) for 12 hours at +4°C. Fixed specimens were rinsed
6 with PBS several times. Then they were incubated in 1-3% bovine serum albumin in PBS (BSA, MP
7 Biomedicals 0216006980) and permeabilized by 0.5% Triton X-100 (Sigma-Aldrich T8787) in PBS.
8 Click-reaction (visualization of EdU) was performed for 1 hour at RT in a solution of 4 mM CuSO₄, 10
9 μ M Sulfo-Cyanine3 Azide (Lumiprobe A1330), 20 mg/ml sodium L-ascorbate (Sigma-Aldrich 11140)
10 in PBS. Click-reaction and subsequent manipulations were carried out in the dark. Specimens were
11 blocked with a blocking solution of 1% BSA, 0.1% gelatin from cold-water fish skin (Sigma-Aldrich
12 G7041), 0.5% Triton X-100, 0.05% Tween-20 (Sigma-Aldrich P1379) in PBS and then incubated in
13 the primary antibodies (Rabbit Anti-phospho-histone H3, Sigma-Aldrich H0412 and Mouse Anti-
14 acetylated- α -tubulin, Sigma-Aldrich T6793, both diluted 1:1000 in blocking solution) overnight at +4°C.
15 After that, specimens were rinsed twice with the blocking solution and treated with the secondary
16 antibodies (Donkey anti-Mouse IgG Alexa Fluor 488, ThermoFisher Scientific A21202 and Donkey
17 anti-Rabbit IgG Alexa Fluor 647, ThermoFisher Scientific A-31573 in blocking solution, both final
18 concentration 1 μ g/ml) overnight at +4°C. Cell nuclei were stained with 4',6-diamidino-2-phenylindole
19 (DAPI, Acros 202710100) in PBS (final concentration 2 μ g/ml). Spicules of *L. variabilis* were dissolved
20 by 5% ethylenediaminetetraacetic acid (EDTA) in distilled water for 30 min at RT. Finally, specimens
21 rinsed with PBS were mounted in 90% glycerol (MP Biomedicals 193996) with 2.5% 1,4-
22 diazabicyclo[2.2.2]octane (DABCO, Sigma-Aldrich D27802) and studied with a confocal laser
23 scanning microscope.

24 Cell proliferation was studied in several body regions of sponges. Three regions were studied
25 in *H. dujardinii*: 1) the endosome adjacent to an oscular tube (n=8), 2) the endosome at some
26 distance (approximately half the body radius) from an oscular tube (n=8), 3) the oscular tube (n=4).
27 Seven regions were studied in *L. variabilis*: 1) the oscular rim (n=10), 2) the distal part of the oscular
28 tube (n=10), 3) the middle part of the oscular tube (n=10), 4) the proximal part of the oscular tube
29 (n=10), 5) the distal part of the diverticulum (n=14), 6) the proximal part of the diverticulum (n=14), 7)

1 the cormus tube (n=11). Studied fields of view within each region were chosen randomly. For
2 subsequent analysis, a Z-stack was obtained from each field of view.

3 Z-stacks were obtained via CLSM Nikon A1 (Nikon, Shinagawa, Japan) using lasers of
4 wavelength 405 nm (DAPI), 488 nm (Mouse Anti-acetylated- α -tubulin ABI + DAM IgG Alexa Fluor 488
5 ABII), 561 nm (EdU + Sulfo-Cyanine3 Azide) and 648 nm (Rabbit Anti-phospho-histone H3 ABI +
6 DAR IgG Alexa Fluor 647). All Z-stacks were obtained with a 1- μ m Z-step, 48 μ m (*H. dujardinii*) and
7 68 (*L. variabilis*) μ m thick on average.

8 During subsequent Z-stack analysis, we estimated the total fractions of EdU- and pH3-
9 positive nuclei (as a proportion of EdU-/pH3-positive nuclei to all nuclei stained with DAPI), the
10 fractions of EdU- and pH3-positive choanocytes and mesohyl cells (as a proportion of EdU-/pH3-
11 positive choanocytes/mesohyl cells to all nuclei stained with DAPI) and the fraction of choanocytes
12 among all EdU/pH3-positive cells (as a proportion of EdU-/pH3-positive choanocytes to all EdU-/pH3-
13 positive cells).

14 Z-stack analysis was performed with Bitplane Imaris v7.2.1 software. Each stack of *H.*
15 *dujardinii* and *L. variabilis* contained 6000-7000 and 2000-4000 cells, respectively. Choanocytes were
16 identified by the presence of flagella and the characteristic shape of choanoderm/choanocyte
17 chambers. Mesohyl cells were separated from choanocytes via the «Surface» tool. Cells of interest
18 (e.g., EdU- or pH3-positive cells) were counted by the «Spots» tool; we combined automatic
19 identification of nuclei via Quality/Intensity Median thresholds with manual correction of spots. Such
20 counting procedure implies 3D visualization of a whole Z-stack and allows us to precisely distinguish
21 between labeled cells of choanoderm and mesohyl.

22 ***EdU tracking experiment in H. dujardinii***

23 Intact *H. dujardinii* individuals were incubated in 5 ml of FSW with 200 μ M EdU for 12 hours
24 and then transferred into a running seawater aquarium. Sponges incubated in 5 ml of FSW with 100 μ l
25 of DMSO were used as negative controls. Sponges were fixed with 4% PFA PBS at 1, 5 and 7 days
26 after transferring into the aquarium (3, 3, and 5 individuals, respectively). Individuals fixed immediately
27 after the EdU incubation served as the initial time point – 0 days. All specimens were treated for

1 CLSM studies as described above (see “Study of cell proliferation with CLSM” section), but no
2 antibody staining was performed in this experiment. We studied areas of the endosome at some
3 distance from an oscular tube. One randomly chosen field of view within the region was analyzed in
4 each individual. For subsequent analysis, a Z-stack was obtained from each field of view. We
5 estimated the fractions of choanocytes and mesohyl cells among all EdU-positive cells (as a
6 proportion of EdU-positive choanocytes/mesohyl cells to all EdU-positive cells). As a result, the main
7 parameter of the analysis was the mean proportion of choanocytes and mesohyl cells in the total
8 number of EdU-positive cells. Details of data acquisition, nuclei counting, and further analysis have
9 been described above (see “Study of cell proliferation with CLSM” section). In this experiment,
10 choanocyte chambers were identified by their characteristic shape visualized by DAPI staining.

11 ***Evaluation of cell cycle distribution in intact tissues***

12 We combined calcium and magnesium-free seawater (20 mM KCl, 300 mM NaCl, 10 mM
13 Tris-HCl and 15 mM EDTA in distilled water) (CMFSW-E) and mechanical tissue dissociation to obtain
14 single-cell suspensions. Sponges were cleaned from epifauna, soaked in CMFSW-E for 30 min and
15 squeezed into CMFSW-E through a sterile gauze with a mesh size of 30 µm. After 30 min,
16 suspensions were centrifuged and resuspended in a new portion of CMFSW-E. After another 30 min,
17 suspensions were fixed with 70% cold ethanol at -20°C. Such dissociation technique resulted in poor
18 quality of the cell cycle curve in case of *L. variabilis*. An alternative fixation method was used: intact
19 pieces of *L. variabilis* were first fixed in cold 70% ethanol. After the fixation, tissues were soaked in
20 CMFSW-E and mechanically dissociated through gauze with a mesh size of 30 µm.

21 Cell suspensions from five *H. dujardinii* and five *L. variabilis* were studied for cell cycle
22 distribution, using propidium-iodide (PI) staining since PI fluorescence is proportional to DNA
23 content³⁷. Cell suspensions were resuspended in PBS and stained with 1 µg/ml PI (P1304MP,
24 ThermoFisher Scientific) for at least 12 hours. Unstained suspensions served as a negative control.
25 Suspensions were analyzed by a FACSAria SORP instrument (BD Biosciences, USA) using an
26 excitation laser wavelength of 561 nm (PI excitation); 50 000 events were collected per sample. The
27 data were processed via FlowJo V10. Primary gating was performed according to forward (FCS-A)
28 versus side scatter (SSC-A) parameters; secondary gating on singlet cells - according to FSC-W
29 versus FSC-H parameters.

1 To obtain cell cycle distribution, we used the internal FlowJo CellCycle plugin. The Watson
2 model approximates DNA content curve as a sum of two Gaussian distributions represented by
3 G0/G1 and G2/M cells, considering cells between them as cells in the S phase. In most cases,
4 standard parameters were used; when the G2/M peak was hard to distinguish, we constrained its
5 position depending on the relative positions of the G0/G1 and G2/M peaks. No CV constraint was
6 performed.

7 ***Study of apoptotic activity in intact tissues***

8 Tetramethylrhodamine ethyl ester (TMRE, ThermoFisher Scientific, T669) and CellEvent
9 Caspase-3/7 Green ReadyProbes Reagent (ThermoFisher Scientific, R37111) (CellEvent) were used
10 as markers of apoptotic activity for both CLSM and flow cytometry studies. The absence of TMRE
11 fluorescence serves as a sign of the mitochondrial inner membrane permeabilization, which is
12 characteristic of the early stages of apoptosis. CellEvent is an indicator of activated effector caspases
13 present in cells during the middle and late stages of apoptosis⁶³. In each experiment, we used
14 unstained specimens as negative controls.

15 *CellEvent staining of intact tissues for CLSM.* Specimens (3 sponges per species) were
16 incubated in 5 ml of FSW with CellEvent (dilution 1:100) for 2 hours at +10°C in the dark. After the
17 incubation, they were fixed with 4% PFA PBS. Then specimens were fixed with 4% PFA PBS, washed
18 with PBS and blocking solution for several hours, and stained with DAPI as described above (see
19 “Study of cell proliferation with CLSM” section). Specimens of *L. variabilis* were stained with
20 antibodies to acetylated- α -tubulin to distinguish choanocytes. In case of *H. dujardinii*, no antibody
21 staining was performed, and choanocyte chambers were identified by their characteristic shape
22 visualized by DAPI staining. During this experiment, we used lasers of 405 nm (DAPI), 488 nm
23 (CellEvent) and 648 nm (Mouse Anti-acetylated- α -tubulin ABI + DAM IgG Alexa Fluor 647 ABII)
24 wavelengths. Details of data acquisition and nuclei counting are provided in the “Study of cell
25 proliferation with CLSM” section. Any nuclei displaying bright CellEvent staining was considered an
26 apoptotic; during the analysis, we estimated the total fraction of CellEvent-positive cells (as a
27 proportion of CellEvent-positive nuclei to all nuclei labeled with DAPI) and the proportion of

1 choanocytes/mesohyl cells among all CellEvent-positive cells (as a proportion of CellEvent-positive
2 choanocytes/mesohyl cells to all CellEvent-positive cells).

3 *CellEvent staining of suspensions for flow cytometry.* We took samples of 250 ml containing
4 1.5×10^6 cells from non-fixed CMFSW-E suspensions of *H. dujardinii* and *L. variabilis* (4 samples from
5 4 sponges per species). The suspensions were obtained as described in the “Evaluation of cell cycle
6 distribution in intact tissues” section. The samples were incubated in CellEvent (dilution 1:100) at
7 +10°C for 30 min in the dark. The samples were then centrifuged and fixed with 4% PFA PBS at +4°C
8 and stored for 2 months. Before the flow cytometry analysis, cells were washed thrice with PBS.
9 Suspensions were analyzed by a FACSAria SORP instrument (BD Biosciences, USA) using an
10 excitation laser wavelength of 488 nm (CellEvent excitation). Primary gating was described above
11 (see the “Evaluation of cell cycle distribution in intact tissues” section); the final quadrant gating to
12 distinguish CellEvent-positive cells was set according to unstained control. Any event exceeding the
13 autofluorescence of the unstained control was considered a CellEvent-positive (apoptotic) cell.
14 Subsequent flow cytometry studies showed that *L. variabilis* suspensions contained an anomalously high
15 number of CellEvent-positive (apoptotic) cells. Thus, the data obtained from these samples were not
16 used in the main analysis.

17 *Vital staining of suspensions for CLSM and flow cytometry.* For vital CLSM study, the
18 suspensions were obtained as described in the “Evaluation of cell cycle distribution in intact tissues”
19 section. The suspensions were stained with TMRE (final concentration - 100 μ M), CellEvent (dilution
20 1:100), and Hoechst 33342 (dilution 1:100) at +10°C for 30 min in the dark. During this experiment,
21 we used lasers of wavelength 405 nm (DAPI), 488 nm (CellEvent), and 561 nm (TMRE). Data
22 acquisition has been described above (see “Study of cell proliferation with CLSM” section). No
23 quantitative analysis was performed; acquired images were used to illustrate flow cytometry data.

24 For vital flow cytometry study, three individuals of *H. dujardinii* (August 2020) and two
25 individuals of *L. variabilis* (February 2020) were transported to Moscow in thermoses with natural
26 seawater at +10°C. Sponges were dissociated in CMFSW-E for 30 min and stained with TMRE and
27 CellEvent as described above. After the staining, the suspensions were analyzed by a FACSAria
28 SORP instrument (BD Biosciences, USA) using excitation laser wavelength of 488 nm (CellEvent
29 excitation) and 561 nm (TMRE excitation). Primary gating was described above (see the “Evaluation

1 of cell cycle distribution in intact tissues” section); the final quadrant gating to distinguish between
2 different cell populations was performed according to unstained control. Any event exceeding the
3 autofluorescence of unstained control was considered a TMRE/CellEvent-positive cell.

4 ***Statistical analysis and plotting***

5 Statistical analysis and plotting were performed in GraphPad PRISM v8.0.1. Mean value and
6 standard deviation were calculated for every parameter (the proportions of EdU-positive cells, pH3-
7 positive cells, etc.). To compare these parameters between different body parts or time points, we
8 used Kruskal-Wallis test followed by Dunn test since it is a distribution-free test appropriate for
9 multiple comparisons between small data subsets. The significance level was 0.05. All p-values
10 represented in the text are Dunn test p-values. Tables with exact p-values for all tests are provided in
11 the Mendeley Data root directory (see “Availability of data and material” section).

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21 advice.

22 **Authors' contributions**

23 NM, AL and AS designed the study. NM, AL, FB, KS and VF collected the material, carried out CLSM
24 proliferation studies and analyzed data via Bitplane Imaris. NM conducted experiments and analyzed
25 data from the pulse-chase EdU studies, apoptosis investigations in intact tissues, DNA content and

1 apoptosis evaluation in cell suspensions, and performed the statistical analysis. AL, NM, AE and AS
2 prepared the manuscript with contributions from all authors. All authors reviewed and approved the
3 final manuscript.

4 **Availability of data and materials**

5 The draft dataset supporting the conclusions of this article is available in the Mendeley Data
6 repository, <https://data.mendeley.com/datasets/hhmc8t6gv7/draft?a=ee1a45f9-2b2d-4b65-b863-ec4e84785263>
7 [ec4e84785263](https://data.mendeley.com/datasets/hhmc8t6gv7/draft?a=ee1a45f9-2b2d-4b65-b863-ec4e84785263) (*H.dujardinii*; doi:10.17632/hhmc8t6gv7.1) and
8 <https://data.mendeley.com/datasets/vbk6h8vcyy/draft?a=97e2ee7f-68e0-455f-b36b-2ad25872ff9a> (*L.*
9 *variabilis*; doi:10.17632/vbk6h8vcyy.1).

10 **List of abbreviations**

- 11 FSW - filtered seawater
- 12 EdU - 5-ethynyl-2'-deoxyuridine
- 13 pH3 - Ser10-phosphorylated histone 3
- 14 DMSO - dimethyl sulfoxide
- 15 PFA - paraformaldehyde
- 16 PBS - phosphate-buffered saline
- 17 CMFSW-E - calcium and magnesium-free seawater
- 18 TMRE - tetramethylrhodamine ethyl ester
- 19 CellEvent or CE - Caspase-3/7 Green ReadyProbes™ Reagent
- 20 PI - propidium iodide
- 21 CLSM - confocal laser scanning microscopy

1 MOMP - mitochondrial outer membrane permeabilization

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11

12

1 Figure legends

2 **Figure 1.** Anatomy and histology of *Halisarca dujardini*. A – general appearance; B – schematic
3 drawing of the aquiferous system representing regions of interest; C – section through the endosome
4 and ectosome, light microscopy; D – area of endosome distant from the oscular tube, CLSM
5 (maximum intensity projection of several focal planes); blue color – DAPI, cell nuclei; green –
6 acetylated α -tubulin, flagella. Black dotted line marks the border between the ecto- and endosome.
7 White dotted lines mark the borders of several choanocyte chambers. ar – archaeocyte, cc –
8 choanocyte chamber, cf – collagen fiber, en – endosome, ec – ectosome, fl – flagella, gr – granular
9 cell, mes – nucleus of mesohyl cell, sph – spherulous cell. Scale bars: A – 1 mm, C, D – 50 μ m.

10 **Figure 2.** Cell proliferation in different body regions of *Halisarca dujardini*, CLSM. A - endosome
11 distant from the oscular tube, B - endosome adjacent to the oscular tube, C – the oscular tube. All
12 images are maximum intensity projections of several focal planes. Insets schematically show position
13 of the studied regions in the sponge body. Blue color – DAPI, cell nuclei; red – EdU, nuclei of DNA
14 synthesizing cells; yellow – pH3, chromatin of late G2/M cells; green – acetylated α -tubulin, flagella.
15 Dotted lines mark the borders of choanocyte chambers. White arrowheads mark cells in the M-phase
16 of the cell cycle; red arrowheads mark EdU-positive cells outside the choanocyte chambers. cc -
17 choanocyte chambers (outlined by the dotted lines). Scale bars: A, B, C - 50 μ m.

18 **Figure 3.** Quantitative analysis of cell proliferation in intact tissues of *Halisarca dujardini*. A, B -
19 fractions of EdU- (A) and pH3-positive (B) cells in different body regions. Data are shown with median
20 values (thick horizontal lines), interquartile ranges (boxes), total ranges (whiskers), and outliers (dots).
21 Asterisks mark significantly different regions of the body. C, D – fractions (mean values) of
22 choanocytes/mesohyl cells among the total number of EdU- (C) and pH3-positive (D) cells.

23 **Figure 4.** Anatomy and histology of *Leucosolenia variabilis*. A – general appearance; B – schematic
24 drawing of the aquiferous system representing regions of interest; C – longitudinal section through the
25 distal part of the oscular tube (including the oscular rim), light microscopy; D, E – cross sections
26 through the middle part of the oscular tube, light microscopy; F – the proximal part of the divericulum,
27 CLSM (maximum intensity projection of several focal planes); G – orthogonal XZ projection of the
28 same Z-stack, CLSM (maximum intensity projection of several focal planes); blue color – DAPI, cell
29 nuclei; green – acetylated α -tubulin, flagella. Osc. rim – the oscular rim; Osc. dist - distal part of the

1 oscular tube; Osc. mid. - middle part of the oscular tube; Osc. prox. - proximal part of the oscular tube;
2 Div. dist. - distal part of the diverticulum; Div. prox. - proximal part of the diverticulum; Cormus tube -
3 cormus tube. ch – choanoderm; en – endopinacocytes, ex – exopinacocytes; ex+mes –
4 exopinacoderm/mesohyl layer; or – oscular rim; os – ostium; pc – porocyte; sp – spicule. Dotted lines
5 mark the borders between the choanoderm and exopinacoderm/mesohyl layers. Scale bars: A – 0.2
6 mm; C, D – 50 μ m; E – 25 μ m; F – 50 μ m.

7 **Figure 5.** Cell proliferation in different body regions of *Leucosolenia variabilis*, CLSM. A – oscular rim;
8 B – the distal part of an oscular tube; C - the middle part of an oscular tube; D - the proximal part of an
9 oscular tube; E - the proximal part of a diverticulum; F - the distal part of a diverticulum; G - a cormus
10 tube; E – metaphase plate in the choanoderm of the middle part of the oscular tube. Images with the
11 “xz” index represent XZ orthogonal projections of the respective Z-stack. All images are maximum
12 intensity projections of several focal planes. Insets schematically show position of the studied regions
13 in the sponge body. Blue color – DAPI, cell nuclei; red – EdU, nuclei of DNA synthesizing cells; yellow
14 – pH3, chromatin of late G2/M cells; green – acetylated α -tubulin, flagella. Arrowheads mark cells in
15 the M-phase of the cell cycle. Scale bars: A, B, C, D, E, F, G – 50 μ m.

16 **Figure 6.** Quantitative analysis of cell proliferation in different body regions of *Leucosolenia variabilis*.
17 A, B - fractions of EdU- (A) and pH3-positive (B) cells in different body regions. Data are shown with
18 median values (thick horizontal lines), interquartile ranges (boxes), total ranges (whiskers), and
19 outliers (dots). C, D – fractions (mean values) of choanocytes/mesohyl cells among the total number
20 of EdU- (C) and pH3-positive (D) cells, respectively. Asterisks mark significantly different regions of
21 the body. Osc. rim - oscular rim; Osc. dist - distal part of an oscular tube; Osc. mid. - middle part of an
22 oscular tube; Osc. prox. - proximal part of an oscular tube; Div. dist. - distal part of a diverticulum; Div.
23 prox. - proximal part of a diverticulum; Cormus - cormus tube.

24 **Figure 7.** EdU tracking experiment in *Halisarca dujardini*, CLSM. A - 0 days after the incubation in
25 EdU (maximum intensity projection of several focal planes); B - 7 days after the incubation in EdU
26 (maximum intensity projection of several focal planes); blue color – DAPI, cell nuclei, red – EdU,
27 nuclei of DNA synthesizing cells. C - fractions of labeled choanocytes and mesohyl cells among all
28 EdU-positive cells (mean values). Dotted lines mark the borders of choanocyte chambers.
29 Arrowheads in B mark EdU-positive nuclei in mesohyl. Scale bars: A, B - 50 μ m.

1 **Figure 8.** Single-parameter histograms of PI fluorescence (DNA content) in *Halisarca dujardini* (A)
2 and *Leucosolenia variabilis* (B) cell suspensions. C, D – an example of light-scatter primary gating to
3 distinguish single cells in a suspension of *L. variabilis*. G0/G1 (red) are diploid cells, G2/M (blue) are
4 tetraploid cells, S (green) are DNA-synthesizing cells. SubG1 cells are cells with fragmented DNA.

5 **Figure 9.** Apoptosis in intact tissues of *Halisarca dujardini* and *Leucosolenia variabilis*, CLSM. A – the
6 endosome of *H. dujardini*; B, C – enlarged single-channel images of the zone in the white rectangle in
7 A; D – the proximal part of the oscular tube of *L. variabilis*; E, F, G – enlarged single-channel images
8 of the zone in the white rectangle in D. Several focal planes are combined into one picture. Blue color
9 – DAPI, cell nuclei; magenta – CellEvent, nuclei of apoptotic cells; green – acetylated α -tubulin,
10 flagella. Magenta arrowheads mark CellEvent-positive cells. Scale bars: A, D - 50 μm ; B, C, E, F, G -
11 25 μm .

12 **Figure 10.** Apoptosis in cell suspensions of *Halisarca dujardini* (A, B) and *Leucosolenia variabilis* (C,
13 D), flow cytometry. A, C - Pacific Blue-A (autofluorescence) vs FITC-A (CE fluorescence) dot plots; B,
14 D – single-parameter histograms of CellEvent fluorescence. CE(+) population gate is set according to
15 the negative control.

16 **Figure 11.** *In vivo* TMRE and CellEvent staining of *Halisarca dujardini* and *Leucosolenia variabilis*
17 suspensions. A - CLSM of *L. variabilis* suspension, scale bar 10 μm ; blue color - Hoechst 33342, cell
18 nuclei; orange - TMRE, functional mitochondria; magenta - CellEvent, nuclei of apoptotic cells; B -
19 scatter plot of FSC-A (cell size) vs SSC-A (cell granularity), an example of primary gating; C, D -
20 TMRE vs CellEvent dot plot of *L. variabilis* (C) and *H. dujardini* (D) suspensions. The borders of
21 quadrants (Q1-Q4) are set by the negative controls.

22

1 Tables

2 **Table 1.** Fractions of proliferating cells in intact tissues of *Halisarca dujardini*, CLSM.

Body region	Distant from the oscular tube	Adjacent to the oscular tube	Oscular tube
n	8	8	4
Fraction of EdU-positive cells among all cells	8.10±1.64%	8.44±3.08%	0.61±0.64%
Fraction of EdU-positive choanocytes among all cells	7.39±1.52%	7.76±3.03%	
Fraction of EdU-positive mesohyl cells among all cells	0.71±0.56%	0.68±0.32%	
Fraction of choanocytes among EdU-positive cells	92.08±6.11%	90.27±7.03%	
Fraction of pH3-positive cells among all cells	0.11±0.07%	0.11±0.07%	No pH3-cells were found
Fraction of pH3-positive choanocytes among all cells	0.10±0.06%	0.10±0.06%	
Fraction of pH3-positive mesohyl cells among all cells	0.01±0.02%	0.01±0.02%	
Fraction of choanocytes among pH3-positive cells	88.33±11.18%	94.64±9.83%	

3 Each fraction is given as mean value and SD. Eight individuals have been examined; n is a number
4 of analyzed stacks.

5

1 **Table 2.** Fractions of proliferating cells in different body regions of *Leucosolenia variabilis*, CLSM.

Body region	Cormus tube	Proximal part of the diverticulum	Distal part of the diverticulum	Proximal part of the oscular tube	Middle part of the oscular tube	Distal part of the oscular tube	Oscular rim
n	11	14	14	10	10	10	10
Fraction of EdU-positive cells among all cells	9.44±1.58%	9.84±2.60%	6.53±2.08%	9.76±1.91%	11.01±1.76%	10.58±1.66%	0.40±0.96%
Fraction of EdU-positive choanocytes among all cells	9.00±1.59%	9.14±2.33%	6.07±2.16%	9.43±1.95%	10.62±1.55%	10.19±1.57%	
Fraction of EdU-positive mesohyl cells among all cells	0.44±0.22%	0.71±0.68%	0.46±0.55%	0.33±0.25%	0.40±0.73%	0.39±0.38%	
Fraction of choanocytes among EdU-positive cells	95.20±2.61%	93.00±4.73%	92.08±9.89%	96.48±2.91%	96.68±5.11%	96.36±2.96%	
Fraction of pH3-positive cells among all cells	0.56±0.18%	0.64±0.24%	0.50±0.20%	0.71±0.36%	0.69±0.18%	0.74±0.17%	0.17±0.20%
Fraction of pH3-positive choanocytes among all cells	0.43±0.16%	0.41±0.17%	0.21±0.15%	0.56±0.30%	0.54±0.16%	0.52±0.16%	
Fraction of pH3-positive mesohyl cells among all cells	0.13±0.07%	0.22±0.13%	0.29±0.15%	0.15±0.07%	0.15±0.10%	0.22±0.09	
Fraction of choanocytes among pH3-positive cells	78.31±12.93%	65.30±15.43%	40.64±22.29%	79.09±7.06%	78.70±11.22%	70.25±10.94%	

2 Each fraction is given as mean value and SD. Six individuals have been examined; n is the number of
 3 analyzed stacks.

1 **Table 3.** Fractions of cells in different phases of the cell cycle, flow cytometry.

Species	N	SubG1	G0/G1	S	G2/M
<i>Halisarca dujardinii</i>	5	23.41±12.22%	38.94±5.9%	20.18±6.14%	9.28±1.78%
<i>Leucosolenia variabilis</i>	5	26.42±3.43%	53.88±2.59%	12.58±0.76%	4.63±1.79%

2 Each proportion is given as mean value and SD.

3 **Table 4.** Fractions of apoptotic cells in the intact tissues of *H. dujardinii* and *L. variabilis*, CLSM.

Species	Body region	n	CE-positive cells (% of all cells)
<i>H. dujardinii</i>	Distant from the oscular tube	7	0.03±0.05%
<i>L. variabilis</i>	Oscular tube	9	0.22±0.19%
	Cornus tube	6	0.44±0.66%
	Diverticula	3	0.21±0.16%

4 Each proportion is given as mean value and SD. Three individuals per species have been examined;

5 n is the number of analyzed stacks.

6 **Table 5.** Fractions of cell populations revealed during the *in vivo* apoptosis investigation, flow

7 cytometry.

Species	N	TMRE- CE-	TMRE+ CE-	TMRE+ CE+	TMRE- CE+
<i>Halisarca dujardinii</i>	3	1.67±0.35%	89.67±1.06%	7.51±0.87%	0
<i>Leucosolenia variabilis</i>	2	13.7±3.25%	70.55±1.48%	8.58±0.77%	0.06±0.01%

- 1 The fraction of each population is represented by mean value and SD. N is the number of analyzed
- 2 individuals.