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PRIMER

Model systems for regeneration: *Hydra*

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ABSTRACT

The freshwater polyp *Hydra* provides a potent model system for investigating the conditions that promote wound healing, reactivation of a developmental process and, ultimately, regeneration of an amputated body part. *Hydra* polyps can also be dissociated to the single cell level and can regenerate a complete body axis from aggregates, behaving as natural organoids. In recent years, the ability to exploit *Hydra* has been expanded with the advent of new live-imaging approaches, genetic manipulations that include stable transgenesis, gene silencing and genome editing, and the accumulation of high-throughput omics data. In this Primer, we provide an overview of *Hydra* as a model system for studying regeneration, highlighting recent results that question the classical self-enhancement and long-range inhibition model supposed to drive *Hydra* regeneration. We underscore the need for integrative explanations incorporating biochemical as well as mechanical signalling.

KEY WORDS: Hydra model system, Genetic manipulations, Organizer centre, Organoid, Reaggregation, Regeneration

Introduction

Hydra is a freshwater polyp of the phylum Cnidaria and class Hydrozoa that exhibits remarkable regenerative capabilities (Fig. 1). For example, when a *Hydra* polyp is bisected, the head and foot regenerate within a few days. In fact, Abraham Trembley, a mathematician born and raised in Geneva, accidently discovered the regenerative capacity of *Hydra* in 1740. He found a green polyp-shaped organism in pond water and was initially uncertain as to whether it might be a plant or an animal. To be able to classify it, he cut the organism into two parts and reasoned that such an amputation would kill an animal but not a plant. After a couple of days, Trembley observed that each half regenerated until the two pieces looked like the original organism (Trembley, 1744). However, he also observed that the organism rapidly contracted upon touch, possessed tentacles that moved and buds that separated from the parent organism, characteristics that are not typical for a plant and that raised doubts about the classification of this organism as a plant. In 1741, he sent a letter describing his findings to René Antoine Ferchault de Réaumur, who agreed that the organism should be classified as an animal. Trembley subsequently performed many different regeneration experiments and also obtained seven-headed 'monsters' that later on inspired Linnaeus and Pallas, who named these polyps *Hydra* based on the many-headed Greek mythological monster (Linnaeus, 1758; Pallas, 1766). In 1744, Trembley published his famous book *Mémoires, pour server à l'histoire d'un genre de polypes d'eau douce, à bras en forme de cornes*, which describes several key aspects of *Hydra* regeneration but also their feeding, walking and budding (Trembley, 1744). Importantly, his manipulations and careful observations foreshadowed the modern era of experimental developmental biology (Galliot, 2012).

Since Trembley's early studies, *Hydra* has been used increasingly as a model system for exploring the principles of regeneration. *Hydra* also displays an amazing feature, which is the ability to regenerate complete polyps from dissociated tissues (Noda, 1971; Gierer et al., 1972). Here, we provide an overview of *Hydra* as a potent model system for stem cell biology and regenerative studies. We review how studies of regeneration in *Hydra* have provided key insights into processes such as patterning, self-organization, mechanical signalling and nervous system regeneration.

An overview of *Hydra* as a model system

Anatomy and reproduction

Hydra animals display a tube shape with a head at their apex that is composed of tentacles and a dome-shaped structure called a hypostome that surrounds the mouth opening (Fig. 1B). At their base, the animals possess a foot called a basal disc, with the body column separating the head from the foot (Fig. 1B). *Hydra* consist of two cell layers, the epidermis and the gastrodermis, that are separated by an extracellular matrix (ECM) named the mesoglea (Fig. 1C). Cell processes from the epidermis and gastrodermis cross the mesoglea to mediate cell-cell interactions (Sarras, 2012).

Hydra can reproduce asexually as well as sexually. To reproduce asexually, the animals develop a bud in the body wall (Fig. 1B) that grows as a complete polyp within 3 days and eventually detaches from the parent (Otto and Campbell, 1977). In contrast, during sexual reproduction, the body wall thickens and either testes or ovaries differentiate within the epidermis. Sperm cells are released from mature testes and can then fertilize the exposed oocytes from either the same or another animal, depending on whether the species in question is hermaphroditic or gonochoristic (Martínez and Bridge, 2012). After the fully grown oocyte ruptures through the

Model systems for regeneration

This article is part of a series entitled 'Model systems for regeneration'. This series of articles aims to highlight key model systems and species that are currently being used to study tissue and organ regeneration. Each article provides background information about the phylogenetic position of the species, its life-cycle and habitat, the different organs and tissues that regenerate, and the experimental tools and techniques that are available for studying these organisms in a regenerative context. Importantly, these articles also give examples of how the study of these models has increased our understanding of regenerative mechanisms more broadly, and how some of the open questions in the field of regeneration may be answered using these organisms. To see the full collection as it grows, please visit: https://dev.biologists.org/collection/regeneration_models.

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ectoderm, thus becoming exposed to the water around the animal, and completes meiosis, the egg has to be fertilized within 2 h for normal embryogenesis to occur. Gastrulation then takes place within 12 h post-fertilization. This is followed by the formation of a thick cuticle that protects the embryo until hatching, which can take place from 2 to 24 weeks later, after a period of dormancy that precedes gut formation and intense neurogenesis during the two days before hatching (Martin et al., 1997).

Experimental accessibility and tools

Hydra can be easily maintained in the laboratory as mass cultures (Loomis and Lenhoff, 1956). The animals are kept in glass or plastic dishes at 18°C and fed with brine shrimp (*Artemia nauplii*) three to four times per week. *H. vulgaris*, *H. oligactis*, *H. braueri* and *H. viridissima* are different *Hydra* species that are all capable of regenerating equally well, while strains of *H. vulgaris* are most commonly used (Kawaida et al., 2010; Martínez et al., 2010). A number of molecular tools exist to analyse gene function in adult and regenerating animals. Stable transgenesis was established in 2006 (Wittlieb et al., 2006), allowing gene overexpression (Gee et al., 2010; Klimovich et al., 2018) as well as gene knockdown with constructs containing shRNAs (Klimovich et al., 2019). Gene knockdown can also be achieved by electroporating small interfering or small hairpin RNAs (siRNAs, shRNAs) into animals or aggregates (Watanabe et al., 2014; Klimovich et al., 2018; Vogg et al., 2019). The *Hydra* genome was made available in 2010 (Chapman et al., 2010), and this was soon followed by the establishment of a reference transcriptome (Wenger and Galliot, 2013), quantitative RNA-sequencing (Hemmrich et al., 2012; Wenger, 2014; Petersen et al., 2015; Wenger et al., 2016, 2019), quantitative proteomics (Petersen et al., 2015; Tomczyk et al., 2017), genome editing (Lommel et al., 2017 preprint) and single cell sequencing (Siebert et al., 2019). All of these tools allow the study of a variety of genes in adult and regenerating animals. In addition, the visualization of *Hydra* regeneration has advanced in recent years, with the addition of fluorescent reporters and sophisticated live-imaging approaches (Aufschnaiter et al., 2011; Carter et al., 2016; Tomczyk et al., 2017; Dupre and Yuste, 2017; Szymanski and Yuste, 2019).

Stem cell populations and regeneration

Hydra homeostasis and regeneration relies on three distinct stem cell populations – unipotent epidermal or gastrodermal epithelial stem cells (eESCs and gESCs, respectively) and multipotent interstitial stem cells (ISCs), which are frequently seen as pairs (Bode, 1996; Hobmayer et al., 2012). ISCs, which give rise to a dozen of different cell types, cycle quickly (every 24-30 h) and are located in the central body column, intermingled between eESCs. ISCs produce germ cell progenitors that differentiate into gametes only when animals become sexual. On a constitutive basis, ISCs produce somatic progenitors, which proliferate as syncytial clusters to differentiate as stinging cells (nematocytes, also named cnidocytes),

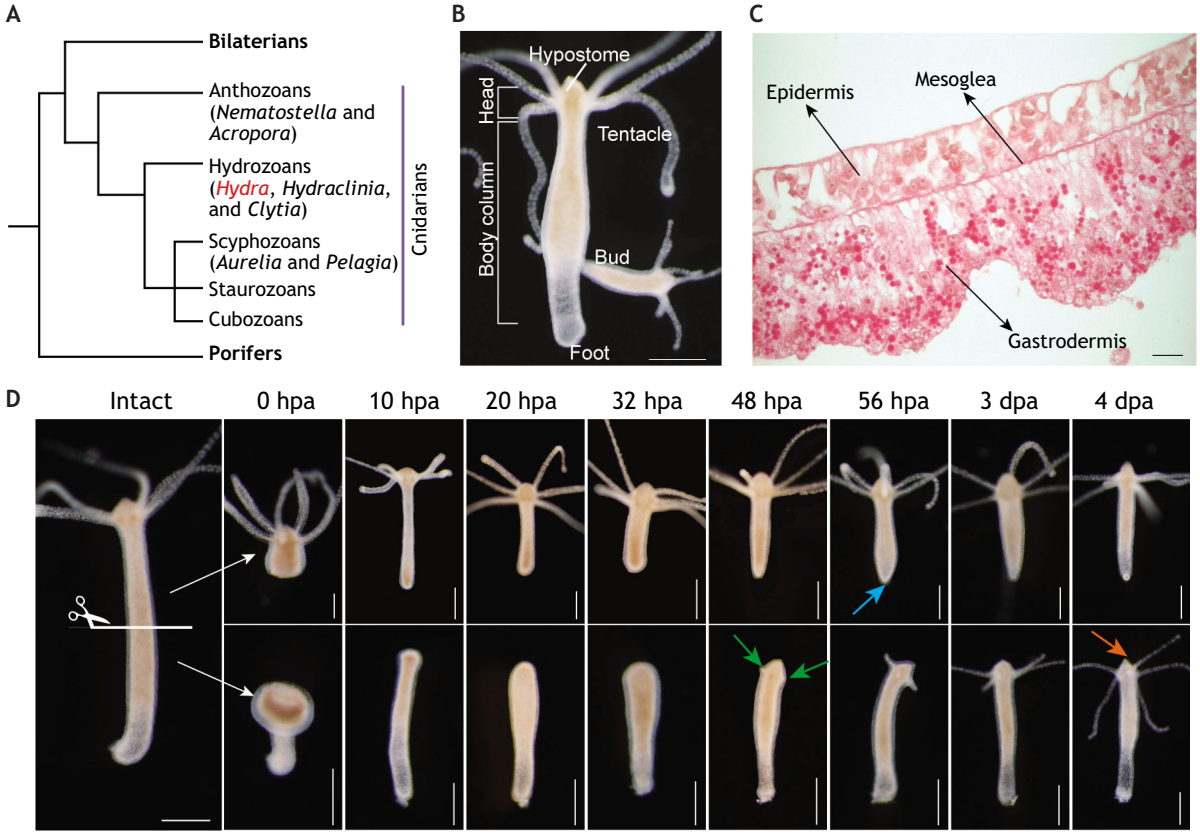

Fig. 1. Phylogenetic position and regenerative capabilities of *Hydra*. (A) Phylogenetic position of *Hydra* within the phylum Cnidaria and the class Hydrozoa. (B) *Hydra* anatomy. On their apical end, animals possess a head consisting of the hypostome and tentacles. The body column separates the head from the foot, which is located at the basal end. (C) Haematoxylin and Eosin staining of paraffin embedded sections through a *Hydra* animal, highlighting the two distinct body layers (the epidermis and the gastrodermis) and the ECM layer (the mesoglea) that separates them. (D) *Hydra* head and foot regeneration. Regenerating animals after mid-gastric bisection at the indicated time points are shown. Blue arrow indicates the fully regenerated foot. Green arrows indicate the emergence of tentacle rudiments. Red arrow indicates a fully regenerated head. Scale bars: 500 µm in B,D; 20 µm in C.

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migrate towards the extremities where they terminally differentiate into neurons or traverse the mesoglea to differentiate as gland cells in the gastrodermis (David and Plotnick, 1980; Bode, 1996). In contrast, unipotent gESCs and eESCs cycle slowly (every 3 to 4 days) and become passively displaced towards the extremities, where they abruptly stop cycling and terminally differentiate into more specialized epithelial cells, such as battery cells in the tentacles or mucous cells in the basal disc.

The fact that all stem cells along the body column are cycling, either paused in G2 or traversing S phase, imposes striking features on regeneration (Buzgariu et al., 2014, 2018). Indeed, all of these cycling cells are under injury-induced regulation, with G2-paused cells undergoing mitosis locally (Cummings and Bode, 1984; Chera et al., 2009; Buzgariu et al., 2018) or directly differentiating into head or foot cells (Dübel and Schaller, 1990), and with interstitial progenitors migrating towards the wound (Tardent and Morgenthaler, 1966; Chera et al., 2009, 2011; Boehm and Bosch, 2012). In a way, the situation is rather similar to that observed in wounded planarians in which proliferative stem cells (termed 'neoblasts') are recruited to migrate towards the wound, where they form a non-proliferative regenerating tissue mass known as a 'blastema' (Reddien and Sanchez Alvarado, 2004). In *Hydractinia*, the proliferating ISCs also migrate towards the wound where they accumulate to form a blastema-like structure, an accumulation not seen in foot regeneration (Bradshaw et al., 2015). In *Nematostella*, and more generally in anthozoans, ISCs have not been identified (Gold and Jacobs, 2013), and both *Nematostella* and *Hydractinia* (which are hydrozoans) require the induction of epithelial proliferation for the regeneration of their oral structures (Passamaneck and Martindale, 2012; Amiel et al., 2015; Bradshaw et al., 2015). These results indicate that proliferating cells play an important role in cnidarian regeneration, although distinct cell types are implicated in different cnidarians, highlighting the importance of investigating several cnidarian models.

Insights gained from studying regeneration in *Hydra*

Principles of homeostatic and regenerative patterning

A key concept in developmental biology is that of the organizer, which was first discovered in 1909 by Ethel Browne using *Hydra*. By transplanting non-pigmented head tissue into the body column of a pigmented host, she observed the induction of a secondary axis that was predominantly made of host cells. She could thus conclude that the *Hydra* head has the ability to instruct and recruit the host tissue to alter its identity, a property later named organizer capacity (Fig. 2A,B) (Browne, 1909; reviewed by Webster, 1966; Vogg et al., 2016). This inductive activity is restricted to the head in intact animals (Broun and Bode, 2002) but Browne also identified an organizer activity in the apical-regenerating tips and in the presumptive head region of the growing bud, indicating that organizers are active in two distinct settings: homeostatic (i.e. in apical tissue from an intact animal) and developmental (i.e. in a budding or regenerating tissue). There is evidence that these experiments influenced the renowned experiments performed by Hans Spemann and Hilde Mangold in 1924 (Lenhoff, 1991). By transplanting the dorsal blastopore lip of an unpigmented newt embryo into a pigmented host, Spemann observed cell fate changes in the host embryo that led to the induction of a Siamese twin (Spemann and Mangold, 1924). Spemann termed the dorsal blastopore lip an 'organizer'.

Over the following decades, it actually turned out that *Hydra* has two distinct organizers: the head organizer located at the apical tip; and a foot organizer located in the basal region (Fig. 2C) (Browne, 1909; Yao, 1945; Webster, 1971; Hicklin and Wolpert, 1973). Moreover, a series of axial and lateral transplantation experiments demonstrated that the head and foot organizers produce activator and inhibitor substances, the respective activities of which are graded along the *Hydra* body axis (Fig. 2C) (Rand et al., 1926; Hicklin and Wolpert, 1973; McWilliams, 1983a,b; Takano and Sugiyama, 1983; Broun and Bode, 2002; Shimizu, 2012). Evidence for a head activation gradient came from Webster and Wolpert, when they transplanted tissue from different positions along the *Hydra* body axis into the mid-digestive zone and observed that secondary axis formation decreases as the distance from the apical tip increases (Webster and Wolpert, 1966). In addition, Webster observed that the transplantation of head tissue into different regions along the axis induces a secondary body axis more frequently as the distance from the

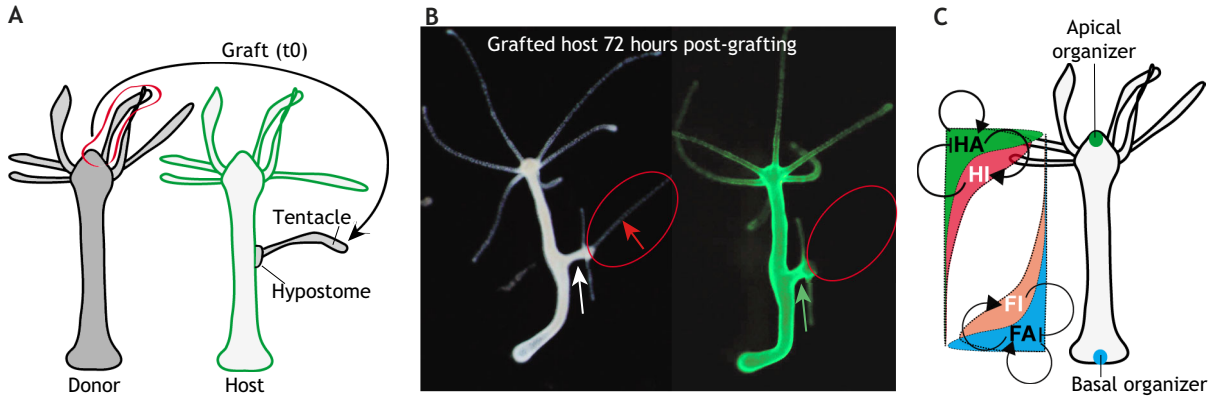

Fig. 2. The *Hydra* head organizer. (A) Schematic representation of Ethel Browne's transplantation experiments from 1909. She grafted a piece of hypostome together with a tentacle (outlined in red), which by itself does not have any organizer activity but is used as a marker of the graft, onto the body column of a host animal. The donor (left) was depigmented, while the host (right) was pigmented green using symbiotic algae, thereby allowing host and donor tissues to be discerned. (B) Reproduction of the Browne lateral grafting experiment, in this case using a wild-type *Hv* animal as the donor and a transgenic host animal that expresses *GFP* under the control of the actin promoter in epidermal cells. The grafted tissue, consisting of hypostomal tissue and a tentacle (red arrow), is outlined in red. The bright-field (left) and fluorescent (right) images shown here highlight how a secondary body axis is induced 72 h after transplantation. GFP-positive cells are recruited from the host (green arrow) into the newly induced body axis (white arrow). (C) Representation of the head activation/head inhibition gradients (HA/HI, green and red) and the foot activation/foot inhibition gradients (FA/FI, blue and orange). They have inverted distribution, maximal at the apical pole for HA/HI and maximal at the basal pole for FA/FI.

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apical tip increases, suggesting an axial head inhibition gradient (Webster, 1966).

Both head and foot activation/inhibition gradients fit into Turing's reaction-diffusion model, which was subsequently adapted by Meinhardt and Gierer to explain pattern formation through local self-enhancement and long-range inhibition (Turing, 1952; Gierer and Meinhardt, 1972). In short, this model suggests that pattern formation is properly achieved when a short-range autocatalytic activator triggers patterning but at the same time is antagonized by a long-range fast-diffusing inhibitor produced under the control of the activator (Fig. 2C). This model is useful to explain the two types of organizers mentioned above: homeostatic, with a stable activity in intact animals; and developmental, which is progressively established in the regenerating tip or the bud spot.

Gierer and Meinhardt also added the concept of 'source density', which they defined as follows: 'The theory is based on short-range activation, long-range inhibition, and a distinction between activator and inhibitor concentrations on one hand, and the densities of their sources on the other. While source density is expected to change slowly, e.g. as an effect of cell differentiation, the concentration of activators and inhibitors can change rapidly to establish the primary pattern: this results from auto- and cross catalytic effects on the sources, spreading by diffusion or other mechanisms, and degradation'. But how does this apply to *Hydra*? In intact animals, the source densities at the tip of the head are stably established, while along the body column, the very same region can remain identical when not injured, or it can produce a head or a foot organizer depending on the level of the cut. As stated by Gierer, this implies that 'no pre-existing local property of the tissue (such as a polarity-defining gradient determining the orientation of regenerates) can per se decide where a head is formed; this can be decided only by the formation of a new morphogenetic gradient after the onset of regeneration' (Gierer, 2012). The challenge for a regenerating *Hydra*, therefore, is to convert a piece of bilayered gastric tissue with no organizer activity into a *de novo* organizer that will lead to patterning, with this conversion taking place at any level along the apical/basal axis. Indeed, we know from transplantation experiments that the equilibrium between the activator and the inhibitor is disrupted upon bisection and is re-established within 2 days of amputation, whatever the bisection level (MacWilliams, 1983a,b). Within the first 10 h after mid-gastric bisection, the activity of the head activator is rapidly restored while that of the head inhibitor slowly increases to its original level, leaving enough time to establish a new head activator with maximal activity at the regenerating tip.

Wnt/β-catenin signalling as an activator of the homeostatic head organizer

At the molecular level, several lines of evidence suggest that Wnt/β-catenin signalling plays a central role in maintaining the activity of the *Hydra* head organizer. First, β-catenin is mainly nuclear in the head region compared with the body column (Broun et al., 2005). Second, head organizer capacity is conveyed on body column tissue upon ectopic activation of Wnt/β-catenin signalling either genetically by overexpressing β-catenin or pharmacologically by inhibiting GSK3β, a negative regulator of the Wnt pathway, using alsterpaullone (Broun et al., 2005; Gee et al., 2010). Third, seven out of eleven *Hydra* Wnt genes are mainly expressed in the tip of the head region (Hobmayer et al., 2000; Lengfeld et al., 2009). Notably, Wnt3 expression is graded along the body column, as detected by RNA-seq (Vogg et al., 2016, 2019). Fourth, head organizer activity in homeostatic animals relies on β-catenin-dependent regulation of Wnt genes: the expression of *Wnt3* is directly controlled by the β-catenin/TCF complex (Nakamura et al., 2011).

In turn, Wnt3 is believed to act as a paracrine factor that maintains β-catenin active in the head organizer region (Hobmayer et al., 2000; Nakamura et al., 2011). The role of Wnt3 in maintaining and re-launching head organizer activity, together with its auto-regulation via β-catenin (Nakamura et al., 2011), support the assumption that the Wnt3/β-catenin canonical pathway fulfils the criteria of the head activator in *Hydra*. However, treating animals with Wnt3 or with drugs that constitutively activate Wnt/β-catenin signalling does not lead to ectopic heads, at least not initially, but instead gives rise to ectopic tentacles, indicating that the activation of this pathway alone does not suffice to recapitulate the activity of the head organizer.

Injury-induced cell death and Wnt/β-catenin signalling as activators of the regenerative head organizer

In contrast to the situation observed in the head organizer, most Wnt genes are expressed at very low levels in the mid-gastric region (Lengfeld et al., 2009; Wenger et al., 2019). As such, injury signals are required to restore head organizer activity in regenerating animals. In short, mid-gastric bisection leads to an asymmetric activation of ROS signalling (Suknovic, 2019), which is sufficient to activate the MAPK/CREB pathway at a higher level in head- versus foot-regenerating tips (Galliot et al., 1995; Kaloulis et al., 2004; Chera et al., 2011). This triggers the death of ISCs and interstitial derivatives (which are more sensitive to apoptotic signals than are ESCs), the release of Wnt3 (or Wnt3-like) by dying cells and the activation of β-catenin signalling in the surrounding cells, mainly in pairs of ISCs and interstitial progenitors, which pushes them through mitosis (Chera et al., 2009; Buzgariu et al., 2018). In parallel, gESCs act as phagocytes that engulf apoptotic bodies, and they begin to express *Wnt3*. Indeed, *Wnt3* is the first *Hydra* gene to display an immediate sustained upregulation after bisection, which is maintained in head- but not foot-regenerating tips (Lengfeld et al., 2009; Wenger et al., 2019).

In head regeneration-deficient *reg-16* animals, the level of *Wnt3* expression in the head-regenerating tips correlates with their level of head-regeneration deficiency (Hobmayer et al., 2000). Interestingly, blocking apoptosis using caspase inhibitors prevents the release of Wnt3 protein and thus the immediate re-launching of head organizer activity (Chera et al., 2009, 2011). The best evidence of this mechanism was obtained by inducing ectopic head organizer activity in foot-regenerating tips that are briefly exposed to heat to trigger apoptosis (Chera et al., 2009). In summary, injury-induced apoptosis is required to rapidly restore head organizer activity after mid-gastric bisection, but not for the maintenance of organizer activity in homeostatic animals.

Inhibitor(s) of the homeostatic and regenerative organizers

Since the experimental discovery of an inhibitory activity of heads on their own formation (Rand et al., 1926), attempts to categorically characterize the head inhibitor remained unsuccessful. A protease-resistant molecule was proposed but never identified (Berking, 1977, 1979). The Dickkopf secreted proteins have also been proposed as head inhibitors but do not fulfil the expected criteria, as Wnt/β-catenin signalling negatively regulates *hyDkk1/2/4* and loss-of-function assays do not induce a multi-headed phenotype (Augustin et al., 2006; Guder et al., 2006). Similarly, a multi-headed phenotype is not induced upon the silencing of thrombospondin, which was recently suggested to act as a negative-feedback regulator of Wnt/β-catenin-dependent organizer formation (Lommel et al., 2018).

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However, a recent study of candidate β-catenin target genes has indicated that the transcription factor Sp5, the expression of which is maximal in the apical region, acts as a head inhibitor (Vogg et al., 2019). Indeed, *Sp5* knockdown triggers multiple head formation in intact as well as regenerating conditions and, as expected from the reaction-diffusion model (Gierer and Meinhardt, 1972), *Sp5* expression is positively regulated by Wnt/β-catenin signalling while Sp5 directly lowers Wnt/β-catenin signalling by repressing *Wnt3* promoter activity. This study also showed that *Sp5* is excluded from the tip of the hypostome, the region where *Wnt3* expression is maximal, suggesting that another regulator prevents *Sp5* expression in this region. Along the body axis, *Wnt3* expression is exponentially graded, as shown by RNA-seq analysis, and is thus potentially able to trigger a parallel graded expression of *Sp5* cell-autonomously (Vogg et al., 2019). In fact, the graded pattern of *Sp5* expression along the body axis varies, being obvious in 'juvenile' animals taken after budding or head regeneration, and lacking in mature animals, where the rather homogenous *Sp5* expression might result from Sp5 auto-activation (Vogg et al., 2019).

The main issue at present is characterizing how Sp5 works as head inhibitor, either cell-autonomously, or non-cell-autonomously via the production of factors released by Sp5-expressing cells. Even though the inhibitor was predicted to be diffusible (Gierer and Meinhardt 1972; MacWilliams, 1983a,b; Technau et al., 2000), a model relying on the activity of a transcription factor could not have been anticipated at the time Meinhardt and Gierer proposed their model, as the key role of transcription factors in developmental processes had not yet been discovered. If Sp5 works cell-autonomously, i.e. without the intervention of a diffuse substance, the Meinhardt and Gierer model might need to be revisited and additional components taken into account, in line with a recent study showing that realistic reaction-diffusion systems are fundamentally different from the concept originally proposed (Marcon et al., 2016). So far, the role of Sp5 has been tested only in the context of developmental head organizers, and its mode of action might be different in the context of a homeostatic organizer, at least during the period in which the organizer becomes re-established.

The foot organizer

In contrast to head regeneration and the head organizer, little is known about the molecular nature of the foot organizer. Recently it has been shown that Wnt/β-catenin signalling is also required for foot regeneration (Gufler et al., 2018) and that regulators of BMP signalling are expressed early during foot regeneration (Wenger et al., 2019), suggesting that crosstalk between components of the Wnt and BMP pathways might be involved in the regeneration and maintenance of the foot organizer. Altogether, these studies highlight that *Hydra* offers a powerful model to study the maintenance and developmental regulation of organizers and to identify new components of activator-inhibitor systems that play a fundamental role in pattern formation during development and regeneration.

Self-organization and organoids

The extreme capacity of *Hydra* to regenerate is best demonstrated by the ability of dissociated tissues (broken up to the single cell level) to rebuild the animal once re-aggregated (Fig. 3). Early studies showed that, within the first hour following *Hydra* dissociation, cells re-aggregate into a mass in which epidermal and gastrodermal cells become sorted, re-establishing the original two cell layers. Three to five days later, complete polyps with hypostomes, tentacles and basal discs are formed (Gierer et al., 1972). Around day six, the regenerated polyps are functional, i.e. able to feed. Importantly, cells from different positions along the *Hydra* body axis exhibit variable potential in establishing such structures. This early work was a clear demonstration of the self-organizing abilities of *Hydra* cells (Noda, 1971; Gierer et al., 1972).

A deeper characterization of this self-organization phenomenon awaited the breakthrough that established the Wnt/β-catenin pathway as a key regulator of apical identity in *Hydra*. Indeed, these studies then revealed that, early during the development of

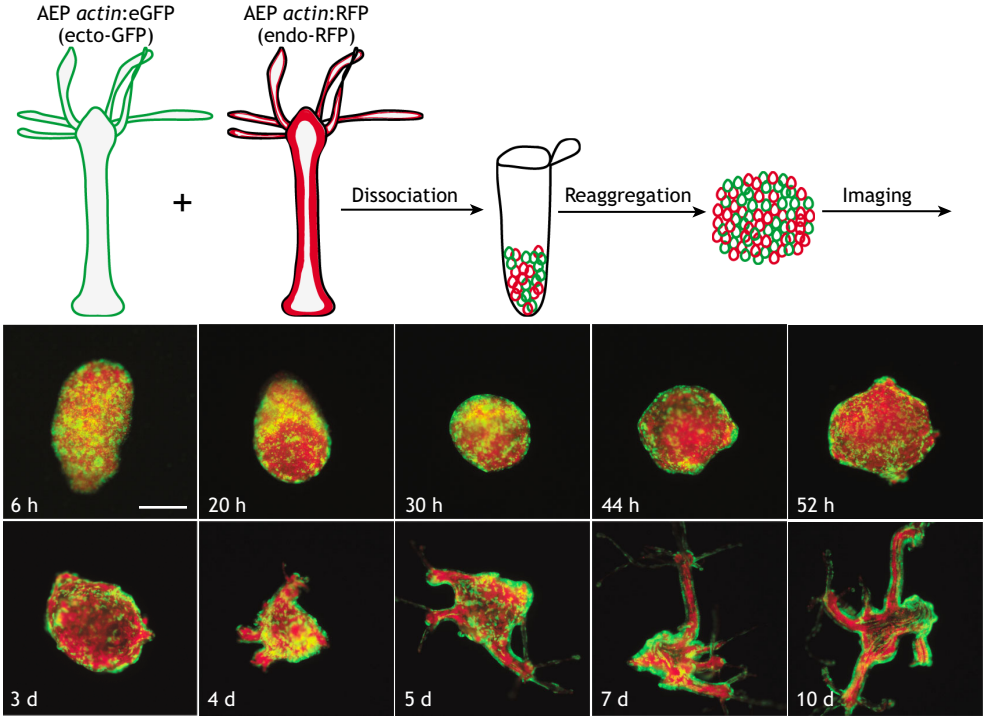

Fig. 3. Regeneration of *Hydra* from reaggregated cells. The reaggregation experiment (top panel) was made with *Hydra* taken from two distinct transgenic AEP strains: one that expresses eGFP under the control of the actin promoter in epidermal cells; and the other that expresses RFP under the control of the actin promoter in gastrodermal cells. Aggregates were imaged as indicated at different time points after re-aggregation (lower panels). The re-aggregated cells are sorted, with gastrodermal cells (red) located inside the aggregate and epidermal cells (green) in the periphery, and there is a subsequent regeneration of a complete *Hydra* animal. Scale bar: 250 µm.

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re-aggregated cells, prior to the morphological appearance of hypostomes or tentacles, *Wnt3* is expressed in specific domains that turn out to become the future oral poles (Technau et al., 2000). Quantitative analysis indicated that a group of 5-15 epithelial cells are capable of forming an organizing centre and establish an inhibition field around them, extending ∼800-900 µm away. However, a critical result, not conforming to the reaction-diffusion dynamics underlying the emergence of organizer centres, was that the number of such centres formed depends on the origin of the cells that give rise to them (i.e. the original location of these cells along the main axis). For example, aggregates made from oral tissue form four times more heads compared with aggregates made from aboral tissue (Technau et al., 2000). Thus, while the precise implementation of reaction-diffusion dynamics remains an unresolved issue, a clear conclusion from this study is that a cell community effect (Gurdon et al., 1993) leads to the emergence of *de novo* organizer centres.

Over the past decade, pluripotent and adult stem cells from mammals have been used in a similar 'self-organizing' manner to generate organoids, which are 3D cellular structures that recapitulate key aspects of tissue/organ function and organization (Kretzschmar and Clevers, 2016). These organoids share fundamental features with *Hydra* aggregates, despite some clear differences (Table 1). Thus, regenerating *Hydra* aggregates can be viewed as forefathers of the now widely studied organoid systems. Importantly, all of these systems can be used to address similar questions regarding how groups of cells self-organize into a functional tissue (Gjorevski et al., 2016). A key step in self-organization is the symmetry-breaking event that leads to a subgroup of cells in an initially mostly homogenous group taking on special properties (Gierer et al., 1972; Rossi et al., 2018). In many organoid systems, with intestinal organoids being a characteristic example, symmetry breaking involves Wnt signalling, as occurs in *Hydra* aggregates (Technau et al., 2000; Clevers, 2016; Serra et al., 2019; Vogg et al., 2019). Indeed, a key step in the development of an intestinal organoid is the establishment of a stem cell niche in the form of a *Wnt3*-expressing Paneth cell (Sato et al., 2011). However, very little is currently known about other genes and pathways that operate during the regeneration of *Hydra* aggregates and that orchestrate self-organization in organoids. Further studies are therefore needed to identify, besides Wnt3, other key players involved in self-organization. Like many organoid systems, *Hydra* aggregates are amenable to cell tracking, as a selection of cell types submitted to genetic or chemical manipulations can be reaggregated in variable proportions (Technau et al., 2000; Cochet-Escartin et al., 2017; Vogg et al., 2019). Moving forward, *Hydra* could thus be used to better understand and improve mammalian organoid formation *in vitro*.

Cell shape changes and mechanical inputs

The recent characterization of *Hydra* mouth opening with cellular resolution led to the conclusion that this process involves cell morphology changes rather than cell repositioning (Carter et al., 2016). As such, questions revolving around the properties of individual *Hydra* cells and their interactions with neighbours are surfacing. Budding and bud detachment in *Hydra* are associated with distinct changes in cell shape, and recently the FGFR and Rho-ROCK-Myosin pathways have been implicated in these events (Holz et al., 2017). In addition, the generation of Lifeact-GFP transgenic *Hydra* (in which F-actin is labelled) has allowed researchers to trace changes in cytoskeletal organization during bud formation (Aufschnaiter et al., 2017). The same transgenic line has enabled observation of the *de novo* establishment of planar cell polarity in the ectodermal layer of regenerating, aggregated *Hydra* cells, showing that this event occurs in defined steps (Seybold et al., 2016). In addition, the recent visualization of actin filaments that traverse a piece of *Hydra* tissue undergoing regeneration uncovered the role of the tissue level organization of such filaments for the proper patterning of the regenerating piece (Livshits et al., 2017). In fact, it seems that the oral/aboral axis follows the orientation of actin filaments, highlighting the importance of the mechanical status of a regenerating piece in determining its fate.

The above results are in accordance with findings suggesting that physical and mechanical properties of regenerating *Hydra* fragments are crucial for their regenerative potential. Indeed, it has been observed that small pieces of *Hydra* undergoing regeneration endure osmotically driven mechanical oscillations (Fütterer et al., 2003). These fragments slowly inflate by pumping excess fresh water into the gastric cavity, and deflate suddenly once a threshold of pressure is reached (Kücken et al., 2008). A change in the oscillation pattern has been associated with *de novo* organizer appearance, while such oscillations were found to be necessary for further development of the *Hydra* fragments (Soriano et al., 2009). A theoretical investigation of these oscillations, which are common in other multicellular cysts, pointed to a possible role for them in size regulation of the regenerating tissue (Ruiz-Herrero et al., 2017). Moreover, a new set of models has extended the existing Gierer-Meinhardt theoretical framework to incorporate mechanical and

Table 1. Comparison between *Hydra* aggregates and organoids

Similarities between *Hydra* aggregates and organoids	Specificities of *Hydra* aggregates	Specificities of organoids
A group of initially similar epithelial cells goes through a symmetry-breaking event to achieve tissue-level patterns	Requires a large number of cells to start (>5000)	Possible to start from a single cell
Symmetry breaking emerges through variations in cell properties and local interactions	End product is one or several animals	End product recapitulates some aspects of an organ
The molecular machinery exploited is similar, with Wnt/β-catenin signalling playing a prominent role in *Hydra* aggregates (Technau et al., 2000) but also in intestinal, stomach and kidney organoids among others (Clevers, 2016)	Does not rely on exogenous factors; the process is true self-organization	Often requires a time schedule of interference/stimulation, with media changes and the addition of factors
Integration of mechanical stimuli is crucial for symmetry breaking not only in *Hydra* (Cochet-Escartin et al., 2017) but also in gut organoids (Gjorevski et al., 2016) where a regeneration program is initiated (Serra et al., 2019)	The process is fast, with symmetry breaking occurring within 24 hours	The process is slow and often requires days, e.g. symmetry breaking in intestinal organoids happens after 3 days
Both are experimental systems amenable to a variety of manipulations, the behaviour of which can be exploited to understand aspects of the original tissue	Gene manipulation so far is restricted to RNAi	Gene manipulation with CRISPR/Cas9 is possible

biochemical communication into the symmetry breaking process (Mercker et al., 2015; Brinkmann et al., 2018). One of the next frontiers for the field will be to understand how cells generate and interpret biophysical signals, and how these signals establish the conditions that allow self-organization to emerge.

Nervous system regeneration

Another field that is undergoing a transformation is the study of *Hydra* nervous system development and regeneration. The *Hydra* nervous system takes on the form of a diffuse nerve net, which is much denser in the apical and basal regions; in some species, a nerve ring is visible at the base of the hypostome (Koizumi, 2007). The behaviour of *Hydra* was a topic of experimentation for Abraham Trembley, who observed their contraction upon mechanical stimulation, habituation and phototaxis phenomena (Lenhoff and Lenhoff, 1986), observations that were later detailed and quantified by Passano and McCullough (1963, 1964, 1965). With the help of computer vision and machine learning techniques, it is now possible to quantify and cluster elementary behavioural patterns in an objective manner (Han et al., 2018). In parallel, Dupre and Yuste recently visualized neuronal activity in the entire animal (Dupre and Yuste, 2017), potentially allowing neuronal activity to be connected to specific behavioural patterns. The expansion of manipulation techniques with new microfluidic approaches (Badhiwala et al., 2018) strengthen arguments in favour of *Hydra* becoming an important model system in the field of neurosciences (Bosch et al., 2017; Rentzsch et al., 2019).

The reappearance of the nervous system during *Hydra* regeneration has also been the subject of investigation (Koizumi et al., 1990). After local destruction due to cell death in head regenerating tips, the nerve net becomes regenerated together with other tissues and, in species that have a nerve ring (e.g. *Hydra oligactis*), the nerve ring reappears (Koizumi et al., 1992; Minobe et al., 1995). The potential to regenerate a nerve net has been exploited in *Hydra* via nervous system transplantation studies (Saffitz et al., 1972), a procedure that is unparalleled in the animal kingdom. *Hydra* can also be treated chemically to kill fast cycling interstitial cells and eliminate all their derivatives, including nerve cells (Tran et al., 2017). In a few weeks, such animals become ‘nerve-free’ and are unable to catch their food but still show regular contractions of their myoepithelial layers and, even more surprisingly, can regenerate after amputation, possibly as a result of the observed genetic plasticity of the myoepithelial cells (Marcum and Campbell, 1978; Wenger et al., 2016). Seeding interstitial cells in a nerve-free animal can rescue these animals, as a new nerve net progressively forms (Minobe et al., 1995). Therefore, the combination of classical approaches and new strategies in *Hydra* neurobiology now allow the functionality of the regenerating nervous system to be probed at each phase of the process. What behaviours are progressively supported by the re-appearing nervous system? How do newly formed nerve cells connect to each other and to the pre-existing nerve net? These are just a few questions that can be asked using *Hydra* to study nervous system regeneration.

Cellular crosstalk, epithelial plasticity and molecular programs of regeneration

The advent of high-throughput omics data in *Hydra* is also shifting our understanding of animal regeneration. For example, time series of transcriptomic and proteomic analyses during head regeneration have become useful resources, as they provide a window into the genetic changes associated with the rebuilding of a truncated head (Wenger, 2014; Petersen et al., 2015; Wenger et al., 2019). Based on the most recent of these transcriptomic studies, a unique resource that provides the spatial, regenerative, cell-type and nerve-free profiles of each *Hydra* gene has now been made publicly available (hydratlas.unige.ch). In addition, a recent cell type-restricted comparative transcriptomic analysis has shed light on the plasticity of *Hydra* epithelial cells: when the epithelial transcriptomic signature was compared between normal and nerve-free animals, several hundreds of genes were found to be upregulated in the epithelial cells of nerve-free animals, implying that epithelial cells change their gene expression profile to compensate for the lack of interstitial cells and nervous system (Wenger et al., 2016). Indeed, among the upregulated genes are neurogenic genes as well as neuronal signalling components including ion channel receptors. These data point to the possibility that ancestral epithelial cells, i.e. those that predate the emergence of neurogenesis, already expressed ‘proto-neuronal’ genetic programs linked to sensing and responding to environmental changes.

These results can also potentially solve apparent contradictions between two observations, on one side the crucial role of *de novo* neurogenesis during head regeneration (Miljkovic-Licina et al., 2007) and on the other side the fact that nerve-free *Hydra* can regenerate, implying that epithelial layers suffice to complete a regeneration program (Marcum and Campbell, 1978). The concept of epithelial plasticity suggests that epithelial cells do not behave identically in intact and nerve-free animals, i.e. plasticity enables them to offset deficiencies due to the lack of a nerve net. This plasticity property might be intrinsically linked to *Hydra* regeneration, as the head-regenerating tip is nerve-free for at least the first 36-40 h after amputation (Chera et al., 2009). The crosstalk between epithelial and interstitial cell lineages indeed plays a key role in *Hydra* regeneration, as identified decades ago (Wanek et al., 1986), but the mechanisms underlying this crosstalk as well as its cellular and developmental impact remain to be further dissected at the genetic and mechanical levels.

Conclusions

Hydra is the oldest model system in experimental developmental biology. Its regenerative abilities are extraordinary, with it being able to regenerate body parts but also regenerate entire animals from a clump of dissociated tissues. New theoretical and experimental tools pave the way for a deeper understanding of these phenomena at the cellular and molecular level. Specific issues, such as the reactivation of organizer centres in aggregates, the crosstalk between cell types and cell layers, nerve net regeneration and emerging behaviours, make *Hydra* a potent and exciting experimental system that can help us understand why and how tissues regenerate or not.

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Competing interests

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