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Review

Using the Carnivorous Sponge *Lycopodina hypogea* as a Nonclassical Model for Understanding Apoptosis-Mediated Shape Homeostasis at the Organism Level

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† n° ISEM 2023-006.

Abstract: The dynamic equilibrium between death and regeneration is well established at the cell level. Conversely, no study has investigated the homeostatic control of shape at the whole organism level through processes involving apoptosis. To address this fundamental biological question, we took advantage of the morphological and functional properties of the carnivorous sponge *Lycopodina hypogea*. During its feeding cycle, this sponge undergoes spectacular shape changes. Starved animals display many elongated filaments to capture prey. After capture, prey are digested in the absence of any centralized digestive structure. Strikingly, the elongated filaments actively regress and reform to maintain a constant, homeostatically controlled number and size of filaments in resting sponges. This unusual mode of nutrition provides a unique opportunity to better understand the processes involved in cell renewal and regeneration in adult tissues. Throughout these processes, cell proliferation and apoptosis are interconnected key actors. Therefore, *L. hypogea* is an ideal organism to study how molecular and cellular processes are mechanistically coupled to ensure global shape homeostasis.

Keywords: sponges; *Lycopodina hypogea*; morphogenesis; homeostasis; apoptosis; proliferation; caspases; stem cells

Citation: Baghdiguian, S.; Le Goff, E.; Paradis, L.; Vacelet, J.; Godefroy, N. Using the Carnivorous Sponge *Lycopodina hypogea* as a Nonclassical Model for Understanding Apoptosis-Mediated Shape Homeostasis at the Organism Level. *Foundations* **2023**, *3*, 220–230. https://doi.org/10.3390/foundations3020018

Academic Editor: Francesco Bellanti

Received: 13 January 2023
Revised: 10 March 2023
Accepted: 29 March 2023
Published: 18 April 2023

1. Introduction

Originally, homeostasis was defined as the process through which an organism tends to maintain constant its biological and physiological features, for instance, those of its internal medium [1,2]. Although this concept generally focuses on the stability of the internal environment (i.e., physiological homeostasis), it can also be generalized and applied to the whole organism and its relationship to its local environment through its shape. In this more general context, apoptosis is a powerful tool to generate, renew, and maintain shapes during embryonic development and throughout adult life [3]. Thus, homeostasis can be extended to all organization levels of living matter. To obtain an integrated view of organism homeostasis in relation to its environment, different organization levels must often be considered (e.g., the molecular level, in relation to the internal medium, and the structural level) in a temporal framework. Accordingly, the changes that ensure the homeostasis of functional structures are slow and prolonged, whereas the homeostasis of the internal environment is maintained through rapid processes. The study of global homeostasis (i.e., the whole-organism homeostasis in relation to its environment) requires biological models that can respond to energy variations in the environment in a measurable manner. This is the case of the carnivorous sponge *Lycopodina hypogea* (Vacelet & Boury-Esnault, 1996) [4]. In this sponge, and apparently in all carnivorous sponges (*Cladorhizidae* family), the response to nutritional stimuli results in a dramatic change in the structures involved in prey capture that requires the complete reorganization of the underlying molecular

networks (see below). The digestive system of these sponges, which has been rarely studied due to their deep-sea habitat, has evolved from a "static" state (filter feeders) to a dynamic state (carnivores) to allow adaptation to their environment. Therefore, these sponges can be defined as structures that maintain their homeostasis through an ordered succession of events in which the apparent persistence of higher levels of organization is supported by the change in subordinated systems. In *L. hypogea*, the recovery of macroscopic structures at the end of a feeding cycle is closely correlated with the return to the initial state of the whole transcriptome and of the molecular networks directly involved in cell turnover [5]. Moreover, *L. hypogea* can easily stay several months without eating macroprey. Therefore, this sponge alternates between states of dynamic equilibrium without any consequence for its global shape (in the absence of nutrients) and drastic changes in shape with a return to the initial state (in the presence of nutrients). *L. hypogea* can adapt its global homeostasis to the presence/absence of food in its environment.

It is well documented how individual cells influence the development of multicellular organisms. Conversely, it is less well understood how the molecular and cellular features of these cells collectively lead to the emergence of robust homeostatic structures [6]. The process of cell renewal is a critical mechanism for ensuring homeostatic morphological and functional structures in adults. This process requires the differentiation of adult stem cells into new differentiated cells and the elimination of old cells by apoptosis. This dynamic equilibrium has been well documented at the cellular level [4–7], but no study has investigated how the shape of a functional structure or of a whole organism is homeostatically controlled.

2. Using *L. hypogea* as a Model to Study Homeostasis at the Organism/Structure Level

To better understand the processes involved in the homeostasis of functional structures during physiological cell turnover, we took advantage of the intriguing biological properties of the carnivorous sponge *L. hypogea* (class Demospongiae) [4]. This sponge was collected in its environment: a submarine cave in the Marseille region (France) that reproduces deep-water conditions (i.e., the classical habitat of carnivorous sponges). Once collected, *L. hypogea* samples could be easily maintained in aquarium conditions. Sponges were fed *Artemia nauplii* shrimps every 1–2 months, but they can survive more than one year without any food. This is probably an adaptation to the low nutrient levels in their natural environment, as suggested by the absence of filtration feeding systems in this sponge. During its feeding cycle, *L. hypogea* undergoes dramatic shape changes. Starved animals are attached to the rock by a 1–2 cm long peduncle (see Figure 1a, 0 h). This peduncle is relayed to an ovoid body covered with many elongated filaments with hook-like spicules that are crucial for capturing prey. After prey capture, filaments rapidly regress until they almost disappear. At the end of the digestion process, filaments slowly reform. The number and length of filaments is maintained between feeding cycles (Figure 1). We previously showed that this succession of morphological changes involves cell apoptosis, phagocytosis, and cell proliferation [5].

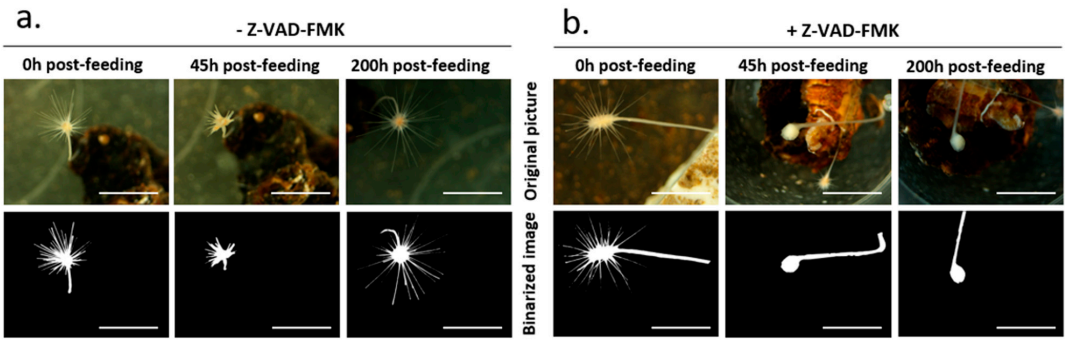

Figure 1. *Cont.*

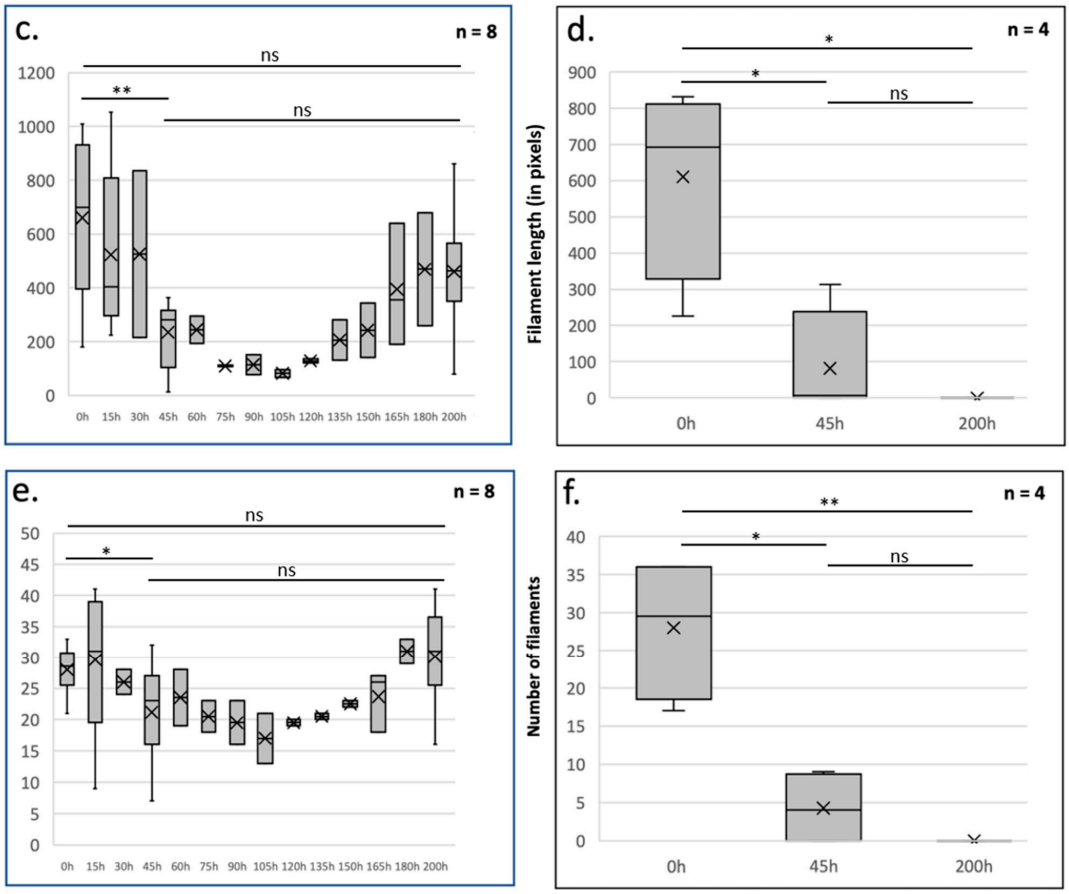

Figure 1. Quantitative approach to investigate caspase-dependent morphofunctional homeostasis during the feeding cycle of *Lycopodina hypogea*. After 2 months of starvation, a feeding cycle was initiated by putting *Artemia nauplii* shrimp on the sponge filaments involved in prey capture, in the absence (**a**,**c**,**e**) or presence of Z-VAD-FMK (pan-caspase inhibitor, Euromedex, 1050A) (**b**,**d**,**f**). Sponges were filmed for 72 h, and images were acquired at the indicated times with a camera (SONY E3CMOS) coupled to a stereoscopic microscope (Nikon SMZ-2B) (upper panels). Scale bar: 5 mm. The original images were binarized with ImageJ (lower panels) [7–9] for the quantitative analysis, expressed in pixels, of the length (**c**,**d**) and number (**e**,**f**) of prey capture filaments. Specifically, images were preprocessed to retain only the green channel (Split channel) [10] in which the sponge was better visualized (brightness, contrast, and grain). The step before the morphological analysis (detecting and delimiting the sponge edge and biomass and detaching it from the rest of the image, such as support, water particles, and prey) was image binarization, performed by adjusting the threshold (*Adjust threshold*) [8,11]. This resulted in an image where the pixels of interest (sponge) had a value of 1 and the background of the image had a value of 0. To permanently remove the background noise, a mask was applied (*Clear outside*). The last step was to segment and quantify the object with a particle analysis (*Analyze particles*) [7,8]. The result was the number of pixels that constituted the object. As there is no scale on the image, the image resolution was previously standardized. Knowing the pixel size of each image, the sponge biomass and the filament length were quantified using the pixels as the unit (*Measure*). In (**c**–**f**), the box and whisker plots represent the 10th and 90th percentiles, with the first, second (median), and third quartiles. X represents the mean. Error bars indicate the standard error of the mean (n = 8 sponges without Z-VAD-FMK and n = 4 sponges with Z-VAD-FMK); ns, not significant (p value > 0.01); * p value < 0.01; ** p value < 0.001 (t test, R software). The first analysis with the t test confirmed the homogeneity of samples/data in each condition. Then, the analysis compared the two conditions.

As the *L. hypogea* genome has yet not been sequenced, we performed a large transcriptomic study that revealed the complex molecular networks implicated in tissue homeostasis [5]. Specifically, we found that the intrinsic caspase-dependent apoptosis pathway is present in the sponge with a quite complex network (Figure 2a). We identified a few apoptotic regulators (i.e., SMAC/Diablo and BH-3-only proteins). Conversely, the extrinsic apoptosis pathway was more limited (without TNFR and with proteins that directly or indirectly interact with FAS only). These observations are in agreement with data obtained in some unicellular eukaryotes [12,13] and in another sponge, *Amphimedon queenslandica* [14]. This suggests that the intrinsic apoptosis pathway is more ancestral than the extrinsic one. We identified the caspase-independent AIF but not ENDOG. The autophagic machinery also displayed a complexity comparable to that observed in humans (Figure 2b). Similarly, cell proliferation actors and the cell cycle regulation were comparable to what is described in mammals but with the absence of cyclin-dependent kinase inhibitors (CDKN) (Figure 2c).

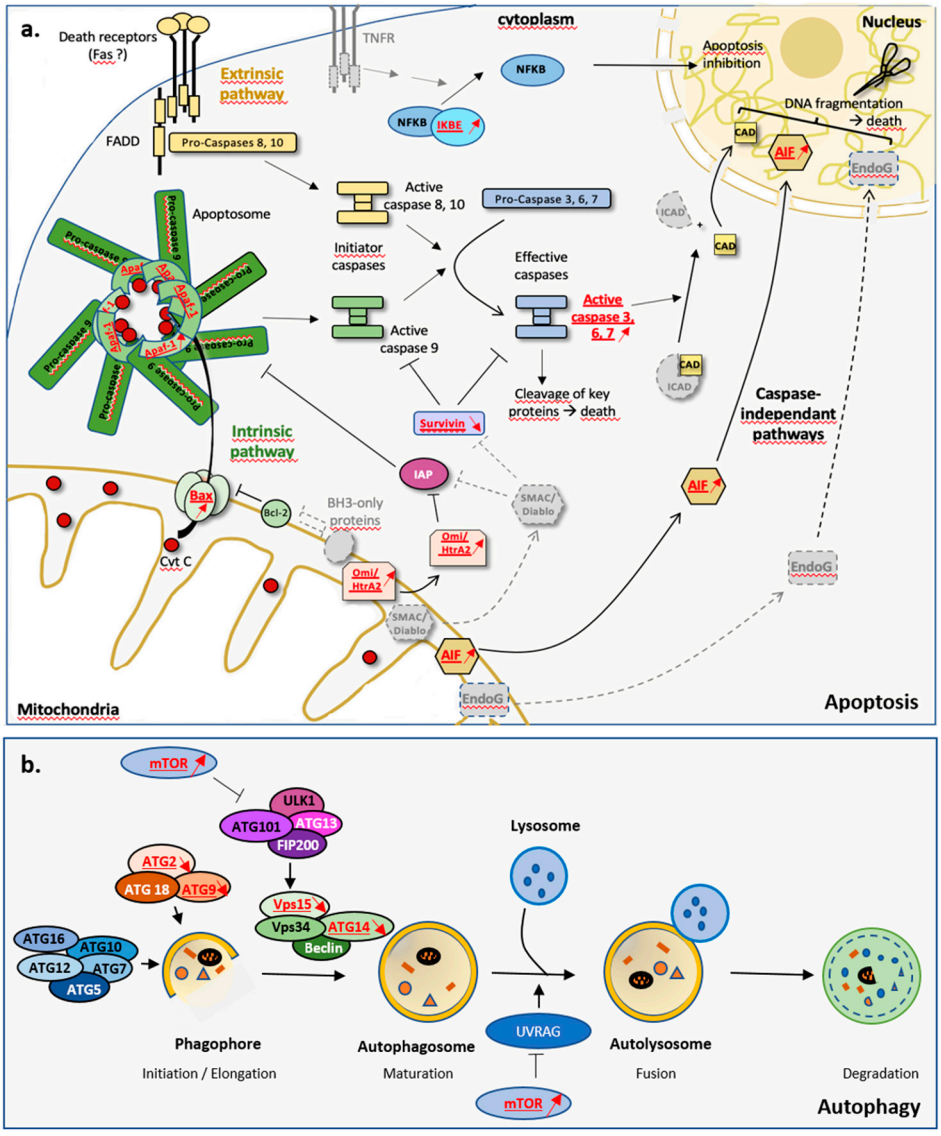

Figure 2. *Cont.*

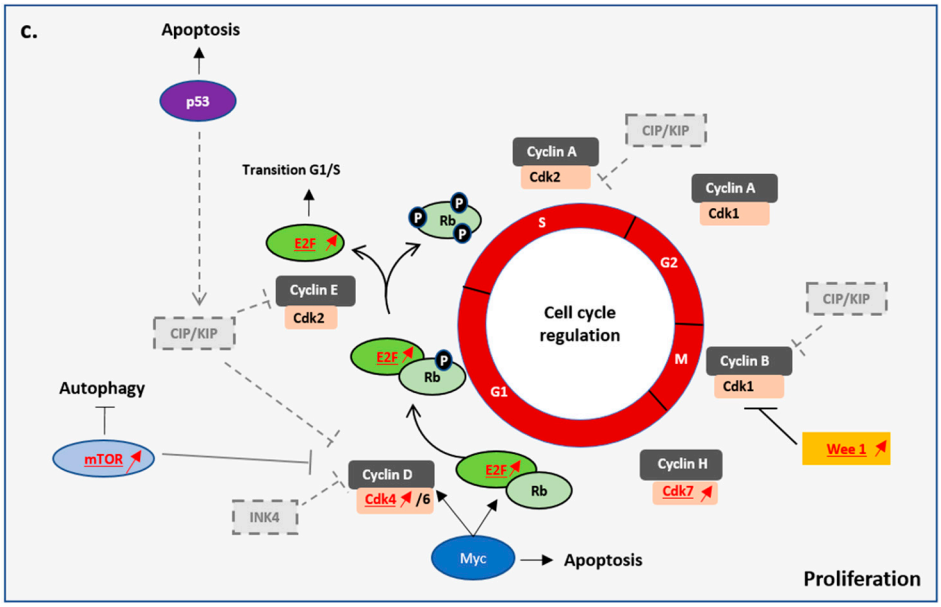

Figure 2. Schematic representation of the main mechanisms involved in tissue homeostasis: apoptosis (**a**), autophagy (**b**), and cell cycle regulation (**c**). Dashed outline and grey color transcripts of proteins not found in any step of the *L. hypogea* feeding cycle in the transcriptomic study; colored names, transcripts of proteins detected in the transcriptomic study; bold, red, and underlined names, transcripts of proteins expression that is regulated during feeding. Red arrows indicate a decrease or increase of the gene expression level.

In *L. hypogea*, all these networks can be monitored with the cell biology methods used in more classical models: BrdU and EdU labeling for cell proliferation, he TUNEL assay for cell apoptosis, and anti-ATG8 antibodies for autophagy. This allows their spatiotemporal visualization in vivo. Moreover, in adult *L. hypogea*, apoptosis can be efficiently inhibited with the caspase inhibitor Z-VAD-FMK [5]. In addition, cell proliferation, apoptosis, and autophagy can be induced in vivo upon feeding in controlled conditions.

Therefore, *L. hypogea* is an ideal model to study how cellular processes are mechanistically coupled to maintain a consistent shape at the whole-organism level. This model also allows studying mechanisms that have been necessary for the acquisition and maintenance of multicellularity in the sister group of all other metazoans [15].

3. Cell Turnover and Adult Tissue Homeostasis in *L. hypogea*

Many adult metazoans remain in a dynamic equilibrium throughout their life, giving the impression of the stability of adult structures. This counterintuitive concept is very old and can be attributed to Heraclitus of Ephesus: *"No man ever steps in the same river twice, for it's not the same river and he's not the same man"*. A living organism is stable and invariant only in appearance: it is actually the manifestation of a perpetual flow of events. Without any interruption, most differentiated cells in living systems "commit suicide" and new stem cells differentiate to replace them. This concept is valid not only for cells but for all levels of life, including at the molecular level because all biomolecules are continuously renewed at varying rates. This dynamic concept of the organism is one of the most important principles of modern biology. Cell death and cell proliferation have been extensively studied separately, but very little is known about the identity of the molecular actors and networks underlying their coupling that maintains morphological homeostasis (i.e., the capacity to return to the initial shape) in adult organs and organisms. In this context, it is crucial to better understand how the regulation of cell turnover integrates the apoptosis of differentiated cells and the division and differentiation of stem cells to maintain and/or form functional structures again (here, the prey capture system of *L. hypogea*). How do these two seemingly opposite processes regulate each other in time and space? Do apoptotic

cells and dividing/differentiating cells cross-talk to maintain an invariant number of cells between two feeding cycles? If this dialogue actually exists, what is the biochemical nature of the involved signals?

Addressing these questions implies identifying the molecules that connect the molecular networks controlling cell proliferation, differentiation, and programmed death at the cell, tissue, and whole-organism levels. To tackle this challenge, the carnivorous sponge *L. hypogea* is an attractive model organism.

This sponge ensures its homeostasis via trans-differentiation processes that involve the proliferation and differentiation of adult stem cells (archeocytes) with a continuous cell turnover mediated by apoptosis [5,16]. In addition to cell proliferation/differentiation and apoptosis, cell migration is implicated. Briefly, after prey capture, the filaments are destroyed and resorbed through a complex mechanism that concomitantly involves apoptosis at the top of the filaments and the active centripetal migration of phagocytic cells from all parts of the sponge toward the prey. At this stage, archeocyte proliferation is already observed. During the digestion process, the filaments reform from newly differentiated cells, including sclerocytes (i.e., cells secreting the skeleton of the new filaments), that adopt a centrifugal migration (Figure 3). In the resting adult sponge, the two processes of creation and destruction (and all their subprocesses) are active and in a dynamic equilibrium to efficiently maintain the filament number and length. Therefore, in *L. hypogea*, homeostasis is achieved at the molecular and cellular levels (restricted homeostasis) and at the structural level to maintain length and number of functional structures (adaptive homeostasis), as demonstrated by the quantitative analysis of filament structures during the feeding cycle (Figure 1).

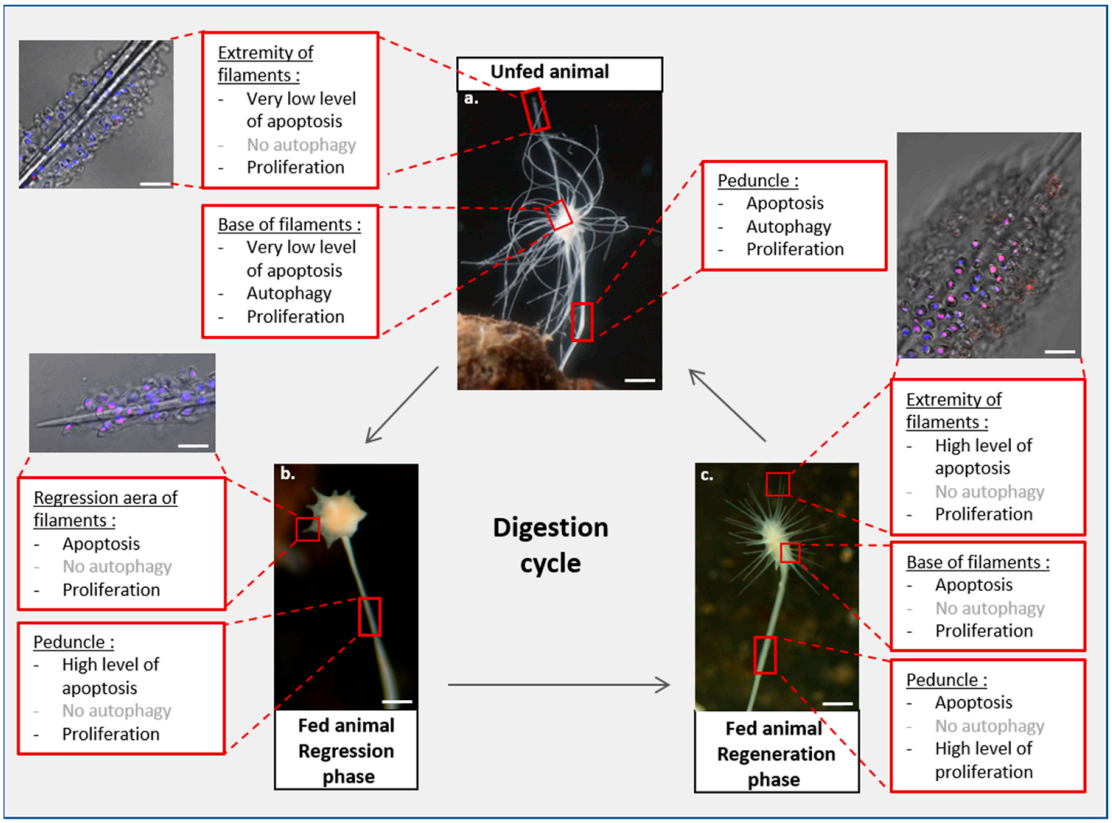

Figure 3. Regulation of apoptosis, autophagy, and proliferation during a feeding cycle in *L. hypogea*. In unfed animals, proliferation is observed in all parts of the sponge. Apoptosis is mainly present in

the peduncle. Autophagy occurs in the peduncle and at the base of filaments (**a**). During the regression phase after feeding, autophagy is inhibited in regressing filaments, and the peduncle undergoes high levels of apoptosis. Proliferation is still present (**b**). The regeneration phase is associated with a moderate level of cell proliferation in filaments and a high level of proliferation in the peduncle. No autophagy is observed. Apoptosis is present everywhere but with higher rates at the tip of the filaments and in the peduncle. (**c**). Examples of immunofluorescent labeling obtained at the end of the filaments are presented together with phase images showing the silica filaments. The sponges were fixed for 30 min with 4% paraformaldehyde in filtered seawater and then permeabilized for 20 min at room temperature with 0.2% Triton X-100 in TS buffer (150 mM NaCl, 25 mM Tris, and pH 7.5). Fixed and permeabilized sponges were labeled with DAPI (Euromedex, 1050A) and TUNEL (Merck, In Situ Cell Death Rhodamine Detection Kit, 12,156,792,910) as previously described [17]. The blue labeling corresponds to a DAPI labeling of nuclei. Red labeling corresponds to TUNEL-positive nuclei. Scale bar: 3 mm (stereoscopic microscope Nikon SMZ-2B images) and 150 µm (confocal microscope Leica TCS-SPE images).

The regulation of homeostasis by cell proliferation and apoptosis is also observed in noncarnivorous sponges, such as *Halisarca dujardinii* (class Demospongiae) and *Leucosolenia variabilis* (class Calcarea) [18]. In these sponges, choanocytes play an important role. Choanocytes have been described as stem cells [19], but this cell type has not been identified in *L. hypogea*. Moreover, inflammation has never been detected at any step of the feeding cycle in *L. hypogea*. This suggests that necrosis and pyroptosis (other types of cell death) are not implicated in the regulation of tissue homeostasis. However, this needs to be confirmed. Similarly, ferroptosis has never been described in *L. hypogea* or in sponges in general. These different types of cell death have been implicated in the regulation of cell homeostasis in various models other than sponges [20–22].

4. Caspase-Dependent Apoptosis Controls Homeostatic Cell Turnover and Tissue Regeneration in *L. hypogea*

Our transcriptomic study of the different steps of the *L. hypogea* feeding cycle allowed us to identify changes in the expression of the genes encoding stem cell markers and the proteins implicated in prey digestion [23], cell cycle control, phagocytosis, autophagy, and apoptosis [5]. For instance, among the four caspases (two of which may be initiator caspases because they present a long pro-domain with CARD and DED domains), one effector caspase is transcriptionally activated by feeding. Several actors of the caspase-dependent intrinsic pathway of apoptosis also are upregulated by feeding: a member of the BCL2 family, APAF-1, and OMI/HTRA2. Conversely, survivin is downregulated upon feeding (Figure 2a). In parallel, different genes encoding proteins implicated in autophagy are downregulated by food ingestion (ATG2, ATG9, ATG14, and VPS15), while mTOR (autophagy inhibitor) is activated (Figure 2b). The expression of some cell cycle regulators is also regulated by feeding (some cyclin-dependent kinases and cyclins, E2F, Wee-1, and IKBE) (Figure 2c). We then extensively monitored cell proliferation, digestive cell differentiation, and apoptosis during the feeding cycle in a large cell-biology study [5]. Surprisingly, apoptosis inhibition by treating *L. hypogea* with Z-VAD-FMK (a pan-caspase inhibitor) did not prevent the complete shortening of filaments but inhibited their regeneration (Figure 1). This unexpected result might be partly explained by caspase-independent apoptosis induction being correlated with the increase in AIF expression (a caspase-independent cell death effector). Concomitantly, the massive cell migration observed in regressing filaments strongly suggests that many cells migrate and differentiate into phagocytic-like cells to ingest food. Apoptotic cells are essentially observed at the filament extremity [5,16]. Unexpectedly, our results also revealed increased cell proliferation in regressing filaments [5]. Our data suggest that during filament regeneration, cell proliferation and migration concomitantly occur to form new filaments. However, they are not enough because the inhibition of apoptosis using the pan-caspase inhibitor Z-VAD-FMK after filament regression is sufficient to prevent filament reformation [5]. Studies in eumetazoans have shown that apoptosis plays an important role in regeneration. This

caspase-dependent regenerative cell death acts by allowing the release of long- or short-distance regeneration signals and by eliminating cells that suppress regeneration [24–26]. In eumetazoans, the stem cell control model is an alternative model for the regulation of cell turnover and regeneration. In this scenario, stem cells control the cell turnover rate by producing apoptotic signals that activate molecular cell death pathways in the surrounding differentiated cells (mainly sclerocytes in sponges) (reviewed in [27]). Our data preferentially support the cell death control model, suggesting that the rate of caspase-dependent apoptosis plays a pivotal role in the cell turnover level. In this case, it could be hypothesized that differentiated cells undergoing apoptosis induce stem cell proliferation, similar to what is observed during compensatory proliferation in *Drosophila melanogaster* [13–16]. In this scenario, the inhibition of cell death should decrease the rate of stem cell division, as observed in *L. hypogea* when caspases are inhibited by Z-VAD-FMK followed by TUNEL and EdU labeling [5]. This type of study in *L. hypogea* allowed us to show that this apoptosis function and the underlying signaling pathways are already functional in sponges. This finding is also supported by the complexity of the signaling pathways involved in apoptosis: the "Janus face" feature. Moreover, we identified a p53 homolog (Lh-p53) that might have a coordinating role in the proliferation/apoptosis processes observed during the destruction/reconstruction of the prey capture structures. Finally, the presence of a c-MYC homolog (Lh-myc) could also link and coordinate the cell cycle entry of quiescent cells and apoptosis [28,29]. With Lh-p53, Lh-myc might increase the robustness of the molecular regulations that ensure organism homeostasis in direct relation to its energetic environment. All these findings show that the apoptosis signaling pathways in sponges are similar in complexity to those in eumetazoans, although sponges have only 5 cell types compared with vertebrates, which have approximately 100 cell types organized in various tissues and organs. This high molecular complexity and the remarkable cell simplicity may help us to improve our understanding of the cell death pathways and their impact on tissue homeostasis.

5. Homeostasis of Functional Structures

In adult tissues, cell renewal involves the permanent removal of old differentiated cells and their replacement by new cells derived from the division of adult stem cells, as observed during the continuous renewal of the intestinal epithelium in vertebrates. Together with many other processes (e.g., cell cycle quality control), this is crucial for tissue homeostasis and the stability of morphogenetic processes throughout adult life [30] and during embryo development [17]. This explains why the term "homeostasis" is used in different contexts, such as adaptive homeostasis in the case of an organism in its close environment and developmental homeostasis during embryogenesis. This fundamental difference highlights the relevance of defining the context and illustrates the importance of in vivo studies focusing on differentiated cell apoptosis and stem cell division/differentiation in adult model organisms. The contribution of cell proliferation and apoptosis to shape modeling has been much studied in development. During prenatal life, apoptosis is implicated in sculpting shapes that mostly remain unchanged after birth. Our model allows studying a transient and functional change of shape in the adult. Therefore, *L. hypogea* is an ideal model because a high level of proliferation/apoptosis coexists throughout the feeding cycle, followed by an extremely low global metabolic level associated with somatic cell stasis between successive feeding cycles. In these intercycle periods, the low metabolic level seems to be mainly mobilized for germ cell maturation (oocytes [31] and spermatophores [32]). Then, spermatophores migrate and accumulate in the filaments (Figure 4) to be released into the environment after long starvation periods or just after digestion. Therefore, filaments have a double function: prey capture/feeding and reproduction. This physiological adaptation, directly linked to the general homeostasis, allows *L. hypogea* to resist starvation for several months while ensuring efficient reproduction, regardless of the level of energy available in the surrounding environment. Moreover, the homeostasis of such a bifunctional structure (feeding and reproduction) contributes to ensuring sponge reproduction

throughout the whole year, as is generally the case for most invertebrates living in deep water [33]. Indeed, the physical environment and temperature are very stable in deep water (the cave in which *L. hypogea* lives is at a temperature of 13 °C all year long with low water movements) [34]. Importantly, *L.* hypogea can be used to better understand the different homeostasis levels that ensure the crucial functions of feeding and reproduction (i.e., restricted, general, and adaptive homeostasis) by simply starting a new feeding cycle (addition of *Artemia nauplii* on the filaments). This perfectly controlled stimulus very efficiently triggers the apoptosis of differentiated cells (mainly sclerocytes) and activates the division/migration/differentiation of totipotent stem cells (archaeocytes that differentiate into phagocytic-like cells and new sclerocytes). Our findings show that cell death and proliferation are much more interconnected than currently described because both mechanisms are active during filament regression and regrowth. These features and the available tools to visualize and manipulate caspase-dependent apoptosis of differentiated cells (i.e., the TUNEL assay and Z-VAD-FMK) and cell regeneration (e.g., BrdU and EdU labeling) form the bases to carry out hypothesis-driven experiments on the regulatory mechanisms implicated in adult tissue turnover and regeneration.

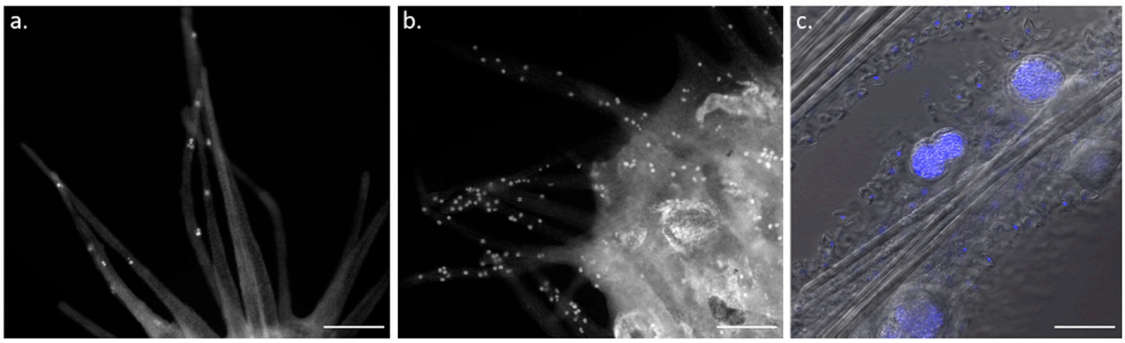

Figure 4. Accumulation of spermatophores in the prey capture filaments after two months of starvation (**a**) and during filament regression after feeding (**b**) in *L. hypogea*. Fluorescence stereomicroscope (Olympus MC5) images after DAPI staining (Euromedex, 1050A) (**a**,**b**). Confocal microscopy (Leica TCS-SPE) image of mature spermatophores on the filament (starved sponge) after DAPI staining of spermatophore nuclei (blue). The sponges were fixed for 30 min with 4% paraformaldehyde in filtered seawater and then permeabilized for 20 min at room temperature with 0.2% Triton X-100 in TS buffer (150 mM NaCl, 25 mM Tris, and pH 7.5). Fixed and permeabilized sponges were labeled with DAPI as previously described [17]. (**c**) (Montpellier RIO Imaging platform, France). Scale bar: 3 mm (**a**,**b**), 35 µm (**c**).

6. Concluding Remarks

Finally, according to Heraclitus' vision of nature, a better understanding of the impact of cell death signaling pathways on the homeostasis of tissues, organs, and organisms requires widening our knowledge of the molecular actors that regulate and coordinate cell death and regeneration. In this context, *SOX* genes play a pivotal role, and their robust induction after a nutritional stimulus in *L. hypogea* strongly suggests their involvement in the maintenance of archeocyte totipotency. The in situ hybridization analysis of these actors (i.e., Lh-caspases, Lh-p53, Lh-myc, and Lh-sox) in Z-VAD-FMK-treated and untreated *L. hypogea* sponges during a feeding cycle should provide insights into the spatiotemporal regulations required to ensure homeostasis at different organization levels (from the molecular to the whole-organism level with its environment). The concept of the organism as an expression of event-driven fluxes leads to the concept of dynamic morphology (perfectly illustrated by *L. hypogea*), the aim of which is to derive organic shapes from a set of homeostatic functions subject to quantitative rules. This will unify the fields of metabolism, growth, and morphogenesis.

Author Contributions: Conceptualization, N.G. and S.B.; methodology, E.L.G. and L.P.; validation, N.G., S.B., E.L.G., L.P. and J.V.; formal analysis, N.G.; investigation, N.G. and S.B.; resources, J.V.; data curation, N.G.; and E.L.G. writing—original draft preparation, N.G., S.B., E.L.G., L.P. and J.V.; writing—review and editing, N.G., E.L.G. and S.B.; supervision, N.G. and S.B.; project administration, N.G.; All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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