A peer-reviewed version of this preprint was published in PeerJ on 26 August 2019.

View the peer-reviewed version (peerj.com/articles/7579), which is the preferred citable publication unless you specifically need to cite this preprint.

Cell proliferation controls body size growth, tentacle morphogenesis, and regeneration in hydrozoan jellyfish *Cladonema pacificum*

Sosuke Fujita¹, Erina Kuranaga¹, Yuichiro Nakajima Corresp.¹, ²

¹ Graduate School of Life Sciences, Tohoku University, Sendai, Japan
² Frontier Research Institute for Interdisciplinary Sciences, Tohoku University, Sendai, Japan

Corresponding Author: Yuichiro Nakajima
Email address: yuichiro.nakajima.d2@tohoku.ac.jp

Jellyfish have existed on the earth for around six hundred million years and have evolved in response to environmental changes. Hydrozoan jellyfish, members of phylum Cnidaria, exist in multiple life stages, including planula larvae, vegetatively-propagating polyps, and sexually-reproducing medusae. Although free-swimming medusae display complex morphology and exhibit increase in body size and regenerative ability, their underlying cellular mechanisms are poorly understood. Here, we investigate the roles of cell proliferation in body-size growth, appendage morphogenesis, and regeneration using *Cladonema pacificum* as a hydrozoan jellyfish model. By examining the distribution of S phase cells and mitotic cells, we revealed spatially distinct proliferating cell populations in medusae, uniform cell proliferation in the umbrella, and local cell proliferation in tentacle bulbs. Blocking cell proliferation by hydroxyurea caused inhibition of body size growth and defects in tentacle branching, nematocyte differentiation, and regeneration. Local cell proliferation in tentacle bulbs is observed in medusae of two other hydrozoan species, *Cytaeis uchidae* and *Rathkea octopunctata*, indicating that it may be a conserved feature among hydrozoan jellyfish. Altogether, our results suggest that hydrozoan medusae possess actively proliferating cells and provide experimental evidence regarding the role of cell proliferation in body-size control, tentacle morphogenesis, and regeneration.
Cell proliferation controls body size growth, tentacle morphogenesis, and regeneration in hydrozoan jellyfish *Cladonema pacifica*

*Sosuke Fujita, Erina Kuranaga and Yu-ichiro Nakajima*

1Graduate School of Life Sciences, Tohoku University, Sendai, Japan
2Frontier Research Institute for Interdisciplinary Sciences, Tohoku University, Sendai, Japan

Corresponding Author: Yu-ichiro Nakajima
Address: 6-3 Aoba, Aramaki-Aza, Aoba-ku, Sendai, 980-8578,
Email address: yuichiro.nakajima.d2@tohoku.ac.jp

Abstract

Jellyfish have existed on the earth for around six hundred million years and have evolved in response to environmental changes. Hydrozoan jellyfish, members of phylum Cnidaria, exist in multiple life stages, including planula larvae, vegetatively-propagating polyps, and sexually-reproducing medusae. Although free-swimming medusae display complex morphology and exhibit increase in body size and regenerative ability, their underlying cellular mechanisms are poorly understood. Here, we investigate the roles of cell proliferation in body-size growth, appendage morphogenesis, and regeneration using *Cladonema pacificum* as a hydrozoan jellyfish model. By examining the distribution of S phase cells and mitotic cells, we revealed spatially distinct proliferating cell populations in medusae, uniform cell proliferation in the umbrella, and local cell proliferation in tentacle bulbs. Blocking cell proliferation by hydroxyurea caused inhibition of body size growth and defects in tentacle branching, nematocyte differentiation, and regeneration. Local cell proliferation in tentacle bulbs is observed in medusae of two other hydrozoan species, *Cytaeis uchidae* and *Rathkea octopunctata*, indicating that it may be a conserved feature among hydrozoan jellyfish. Altogether, our
results suggest that hydrozoan medusae possess actively proliferating cells and provide experimental evidence regarding the role of cell proliferation in body-size control, tentacle morphogenesis, and regeneration.

**Introduction**

Cell proliferation lies at the core of controlling cell number in Metazoa and thus contributes to the growth and the maintenance of animal body and organs (Leevers & McNeill 2005; Penzo-Mendez & Stanger 2015). During development, cell proliferation plays a critical role in body-size increase by adding cells into tissue layers, and it further generates cellular resources for different cell types by multiplying progenitors (Gillies & Cabernard 2011; Hardwick et al. 2015). Later in adults, proliferating cells are required for physiological cell turnover and for the replacement of damaged cells after tissue injury (King & Newmark 2012; Pellettieri & Sanchez Alvarado 2007). These roles of cell proliferation in multicellularity must be conserved throughout evolution: indeed, sponges, one of the earliest metazoan organisms, have acquired mechanisms to allow cell turnover by controlling proliferative capacities (Alexander et al. 2014; Kahn & Leys 2016).

As the sister group of bilaterians and early-branching metazoans, cnidarians have been studied as a model to understand evolutionary development (Genikhovich & Technau 2017). Cnidarians are diploblastic and radially symmetric animals that include diverse species such as corals, sea anemones, hydroids, and jellyfish (Technau & Steele 2011). During the embryonic development of the sea anemone Nematostella vectensis, cell proliferation is coordinated with epithelial organization and is involved in tentacle development (Fritz et al. 2013; Ragkousi et al. 2017). Cnidarians are also known for their regenerative abilities: for instance, Hydra polyps have been used for a century to investigate mechanisms of metazoan regeneration (Fujisawa 2003; Galliot & Schmid 2002). The basal head regeneration of Hydra relies on cell proliferation triggered by dying cells (Chera et al. 2009b; Galliot & Chera 2010). Hydractinia polyps regenerate through cell proliferation and the migration of stem-like cells (Bradshaw et al. 2015; Gahan et al. 2016). Although much has been learned about mechanisms controlling embryogenesis and growth during regeneration, it is unclear how cnidarians
integrate cell proliferation to control their body size and maintain tissue homeostasis under normal physiological conditions.

Among cnidarians, hydrozoan jellyfish have a complex life cycle including planula larvae, sessile polyps, and free-swimming medusae. While polyps undergo asexual reproduction to grow vegetatively, medusae generate gametes to perform sexual reproduction. Despite the limited life span compared to the long-lived or possibly immortal polyps, the size of medusae increases dramatically (Hansson 1997; Miyake et al. 1997). Furthermore, medusae maintain their regenerative capacity for missing body parts by integrating dedifferentiation and transdifferentiation (Schmid & Alder 1984; Schmid et al. 1988; Schmid et al. 1982). Recent studies using the hydrozoan jellyfish Clytia hemisphaerica have provided mechanistic insights into embryogenesis, nematogenesis, and egg maturation (Denker et al. 2008; Momose et al. 2008; Quiroga Artigas et al. 2018). However, little is known about the mechanism that controls body size growth in medusae. It is also unclear whether cell proliferation is required for tentacle morphogenesis and regeneration of hydrozoan jellyfish.

The hydrozoan jellyfish Cladonema is an emerging model, with easy lab maintenance and a high spawning rate, that is suitable for studying diverse aspects of biology including development, regeneration, and physiology (Fujiki et al. 2019; Graziussi et al. 2012; Suga et al. 2010; Takeda et al. 2018b; Weber 1981). Cladonema is characterized by small-sized medusae with branched tentacles. Using specialized adhesive tentacles, Cladonema can adhere to different substrata, such as seaweed, in the field. The species Cladonema pacificum, originally found along coastal areas in Japan, have nine main tentacles with a stereotyped branching pattern (Fig. 1A). During the Cladonema medusa’s maturation, body size increases, and each main tentacle grows and exhibits branching morphology (Fujiki et al. 2019), providing an ideal system to dissect the cellular mechanisms associated with jellyfish growth and morphogenesis.

In this study, we investigate the role of cell proliferation in medusa growth and morphogenesis, using Cladonema pacificum as a model of hydrozoan jellyfish. We show that cell proliferation occurs evenly across the medusa body, including the umbrella and manubrium, with the exception of the tentacles, where cell proliferation is spatially localized to the bulb area. Blocking cell-cycle progression with a
pharmacological assay inhibits the increase of body size, tentacle branching, and nematocyte differentiation, which suggests that cell proliferation is necessary for growth and tentacle morphogenesis. We further show that cell proliferation is required for tentacle regeneration in *Cladonema* medusae. Our findings reveal cell proliferation’s critical roles in the development and maintenance of the *Cladonema* body and appendages and provide a basis for understanding growth-control mechanisms in hydrozoan jellyfish.

**Materials & Methods**

**Animal cultures**

We used *Cladonema pacificum* (strains 6W and UN2), *Cytaeis uchidae* (strain ♀17) and *Rathkea octopunctata* (strain MF-1) medusae for this research. The medusae were cultured in plastic cups (V-type container, V-7 and V-8, AS ONE) at 20°C (*Cladonema* and *Cytaeis*) or 4°C (*Rathkea*), and their polyps were maintained in the cups (V-7) at 20 °C or 4 °C in darkness. Vietnamese brine shrimp (A&A Marine LLC) were fed to medusae and polyps. Artificial sea water (ASW) was prepared by SEA LIFE (Marin Tech, Tokyo). Pictures of medusae were taken through a LEICA S8APO microscope with a Nikon digital camera (D5600).

**Immunofluorescence**

The medusae were anesthetized with 7% MgCl₂ in ASW for 10 min and fixed 4% paraformaldehyde (PFA) in ASW for 1 hr. After fixation, the samples were rinsed in 1x PBS and washed 3 times (10 min each) in PBS containing 0.1% Triton X-100 (0.1% PBT). The samples were incubated in primary antibodies in 0.1% PBT overnight at 4 °C. The antibodies used were rabbit anti-Phospho-Histone H3 (Ser10) (1:500; Upstate, 06-570) and mouse anti-α-Tubulin (1:500; SIGMA, T6199). After the primary antibody incubation, the samples were washed 3 times (10 min each) in 0.1% PBT and incubated in secondary antibodies (1:500; ALEXA FLUOR Goat anti-mouse IgG, ALEXA FLUOR Goat anti-rabbit IgG, Life Technologies) and Hoechst 33342 (1:250; Thermo Scientific) in 0.1% PBT for 1hr in dark. After 4 washes (10 min each) in 0.1% PBT, the samples
were mounted on slides with 70% glycerol. Confocal images were collected through Leica SP8 or SP5 confocal microscopes. Z-stack images were performed using ImageJ/Fiji software.

**EdU labeling**
The medusae were incubated with 20 μM EdU (EdU kit; Invitrogen, 1836341) in ASW for 24 hr or 150 μM for 1hr. After EdU treatment, the medusae were anesthetized with 7% MgCl₂ in ASW for 10 min and fixed 4% paraformaldehyde (PFA) in ASW for 1 hr. After fixation, the samples were rinsed in 1x PBS and washed 3 times (10 min each) in 0.1% PBT. The samples were incubated with a EdU reaction cocktail (1x reaction buffer, CuSO₄, Alexa Fluor azide, and 1x reaction buffer additive; all included in EdU kit; Invitrogen, 1836341) for 30 min in the dark. After the EdU reaction, the samples were washed 3 times (10 min each) in 0.1% PBT and Hoechst 33342 (1:250; Thermo Scientific) in 0.1% PBT for 1hr in dark. The samples were washed 4 times (10 min each) in 0.1% PBT and were mounted on slides with 70% glycerol.

**Hydroxyurea treatment**
The live medusae were incubated with 10mM hydroxyurea (HU) (Wako, LKP3349) in ASW (ASW only for control) (Fig. 2-4). HU incubation was continued for 9 days and HU solution or ASW was changed every other day.

**Measurement of umbrella size and tentacle length**
Pictures of medusae were taken with a Nikon D5600, and umbrella size was measured using polygon selections with ImageJ software (Fig. 2B). We measured the length and width of medusae under the microscope using an ocular micrometer and multiplied the length and width to generate a value for umbrella size (Fig. 2D). Tentacle length was measured daily under the microscope with an ocular micrometer (Fig. 4D).

**DAPI poly-γ-glutamate staining**
This protocol was adapted from (Szczepanek et al. 2002): The medusae were anesthetized with 7% MgCl₂ in ASW for 10 min and fixed with 4% PFA in ASW for 1 hr.
After fixation, the samples were rinsed in 1x PBS and washed 3 times (10 min each) in 0.1% PBT. The samples were incubated in DAPI (1:500; Polysciences, Inc.) in PBT for 60 min. After the DAPI incubation, samples were washed 4 times (10 min each) in PBT and mounted on slides with 70% glycerol in DW. Samples were scanned with a combination of 488nm excitation and 555nm emission filter using either Leica SP8 or SP5 confocal microscopes. Using ImageJ, we performed Z-stacks and counted nematocysts. Empty nematocysts were counted manually.

**Dissection of tentacles for regeneration**

Tentacles’ basal sides were dissected with small scissors, leaving the tentacle bulbs intact. Amputated medusae were fed every other day.

**Results**

**Cell proliferation patterns in the medusa *Cladonema pacificum***

To understand the spatial pattern of cell proliferation in *Cladonema* medusa, we performed 5′-ethynyl-2′-deoxyuridine (EdU) staining (*Salic & Mitchison 2008*). EdU-positive cells, which indicate S-phase or the former S-phase cells, were broadly detected in the whole medusa body including the umbrella, the manubrium (a supporting organ of the oral in medusae), and the tentacles (Fig. 1B and 1C). In the tentacles, large numbers of EdU positive cells were located at their base, called tentacle bulbs, suggesting that tentacle bulbs might behave as a proliferation zone (Fig. 1D). We confirmed that these EdU-positive cells were proliferating cells using the mitotic marker, anti-Phospho-Histone 3 (PH3) antibody. PH3-positive cells were detected in both the umbrella and the tentacle bulbs (Fig. 1E and 1F). We further observed mitotic spindles, detected with an anti-α Tubulin antibody in PH3-positive cells (Fig. 1E). These results suggest that cell proliferation may occur uniformly in the medusa body, while a subset of cell proliferation could occur locally in the tentacle bulbs. Based on these observations, we hypothesized that uniform cell proliferation may control body size growth while local cell proliferation in the tentacle bulbs may contribute to tentacle morphogenesis.
Cell proliferation is necessary for the control of body size

Animal body size increases upon intake of nutrition because nutrition influences cell proliferation and cell growth (Bohnsack & Hirschi 2004). We first monitored the body size of juvenile medusae by focusing on the size of their umbrella because the umbrella grows in direct proportion with whole body size. Under normal feeding conditions, the medusa umbrella size increased dramatically by 54.8%, from 0.62±0.02 mm$^2$ to 0.96±0.02 mm$^2$ during the first 24 hours, with a subsequent minor increase observed over the following 5 days (0.98±0.03mm$^2$) (Fig. 2A and 2C). By contrast, under starved conditions, the size of medusa umbrella did not increase, compared to controls, and rather gradually decreased over the following 5 days. Moreover, fewer EdU positive cells were detected in the starved medusae than in fed controls (Fig. 2B), suggesting that, at the cellular level, nutrition affects cell proliferation in medusae. These results indicate that body-size growth in juvenile medusa depends on available nutrition.

To test the hypothesis that uniform cell proliferation in medusa contributes to body-size increase, we performed a pharmacological assay to block cell-cycle progression using hydroxyurea, a cell-cycle inhibitor that causes G1 arrest (Koc et al. 2004). Under hydroxyurea treatment, S phase cells detected by EdU staining disappeared from the medusa body (Fig. 2D). By tracking the size of umbrella, we found that hydroxyurea-treated medusae did not exhibit the size increase that was observed in controls (Fig. 2E). Together, these results suggest that cell-cycle progression affects body size in Cladonema medusae.

Cell proliferation is necessary for tentacle morphogenesis

In Clytia hemisphaerica, another hydrozoan jellyfish, stem-like cells or progenitors are proposed to exist in tentacle bulbs (Denker et al. 2008). The local cell proliferation observed in the tentacle bulbs of Cladonema medusa may reflect such stem or progenitor cell populations (Fig. 1D and 1F). To test the hypothesis that local cell proliferation in tentacle bulbs contributes to tentacle morphogenesis, we first focused on tentacle branching. Although the initial tentacles have one branch in juvenile medusa, the number of branches gradually increases during medusae maturation (Fujiki et al. 2004).
In our normal feeding condition, the branching number reached approximately three (2.98±0.05 per tentacle) by day 9 (Fig. 3A and 3C). By contrast, when cell proliferation was blocked with hydroxyurea, none of the medusae exhibited the typical increase in branched tentacles; rather, all maintained only one branch (Fig. 3B and 3C). This result points to cell proliferation in tentacle bulbs as a necessary component for normal tentacle branching.

Cnidarian tentacles have nematocysts, organelles specific to the cnidarian phylum that are utilized for food capture and defense against predators (Kass-Simon & Scappaticci 2002). In Clytia hemisphaerica, stem-like cells or progenitors in tentacle bulbs seem to supply nematocysts at the tips of tentacles via cell proliferation, migration to the tip, and differentiation (Denker et al. 2008). This evidence raises the possibility that cell proliferation also controls nematocyte development or nematogenesis in hydrozoan jellyfish. To monitor nematocytes in Cladonema tentacles, we utilized DAPI, a nuclear staining dye that can label poly-γ-glutamate synthesized in the nematocyst wall (Szczepanek et al. 2002). Using poly-γ-glutamate staining, we discovered nematocyte size variations ranging from 2μm²-110μm² (Fig. 3D). Because nematocytes increase in size during maturation, small nematocytes tend to be immature nematocysts. We also found that some of the nematocysts were empty, suggesting that such nematocytes had been depleted (Fig. 3D). In order to investigate whether cell proliferation in tentacle bulbs also contributes to nematocyte maturation, we examined the size distribution and emptiness of nematocytes after cell-cycle blocking with hydroxyurea. Compared to controls, the rate of small nematocysts significantly reduced in the medusae under the treatment of hydroxyurea (HU+: 65.1±3.6%; HU-: 85.3±2.3%, Fig. 3D and 3E). We further detected that the rate of the empty nematocysts was higher in the medusae with hydroxyurea treatment than in controls (HU+: 25.7±3.1%; HU-: 14.1±3.1%, Fig. 3D and 3F). These results indicate that even after discharge, nematocytes are still actively supplied by progenitor cell proliferation and that this refill is prevented when cell proliferation is blocked. Taken together, our data suggest that cell proliferation in tentacle bulbs plays an important role in both tentacle branching and nematogenesis.
Cell proliferation is necessary for tentacle regeneration

Cnidrians are known to have a high regenerative capacity (Galliot & Schmid 2002; Holstein et al. 2003), and the hydrozoan jellyfish Cladonema species exemplifies this typical regenerative ability (Weber 1981). Given the localization of proliferative cells in the Cladonema tentacle bulb, we decided to investigate the nature of tentacle regeneration. After dissecting tentacles at their base, we monitored the process of tentacle regeneration (Fig. 4A). During the first 24 hours, wound healing occurred at the dissected area. Subsequently, the tip of tentacle became elongated and started branching on day 2 (Fig. 4A). At day 4, fully branched tentacles were observed (Fig. 4A), suggesting that tentacle regeneration may follow normal tentacle morphogenesis after elongation.

To examine the initial stage of tentacle regeneration, we examined the distribution of proliferating cells using PH3 staining to visualize mitotic cells. While dividing cells were frequently observed near the amputated area, mitotic cells were dispersed in uncut control tentacle bulbs (Fig. 4B). We quantified the number of PH3-positive cells present in the tentacle bulbs and found a significant increase in PH3-positive cells in the tentacle bulbs of amputee medusae, compared to controls (Fig. 4C). These observations indicate that initial regenerative responses accompany the active increase of cell proliferation in tentacle bulbs. In order to test the role of cell proliferation in tentacle regeneration, we blocked cell-cycle progression using hydroxyurea after dissection and monitored the length of regenerating tentacles. While the tentacles continued to elongate from the bulb structure after dissection in controls, tentacles in animals treated with hydroxyurea were not able to elongate despite displaying normal wound healing (Fig. 4D). These results demonstrate that cell proliferation in tentacle bulbs is required for proper tentacle regeneration.

Cell proliferation patterns across different hydrozoan jellyfish

Hydrozoan jellyfish constitute the most broadly varied class of cnidarian jellyfish with approximately 2,700 species worldwide featuring highly diverse morphological and physiological characteristics (Cartwright & Nawrocki 2010; Schuchert 2019). For
instance, *Cytaeis uchidae* has four tentacles, and their polyps live exclusively on one type of shell: *Niotha livescens* (*Takeda et al. 2018a; Takeda et al. 2013*). Another species, *Rathkea octopunctata*, has eight grouped-tentacles, and their juvenile medusae asexually produce medusae that grow out of the manubrium (*Berrill 1952; Schuchert 2007*). To gain insight into the conserved and diversified nature of cell proliferation in hydrozoan jellyfish, we investigated the spatial pattern of cell proliferation in *Cytaeis* and *Rathkea* medusae. In *Cytaeis* medusa, EdU-positive cells were observed in manubrium, tentacle bulbs, and at the top of the umbrella (Fig. 5A and 5B). PH3-positive cells were also detected in the same regions, suggesting that proliferating cells in *Cytaeis* are distributed in a pattern similar to that observed in *Cladonema*. (Fig. 5C and 5D). By contrast, in *Rathkea octopunctata*, EdU-positive cells and PH3-positive cells were mostly restricted to the manubrium and tentacle bulbs (Fig. 5E-G). Of note, proliferating cells were frequently detected in the medusa buds that grew out of the manubrium (Fig. 5E and 5F), which may reflect asexual reproduction in *Rathkea* medusae. These results suggest that cell proliferation may occur in tentacle bulbs across hydrozoan medusae commonly, while cell proliferation patterns may vary in a species-specific manner with physiology.

**Discussion**

In this study, we show that the body size of *Cladonema* medusae is influenced by cell proliferation following uptake of nutrition. Without nutrition and under the blocking of cell-cycle progression, body-size increase is inhibited (Fig. 2). Intriguingly, despite the significant differences between fed and starved animals and between hydroxyurea-treated and -untreated animals, the body size of *Cladonema* medusae increases during the first 24 hours regardless of condition (Fig 2). These results can be explained by cell growth via protein synthesis (*Schiaffino et al. 2013*) or accretionary growth, in which cells secrete extracellular matrix to increase extracellular regions, as has been suggested in the growth of cartilage and bone (*Karsenty et al. 2009; Wang et al. 2014*). Given the large amount of collagen that jellyfish contain (*Khong et al. 2016; Miura & Kimura 1985*), extracellular matrix may increase their size during the initial growth of juvenile medusae. Another interesting feature we observed is that the body size of the
starved medusae gradually decreases after 24 hours (Fig. 2B). Similarly, upon starvation, *Hydra* polyps cease asexual budding and decrease their size (Buzgariu et al. 2008; Chera et al. 2009a), suggesting that cnidarian animals are sensitive to nutrition availability and adapt to metabolic changes. At the organ and tissue level, such size reduction can occur via autophagy or cell death during starvation in diverse phyla (Jeschke et al. 2000; O’Brien et al. 2011; Thongrod et al. 2018; Tracy & Baehrecke 2013). Cnidarians thus may utilize similar mechanisms to reduce cell size and/or cell number to adjust their body size in response to environmental changes. Molecularly, TOR and Hippo signaling are conserved machinery that control organ size, and, as such, these molecules may also play an important role in cnidarian growth control (Coste et al. 2016; Ikmi et al. 2014; Loewith & Hall 2011; van Dam et al. 2011).

Hydrozoan animals are known to possess interstitial stem cell populations, called i-cells. In *Hydra* and *Hydractinia* polyps, i-cells are localized to the body column and have the potential to differentiate into several cell types including nematocytes, nerve cells, and gametes (Gold & Jacobs 2013; Hemmrich et al. 2012; Hobmayer et al. 2012; Kunzel et al. 2010; Muller et al. 2004). By contrast, the current understanding of the localization and roles of stem-like cells or i-cells in hydrozoan jellyfish are limited (Leclere et al. 2012). In *Cladonema* medusae, proliferative cells are distributed in tentacle bulbs (Fig. 1), which have been similarly observed in the tentacle bulbs of the *Clytia* medusa (Denker et al. 2008). Our pharmacological experiments confirmed that cell proliferation contributes to tentacle branching, nematogenesis, and tentacle regeneration in *Cladonema* (Fig. 3 and Fig. 4), suggesting that these proliferative cells may behave as progenitors or stem-like cells. We further found similar distribution of proliferative cells in tentacle bulbs of *Cytaeis uchidae* and *Rathkea octopunctata* (Fig. 5). Together, these results suggest that the distribution of proliferative cells in tentacle bulbs are widely conserved in hydrozoan jellyfish, while such cells might exist in other tissue to allow body-size increase and species-specific life styles.

Acknowledgements
We thank R. Deguchi (Miyagi Education Univ. Japan) for sharing jellyfish species and helpful discussion. We thank H. Takashima for technical assistance. We thank Kuranaga lab members for discussion.

References


Figure 2. Cell proliferation is necessary for body-size growth
(A) Cladonema pacificum newborn medusa (0 day old) and juvenile medusa (8 day old). (B) Distribution of S-phase cells in control medusa and starved medusa with EdU staining (150μM, 1hr). (C) Quantification of umbrella size in control and starved medusae. Control medusae were fed every other day. Error bar: SD, ***p < 0.0005. (D) Distribution of S-phase cells in medusa of control (HU-) and hydroxyurea (HU) treatment detected by EdU staining (20μM, 24hr). No S-phase cells were detected in HU+ medusa. (E) Quantification of body size in control and in HU conditions. HU suppresses body-size growth. HU-: control medusae incubated in ASW, HU+: medusae incubated in HU 10mM ASW. Both HU+ and HU- were fed every other day. Error bar: SD, ***p < 0.0005. Scale bar: (A) 1mm, (B and D) 100μm.
(A) Control (HU-) medusa incubated in ASW for 9 days. The picture shows the representative image of medusae with three branched tentacles. (B) The medusa incubated in 10mM HU (HU+) ASW for 9 days. The picture shows the representative image of medusae with one branched tentacle. (C) Quantification of branching numbers per tentacle at Day 0 and Day 9. HU+: $n=313$, HU- condition: $n=199$. Error bars: SD, ***$p < 0.001$. (D) Nematocytes in tentacles labeled by DAPI (poly-$\gamma$-gultamate) in the 8 day old medusa incubated in ASW (HU-) or 10mM HU ASW (HU+). Arrows indicate small nematocysts, and arrowheads indicate empty nematocysts. (D) The rate of small nematocysts (size: 2–40 $\mu$m$^2$) in HU- and HU+ medusa. HU+: $n=19$, HU-: $n=18$. (E) The rate of empty nematocysts in HU- and HU+ medusa. HU+: $n=19$, HU-: $n=18$.

Figure 4. Cell proliferation is necessary for tentacle regeneration

(A) Tentacale regenerative processes after amputation in an adult medusa. Series of pictures show the growing tentacle over 4 days. (B) Mitotic cells (PH3+) in tentacle bulbs of the unremoved control and the dissected medusa. Arrowheads indicate PH3-positive cells. (C) Quantification of proliferative cells in tentacle bulbs for control and after amputation. Control: $n=26$, Amputation: $n=11$. error bar: SD, ***$p < 0.0005$. (D) Quantification of tentacle length after amputation in control (HU-) and 10mM HU treatment (HU+). Scale bar: (A) 1mm, (B) 100$\mu$m.

Figure 5. Cell proliferation patterns across different hydrozoan jellyfish

(A) Distribution of S-phase cells in the Cytaeis uchidae medusa (30 day old) revealed by EdU staining (EdU: 20$\mu$M, 24hr). (B) Distribution of S-phase cells (EdU+) in Cytaeis medusa (11 day old). (C) Mitotic cells (PH3+) in the umbrella of Cytaeis medusa (30 day old). (D) Mitotic cells in Cytaeis medusa tentacle bulbs (30 day old). (E) Distribution of S-phase cells (EdU+) in the Rathkea octpunctata juvenile medusa (EdU: 20$\mu$M, 24hr). (F) Mitotic cells (PH3+) in a manubrium of Rathkea juvenile medusa. (G) Mitotic cells (PH3+) in Rathkea juvenile medusa tentacles. Arrows indicate PH3-positive mitotic cells. Scale bars: 100$\mu$m.
Figure 1 (on next page)

Cell proliferation patterns in *Cladonema* medusa

(A) Adult medusa of *Cladonema pacificum*. (B) Distribution of S-phase cells in the *Cladonema pacificum* medusa (1 day old) revealed by EdU staining (20μM, 24hr incubation). (C) Uniform distribution of S-phase cells (EdU+) in a medusa umbrella (1 day old). (D) Local distribution of S-phase cells (EdU+) in medusa tentacle bulbs (1 day old). (E) Mitotic cells detected by anti-PH3 in a medusa umbrella (8 day old). (F) Mitotic cells (PH3+) in medusa tentacle bulbs (1 day old). Arrows indicate EdU-positive (C, D) and PH3-positive (E, F) cells, respectively. Scale bar: (A) 1mm, (B-D) 100μm, (E, F) 50μm.
Cell proliferation is necessary for body-size growth

(A) *Cladonema pacificum* newborn medusa (0 day old) and juvenile medusa (8 day old). (B) Distribution of S-phase cells in control medusa and starved medusa with EdU staining (150μM, 1hr). (C) Quantification of umbrella size in control and starved medusae. Control medusae were fed every other day. Error bar: SD, ***(p < 0.0005. (D) Distribution of S-phase cells in medusa of control (HU-) and hydroxyurea (HU) treatment detected by EdU staining (20μM, 24hr). No S-phase cells were detected in HU+ medusa. (E) Quantification of body size in control and in HU conditions. HU suppresses body-size growth. HU-: control medusae incubated in ASW, HU+: medusae incubated in HU 10mM ASW. Both HU+ and HU- were fed every other day. Error bar: SD, ***(p < 0.0005. Scale bar: (A) 1mm, (B and D) 100μm.
Figure 2

(A) Images showing the development of a jellyfish from day 0 to day 8.

(B) Immunofluorescence images of DNA and EdU staining on day 3.

(C) Graph showing the change in umbrella size over time for control and starved groups.

(D) Images showing the effect of HU- and HU+ conditions on jellyfish development.

(E) Graph showing the change in umbilical size over time for HU- and HU+ groups.
Cell proliferation is necessary for tentacle morphogenesis

(A) Control (HU-) medusa incubated in ASW for 9 days. The picture shows the representative image of medusae with three branched tentacles. (B) The medusa incubated in 10mM HU (HU+) ASW for 9 days. The picture shows the representative image of medusae with one branched tentacle. (C) Quantification of branching numbers per tentacle at Day 0 and Day 9. HU+: $n=313$, HU- condition: $n=199$. Error bars: SD, ***$p < 0.001$. (D) Nematocytes in tentacles labeled by DAPI (poly-γ-gultamate) in the 8 day old medusa incubated in ASW (HU-) or 10mM HU ASW (HU+). Arrows indicate small nematocysts, and arrowheads indicate empty nematocysts. (D) The rate of small nematocysts (size: 2-40 μm$^2$) in HU- and HU+ medusa. HU+: $n=19$, HU-: $n=18$. (E) The rate of empty nematocysts in HU- and HU+ medusa. HU+: $n=19$, HU-: $n=18$. 
Figure 3

(A) HU- main tentacle

(B) HU+ main tentacle

(C) The rate of branching tentacles (%)

(D) DNA poly-γ-glutamate α-tub

(E) The rate of small nematocysts (%)

(F) The rate of empty nematocysts (%)

*** number of branching tentacles

1 2 3 4

HU- day 0 HU+ HU- day 9
Cell proliferation is necessary for tentacle regeneration

(A) Tentacle regenerative processes after amputation in an adult medusa. Series of pictures show the growing tentacle over 4 days. (B) Mitotic cells (PH3+) in tentacle bulbs of the unremoved control and the dissected medusa. Arrowheads indicate PH3-positive cells. (C) Quantification of proliferative cells in tentacle bulbs for control and after amputation. Control: n=26, Amputation: n=11. error bar: SD, ***, p < 0.0005. (D) Quantification of tentacle length after amputation in control (HU-) and 10mM HU treatment (HU+). Scale bar: (A) 1mm, (B) 100μm.
Cell proliferation patterns across different hydrozoan jellyfish

(A) Distribution of S-phase cells in the *Cytaeis uchidae* medusa (30 day old) revealed by EdU staining (EdU: 20μM, 24hr). (B) Distribution of S-phase cells (EdU+) in *Cytaeis* medusa (11 day old). (C) Mitotic cells (PH3+) in the umbrella of *Cytaeis* medusa (30 day old). (D) Mitotic cells in *Cytaeis* medusa tentacle bulbs (30 day old). (E) Distribution of S-phase cells (EdU+) in the *Rathkea octpunctata* juvenile medusa (EdU: 20μM, 24hr). (F) Mitotic cells (PH3+) in a manubrium of *Rathkea* juvenile medusa. (G) Mitotic cells (PH3+) in *Rathkea* juvenile medusa tentacles. Arrows indicate PH3-positive mitotic cells. Scale bars: 100μm.