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Past, present and future of *Clytia hemisphaerica* as a laboratory jellyfish

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Abstract

The hydrozoan species *Clytia hemisphaerica* was selected in the mid 2000s to address the cellular and molecular basis of body axis specification in a cnidarian, providing a reliable daily source of gametes and building on a rich foundation of experimental embryology. The many practical advantages of this species include genetic uniformity of laboratory jellyfish, derived clonally from easily-propagated polyp colonies. Phylogenetic distance from other laboratory models adds value in providing an evolutionary perspective on many biological questions. Here we outline the current state of the art regarding available experimental approaches and in silico resources, and illustrate the contributions of *Clytia* to understanding embryo patterning mechanisms, oogenesis and regeneration. Looking forward, the recent establishment of transgenesis methods is now allowing gene function and imaging studies at adult stages, making *Clytia* particularly attractive for whole organism biology studies across fields and extending its scientific impact far beyond the original question of interest.

1) Introduction and historical background

Our original, pragmatic, motivation to select *Clytia hemisphaerica* as a laboratory model was because of unique features suited for studying embryonic axis specification. Specifically, we were seeking an experimentally tractable species in which embryo polarity was established de novo following fertilization, rather than being directed by positional cues ("maternal determinants") in the egg. Such determinants, often as localized mRNAs, are responsible for axis specification in almost all traditional model species, but the key events of symmetry breaking are challenging to image and analyse because they occur over protracted periods of oogenesis within the mother's ovary (Bashirullah et al., 1998). An important body of previous experimental embryology indicated at the time (mid 2000s) that animals from the clades Ctenophora or Cnidaria might offer uniquely accessible material for studying embryo polarity establishment. Dye tracing and egg manipulation experiments on many species from these taxa, including the cnidarian *Clytia* (a.k.a. *Phialidium*) hydrozoan jellyfish species, had indicated that the orientation of their single principle body axis was determined de novo in the fertilized egg, the position of the zygote nucleus dictating that of the future 'oral' pole (Freeman, 1976, 1977, 1981a). Thus embryo polarity establishment mechanisms in cnidarian and ctenophore species could potentially be analyzed in embryos developing in sea water on the bench.

Against expectations it turned out that the embryonic axis in *Clytia* is positioned by localized mRNAs that cause polarized Wnt pathway activation, revealing close and unexpected parallels with axis determination in traditional model species from Bilateria and allowing the historical experiments to be reinterpreted in a new light (see Section 4). By then, *Clytia* had proved its worth as an experimental model, and the initial investment in techniques and resources provided a platform for further studies on many topics. This included early realisation that the ovary of female *Clytia* has particular properties that make it an exceptional model for studying the regulation of oogenesis, oocyte maturation, and spawning, as detailed in Section 3. Furthermore, *Clytia's* phylogenetic distance from other laboratory models, including the increasingly popular cnidarian model *Nematostella vectensis* (Layden et al., 2016), proved increasingly to have significant added value in providing an evolutionary perspective on many processes, as illustrated across different sections of this chapter. In parallel the *Clytia* model has been exploited for more explicit 'EvoDevo' studies to map the genomic changes accompanying animal evolution, notably in the early years by Michael Manuel and colleagues in Paris, with possibilities augmented more recently by availability of the whole genome sequence as outlined in Section 6.

The choice of *Clytia hemisphaerica* among many candidate cnidarian species available in Villefranche was essentially based on ease of culture and suitability for embryo experimentation.

Clytia jellyfish reliably provide a daily supply of large (180 µm diameter), transparent, eggs devoid of extracellular envelopes or jelly, which are robust to manipulate and develop reliably. Crucially, previous studies on the site had shown that *C. hemisphaerica* could be cultured as 'immortal' vegetatively propagating polyp colonies supplying medusae by constant budding (Figure 1; Carré & Carré, 2000). The adults are a convenient size to be maintained in modestly-dimensioned beaker or tank based aquarium systems, and survive on a simple diet of *Artemia* nauplii (Lechable et al., 2020). Other unanticipated practical advantages became apparent as the model developed. The clonal origin of jellyfish by vegetative budding from stable polyp colonies was a huge boon, not only increasing reproducibility between experiments but meaning that when ESTs (Chevalier et al., 2006; Forêt et al., 2010) and later full transcriptome and genome resources (Leclère et al., 2019) were established, the sequences exactly matched those of the laboratory animals. This alleviated the polymorphism problems experienced for many marine animal models collected from wild populations, facilitating successful selection of sequence targets for gene knockdown approaches such as Morpholino antisense and later CRISPR/Cas9 (Section 2). The partial temperature dependence of colony sex determination (Carré & Carré, 2000) allowed a set of highly genetically-related core colonies to be established via self-crossing (Leclère et al., 2019). These colonies can be conveniently distributed to colleagues around the world as cuttings on glass slides (Lechable et al., 2020).

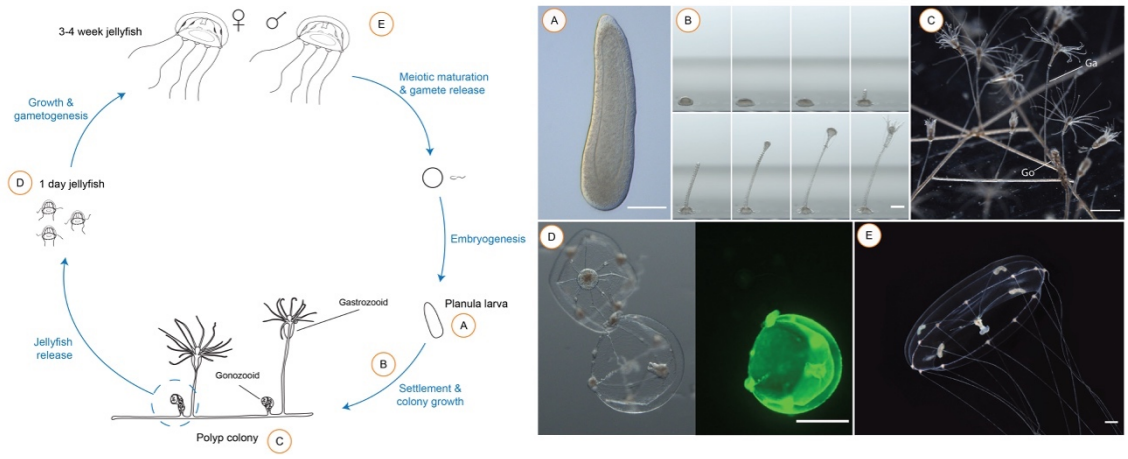

Figure 1 - *Clytia hemisphaerica* life cycle.
On the left is a schematic of the full cycle, as maintained in the laboratory. Images on the right illustrate different stages. (A) 2-day planula larva. Its simple organization comprises two tissue layers: epidermis (ectoderm) and gastrodermis (endoderm) and an elongated "oral-aboral" axis (oral at the top). (B) Images from a time-lapse recording of planula metamorphosis recorded over 2 days following settlement on a substrate. The form drastically changes to generate the primary feeding polyp (gastrozooid). (C) Extension of a stolon from the base of the primary polyp and subsequent emergence of many polyps, both gastrozooids and gonozooids (budding polyps), leads to formation of a colony. Ga - Gastrozooid, Go - Gonozooid. (D) Bright field (left) and fluorescence (right) images of wildtype (bottom) and *GFP1/GFP2* knockout (top) jellyfish shortly following budding. Multiple loci of GFP genes were simultaneously mutated by CRISPR/Cas9. (E) Adult jellyfish. Scale bars 50 µm for A, 200 µm for B, 500 µm for D, and 1 mm for C and E. Lifecycle diagram adapted from Munro (2021a). A-D adapted from Momose (2021a,b,c,d); E adapted from (Lechable et al., 2020).

Since the first 'molecular era' papers on *Clytia* (Chevalier et al., 2006; Jager et al., 2006; Momose & Houliston, 2007; Momose et al., 2008) which introduced ESTs, in situ hybridisation and gene function analysis via Morpholino/mRNA microinjection, technical possibilities have steadily widened and techniques refined. These advances, along with the progressive accumulation of in silico resources, are detailed in Section 2 and Table 1. In parallel, attention has extended from embryos and larvae to the adult medusa stage. This one-centimeter diameter transparent, actively swimming and sexually reproducing animal (Figure 1E) is already showing great promise as a model in the fields of regeneration and neural systems biology (see Section 5). *Clytia* thus illustrates how the impact of a new model organism can extend far beyond the original field of interest.

2) Practical State of the Art

Animal culture and life cycle

Clytia hemisphaerica culture methods have been refined progressively over the last 15 years, and a detailed update is available (Lechable et al., 2020). A specific challenge compared to polyp-only cnidarian model species (notably *Hydra, Nematostella* and *Hydractinia*) is maintenance of the fragile and planktonic jellyfish (medusa) stage (Figure 1D,E), which requires constant gentle water flow. A convenient solution is to use adapted "Kreisel" tanks for juvenile and adult stages. *Clytia* jellyfish reach sexual maturity 2~3 weeks after budding from specialized polyps called gonozooids (Figure 1C). They then release gametes for the rest of their lifespan, typically around 2 months total. Between 400 and 1000 eggs can be collected every day from a batch of 30-40 females in one tank, and successfully fertilized within one hour of spawning by mixing with sperm released in parallel from a few males. Embryogenesis (see Section 3) results in formation of a planula larva (Figure 1A), described in detail in Section 4. After metamorphosis of the planula into a primary feeding polyp (gastrozooid) on glass slides (Figure 1B), extension of the stolon network creates a polyp colony, which is nourished by prey captured by the gastrozooids. Individual gastrozooids and gonozooids periodically atrophy, but colony growth allows their indefinite renewal. The colony is thus an extremely convenient form to amplify, maintain and distribute genetically uniform strains including mutants without need for crossing.

Functional genomics

Functional approaches, notably gene loss- and gain-of-function, enable a range of molecular and genetics studies. For *C. hemisphaerica* as a model, the first successful approach was microinjection of Morpholino antisense oligonucleotide (MO) and synthetic mRNAs into eggs before fertilization. MOs

designed to block mRNA splicing or translation generated reproducible loss-of polarity phenotypes for developmental regulators, starting with the Wnt receptors Fz1 and Fz3 (Momose & Houliston, 2007). Other examples are illustrated in Sections 3-4 below. Injection of in vitro synthesized mRNA allows gene overexpression, verification of MO phenotype specificity, and expression of fluorescent protein markers to visualize protein localization (Momose et al., 2012).

MO and mRNA egg microinjection can address biological events only between early blastula stage and metamorphosis, so other gene manipulation methods are required for studying processes in the polyp and jellyfish stages. Efforts to achieve reliable and effective targeted gene knockout started in the early 2010s, initially exploring the use of TALEN (Zhang et al., 2011) before switching to the CRISPR/Cas9 system (Momose et al., 2018). We were able to establish a protocol that consistently generates gene knock-out *Clytia* strains (ie F0 polyp colonies) with very low mosaicism in a single generation, involving prediction of the dominant mutation that will be induced by each sgRNA (Momose et al., 2018). This method exploits the observation that the Microhomology Mediated End-Joining (MMEJ) pathway is the principal pathway for DNA double strand break repair active in the early *Clytia* embryo. It has so far been used to knock-out endogenous GFPs (Figure 1D) as well as to identify key genes mediating oocyte maturation and spawning (see Section 3).

Development of high-efficiency transgenesis and gene knock-in approaches has been more difficult. The MMEJ pathway likely favours rapid and error-prone DNA repair over homology-mediated insertion, explaining the relatively low incorporation of transgenes achieved in trials with CRISPR/Cas9 methods using various template DNA types. Observed accumulation of injected DNA around nuclei (T.M. and B. Weissbourd unpublished) suggests that effective DNA delivery to the genomic target is also an issue. A recent breakthrough is the successful establishment of a transposon-mediated transgenesis protocol, through extensive collaboration between Caltech (B. Weissbourd and D. J. Anderson) and Villefranche teams. This achieves highly efficient random transgenesis using the Tol2 transposon vector system, co-injected with purified Tol2 protein (Weissbourd et al., 2021). A first study successfully employed this method to create a stable F1 transgenic strain expressing the calcium indicator GCaMP in RFamide-expressing neurons in jellyfish (Weissbourd et al., 2021). The use of polycistronic expression with 2A-peptide (Liu et al., 2017), further allowed metronidazole-inducible specific ablation of RFamide positive neurons by driving expression of nitroreductase with the *ChePP5* promoter. So far, strong and uniform transgene expression has been obtained using promoters from a non-muscle actin gene and from the RFamide precursor gene *ChePP5*. Methods to predict and select promoter sequences are currently not fully established. Further development will be aided by more information concerning the gene regulatory landscape in *Clytia*.

Table 1 Methods and Resources.

1A. METHODS			
Method	**References** - first citation and updated methods	**Target life cycle stage**	**Application**
Animal culture	Lechable et al. 2020	Whole life cycle	Planula, polyp and medusa culture
Morpholino antisense oligo	Momose and Houliston 2007; Amiel et al. 2009	Oocyte to planula	Loss of function
mRNA microinjection	Momose and Houliston 2007; Amiel et al. 2009	Oocyte to planula	Gain of function, protein localization
CRISPR/Cas9 gene KO	Momose et al., 2018; Quiroga Artigas et al. 2018, 2020	Whole life cycle	Loss of function
Transgenesis	Weissbourd et al., 2021	Whole life cycle	Gain of function
Cell type specific ablation	Weissbourd et al., 2021	Whole life cycle	Specific elimination of cells
Pharmacological treatments (NB target specificity is not guaranteed)	Momose and Houliston 2007; Amiel et al. 2009; Sinigaglia et al. 2020	Whole life cycle	Loss- or Gain-of-function
In situ hybridization	Chevalier et al. 2006; Sinigaglia et al., 2018	Whole life cycle	Visualizing gene expression patterns
Immuno/phalloidin staining	Denker et al. 2008b; Fourrage et al; 2010, Sinigaglia et al. 2020	Whole life cycle	Visualizing protein localization
Electron microscopy	Roosen-Runge, 1962, Thomas et al 1987; Carré & Carré, 2000; Kraus et al. 2020	Whole life cycle	Ultrastructural descriptions of cell types and tissue organisation.
Medusa dissection/grafting	Kamran et al. 2017; Sinigaglia et al. 2020	Medusa	Regeneration experiments
DIC microscopy for live imaging	Malamy and Shribak 2018	Medusa	Live imaging of epithelial cells
1B. *In silico* Resources			
Description	**References**	**Database**	**Details**
Whole genome sequence	Leclère et al. 2019	marimba.obs-vlfr.fr NCBI/EBI	Improved genome assembly (N50 > 1Mb)
Genome browser	Leclère et al. 2019	marimba.obs-vlfr.fr ensembl.org	Marimba combines genomic and gene expression data
Transcriptome assembly	Lapebie et al. 2014; Quiroga Artigas et al. 2018; Leclère et al. 2019	marimba.obs-vlfr.fr NCBI/EBI	Genome-guided transcriptome available
Expression profiling (RNAseq)	Lapebie et al. 2014; Quiroga Artigas et al. 2018; Condamine et al. 2019; Leclère et al. 2019, Sinigaglia et al. 2020.	NCBI/EBI	Wnt-manipulated Gastrulae; Ovary tissues; Tentacle bulb regions; Life-cycle stages; Regenerating manubrium time course
Expressed Sequence Tags (EST)	Chevalier et al. 2006; Foret et al. 2010	NCBI/EBI	Mixed stages libraries
Single cell RNA seq: Data and analysis pipelines	Chari et al. 2021	github.com/pachterlab/CWGFLHGCCHAP_2021	Cell types from adult female jellyfish, control and starved
Ontology terms for anatomy and developmental stages	Manuscript in preparation	www.ebi.ac.uk/ols/ontologies/clyh	Reviewed by the OBO Foundry

Medusa cell type atlas and single cell sequencing

The medusa (jellyfish) stage is the sexual phase of the life cycle, and has much more complex anatomy than the polyp stage. *Clytia* jellyfish are tetraradial, with 4 gonads each connected via radial canals to the manubrium (mouth) in the center and the circular canal around the periphery (Figure 2A). The circular canal bears the tentacle bulbs, which continually replenish the tentacles. Adjacent to the circular canal, positioned between each tentacle bulb, are sensory balance organs called statocysts (Singla, 1975). The nervous system consists of various nerve nets and two nerve rings running around the margin of the bell, connected to the statocysts. The jellyfish has both smooth muscle (radially orientated) and striated muscle (circularly orientated). The nervous system and muscles enable complex behaviours such as swimming and feeding (Houliston et al., 2010; Leclère & Röttinger, 2016; Weissbourd et al., 2021). Until recently, the diversity of different cell types comprising the *Clytia* jellyfish was underexplored. A significant addition to available resources is a cell type atlas documenting the presence, distribution and transcriptomes of 36 distinct cell types (Chari et al., 2021). This cell atlas was derived from analysis of single cell transcriptome data (scRNAseq) combined with in situ hybridisation to localize the expression of marker genes.

3) Gametogenesis

The *Clytia* gonad is one of the simplest organs in the animal kingdom. Its basic structure comprises the two canonical cnidarian body layers, with the basal surfaces of the epidermal and gastrodermal epithelial cells facing each other across a common, loosely-structured extracellular matrix (ECM). Within this ECM layer, gametogenic cells proliferate and differentiate. In female medusae, daily batches of small oocytes grow from around 50µm diameter to the full size of around 180 µm ready for the spawning cue (Amiel and Houliston, 2009; Jessus et al 2020; Figure 2A). The four gonads hang as pouches beneath the medusa bell, with the gonad gastrodermis derived from localized thickening of the radial canals and made up of specialized digestive-absorptive and secretory cell types (Figure 2A, D) (Chari et al., 2021).

Two principal features render *Clytia* gonads particularly suited to gametogenesis research compared to the most studied species in this field (notably *Xenopus*, mouse and starfish; see Jessus et al., 2020). Firstly, they are highly transparent, allowing the entire progression of events to be observed in whole animals, live or fixed. Secondly, they function largely autonomously, such that gonads separated by simple dissection from adult mature jellyfish support repeated cycles of gamete production and release for several days until nutritional resources (including recycled somatic tissue) are exhausted (Freeman, 1987; Takeda et al., 2018). So far, molecular studies of oogenesis have focussed mainly on females. This reflects the historical interest in maternal determinants for axis specification, as outlined in sections 1 and 3. Identification of the maternal determinant mRNAs Wnt3, Fz1 and Fz3 allowed the

cellular mechanisms responsible for their localisation to be linked to different oogenesis events: Fz1 mRNA localizes to the animal hemisphere (future oral) cytoplasm by a microtubule-based mechanism during the major daily oocyte growth phase (Figure 2B), while Wnt3 and Fz3mRNA segregate respectively to oral and aboral domains of the oocyte cortex during the two hour process of light-induced oocyte maturation (Figure 2C; Amiel & Houliston, 2009).

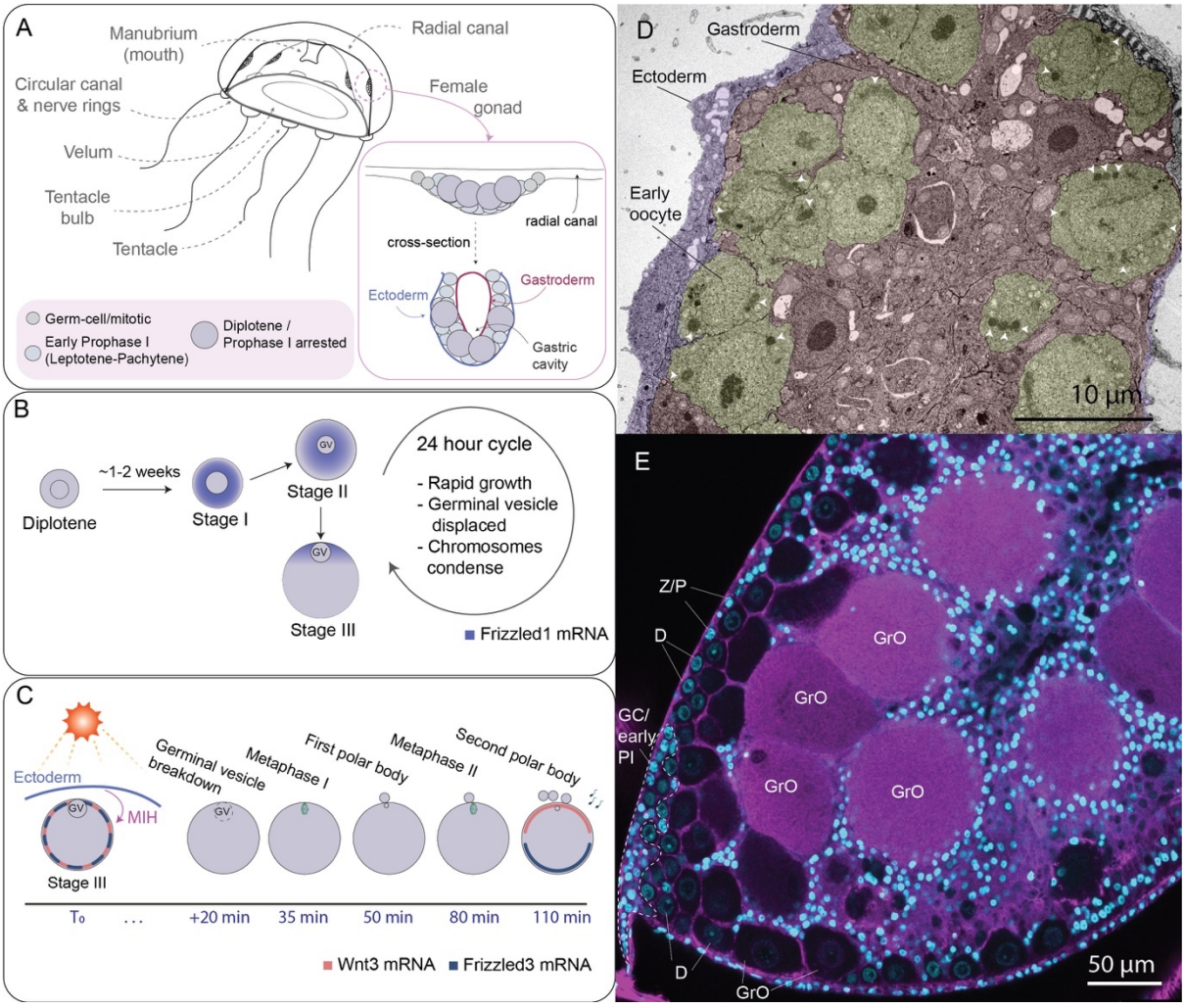

Figure 2: Jellyfish anatomy and gametogenesis

A. Schematic of a female jellyfish, with the organisation of the female gonad highlighted. Adapted from Munro, C. (2021a,b). B. Schematic of oocyte growth in a prophase I arrested oocyte. In fully grown jellyfish, cohorts of stage I oocytes (approx 50µm diameter) grow daily to stage III (approx 180µm diameter) prior to spawning. Fz1 mRNA becomes concentrated in the animal cytoplasm during this growth phase. C. Schematic of oocyte maturation. Light triggers the release of oocyte maturation hormone (MIH) from cells in the gonad epidermis, triggering oocyte maturation and spawning. Wnt3 and Fz3 mRNAs are localized around the periphery of the stage III oocyte. During meiotic maturation, Fz3 becomes localized to the vegetal cortex and then Wnt3 to the animal cortex. D. False coloured electron micrograph from the ovary of a 1 week-old jellyfish. Ectoderm is highlighted in blue, gastroderm in red and early oocytes in green (arrows indicate the perinuclear "nuage" characteristic of the germ cells). E. Confocal microscopy image of a two week ovary by Anna Ferraioli. Nuclei are stained with hoechst (cyan), and actin is stained with phalloidin (magenta). GC/ Early PI = Germ cell, Early Prophase I; Z/P = Zygotene/Pachytene; D = Diplotene; GrO = Growing oocyte.

Oocyte maturation

Oocyte meiotic maturation transforms a fully grown, tetraploid, diplotene oocyte into a haploid gamete ready to be released from the gonad for fertilization. Completion of the first meiotic division terminates in emission of a first polar body containing one set of replicated homologous chromosomes, the second meiotic division separates sister chromatids and expels one set in the second polar body. In *Clytia*, meiotic maturation ends in an unfertilized egg containing a haploid female pronucleus (Figure 2C). These events can be followed in isolated gonads exposed to light after darkness, or in isolated oocytes treated with oocyte maturation hormone (MIH) or with cell-permeable cAMP analogues (Amiel et al., 2009; Takeda et al., 2018). As extensively discussed recently in a recent review (Jessus et al., 2020) they are regulated by core kinase systems highly conserved across the animal kingdom: the Cdk1/Cyclin B complex (also known as MPF) active during each M phase, MAP-kinase which progressively activates as maturation proceeds, and cAMP-dependent kinase (PKA) activation during the initial triggering mechanism (Freeman & Ridgway, 1988; Takeda et al., 2006). A study focussed on *Clytia* orthologues of the animal oocyte cytoplasmic kinase Mos, which initiates MAP-kinase activation by phosphorylating the MAP-kinsase kinase MEK in animal oocytes, illustrates how *Clytia* can reveal both core and novel features of this regulation (Amiel et al., 2009). Mos activity is regulated by the timing of its translation during meiotic maturation, as extensively analysed in the *Xenopu*s and mouse vertebrate models (Gebauer & Richter, 1997). In *Clytia*, hydrozoan Mos gene family expansion has generated several paralogs, including two expressed in oocytes. Morpholino-mediated inhibition of their translation revealed a conserved role for *Clytia CheMos1* in the oocyte-specific processes of polar body formation and post-meiotic arrest, but suggest that *CheMos2* has evolved a distinct function in preparing oocytes for maturation. Since the kinase activities of the two proteins appear equivalent, this difference appears to be mediated by evolution of UTR sequence mediating translation timing (Amiel et al., 2009). It will be of interest to explore in *Clytia* the role and evolution of other oocytes regulatory kinases such as PKA and Greatwall, to help understand how oocyte maturation regulation has adapted to different species-specific requirements to synchronize gamete release and thus optimize reproductive success.

Triggering oocyte maturation and spawning

Studies exploiting CRISPR/Cas9-mediated gene knockdown have dissected the initiation mechanism for oocyte meiotic maturation in *Clytia*. This process takes about two hours and ends with the release of unfertilized eggs via localized rupturing of the gonad epidermis. This spawning process in females, and also the parallel process of sperm activation and release in males, is initiated by a light cue after darkness (Roosen-Runge, 1962; Freeman, 1987; Miller, 1979; Takeda et al., 2018). Specialized cells with neural-like morphology in the gonad ectoderm co-express one of 10 *Clytia* opsin proteins, Opsin9, and precursors of the oocyte maturation-inducing neuropeptide hormone MIH (Quiroga Artigas et al., 2018; Takeda et al., 2018). Upon light stimulation, Opsin9 mediates the release of MIH

both inside and outside the gonad. Subsequent binding of MIH to a specific G-protein coupled receptor in the oocyte membrane initiates activation of meiotic maturation via GalphaS, cytoplasmic cAMP rise and PKA activation (Figure 2C) (Quiroga Artigas et al., 2020; Takeda et al., 2006).Thus medusae from F0 female polyp colonies carrying mutations in the *Opsin9* or MIH receptor (*MIHR*) genes accumulate fully grown oocytes but fail to spawn. In both cases the absence of light-induced oocyte maturation can be overridden by treatment with cell-permeable cAMP analogues.

Origins of the germ cells

In both male and female medusae, the germ cells originate from a population of cells positioned within the gastrodermis close to the canal. These are characterized by small cytoplasm: nuclear ratio, expression of a "Germ line multipotency" gene set (Juliano et al., 2010) including Piwi and Nanos genes (Leclère et al., 2012), and perinuclear clumps of "nuage" material (Figure 2E). Knowledge from other hydrozoans, notably *Hydra*, suggests that this cell population likely includes both i-cells ("interstitial cells"), the classic hydrozoan multipotent stem cells (Hobmayer et al., 2012), and their derivatives that have embarked towards a germ cell fate. Whether a separate germ-line stem cell population derived from i-cells undergoes self renewal in the adult, as suspected in *Hydra* (Nishimiya-Fujisawa & Kobayashi, 2012), remains to be addressed.

Entry into meiosis

Clytia gonads, through their transparency and accessibility within the living animal, have excellent potential for studying the very early events of meiosis including the transition from mitotic to meiotic cycles, synapsis, and recombination. In female jellyfish oocytes are produced continuously, and early-stage meiotic oocytes are present in the gonads of female medusae throughout its lifespan. Meiosis progresses through leptotene, zygotene and pachytene before arresting in diplotene during oocyte growth (Jessus et al., 2020). The early meiotic stages are most accessible for study inside young jellyfish within the first 1 to 2 weeks following their release from the gonozooid. During this period they dominate the population compared to growing and fully grown oocytes. On the first day of jellyfish release, a handful of germ cells are found in a patch along the radial canal at the future site of the gonad. After several days, the number of cells has increased and oocytes at zygotene and pachytene stages can be recognized. In one week old female jellyfish, the gonad is full of early oocytes at all stages of prophase I (Figure 2D). After 2 weeks, diplotene stage oocytes have already started to grow (Figure 2B,E), while oocytes of earlier stages are still present in the periphery of the gonad (Figure 2E) (Amiel et al., 2010; Amiel & Houliston, 2009; Jessus et al., 2020). In a collaboration between the Villefranche lab and Jean-René Huynh at Collège de France, we have recently initiated studies of entry into meiosis and the early meiotic events in prophase I. CRISPR/Cas9 is being used to target key genes, including the highly conserved meiotic gene Spo11, which is responsible for initiating meiotic double stranded breaks.

Recent technical advances open up exciting possibilities for future studies of gametogenesis in *Clytia* based on live imaging, particularly of the cellular mechanisms of early meiotic entry where cells are inaccessible for injection. As well as studying meiosis in females, many issues concerning spermatogenesis could also be addressed using the transparent and highly structured male gonads (Carré & Carré, 1992; Roosen-Runge & Szollosi, 1965; Szollosi, 1964).

4) Embryogenesis

The daily spawning from laboratory cultures of hundreds of transparent eggs provides ideal material for studies of embryogenesis, allowing the comparison of mechanisms within Cnidaria as well as more widely across animals. The cnidarian larval form, the planula, is typically elongated along the principle "oral-aboral" body axis, and made up of the two basic cnidarian tissue layers: epidermis and gastrodermis. Unlike as in some other cnidarian larvae, the oral end of the *Clytia* planula is not marked by a blastopore or later by development of a mouth opening, but it transforms into the feeding "head" of the polyp following the drastic morphological transformations of metamorphosis (Freeman, 2009). Coordinated ciliary beating propels the larva with the aboral pole in front. It becomes competent to metamorphose after about three days of development, and at that time will settle on a suitable substrate and metamorphose into a primary polyp (Figure 1B). The different cell types of the planula have not yet been fully characterized. Nematocytes (cnidocytes) are scattered through the lateral epidermis and concentrated at the oral pole, while specialized secretory cells involved in substrate adhesion are concentrated at the aboral pole (Bodo and Bouillon, 1969). Nematocytes are the stinging cells that characterize Cnidaria, whose pressurized stinging capsules include products of genes acquired by horizontal transfer from bacteria (Denker et al., 2008a). Various neural cell types classed as sensory and ganglionic are distributed within the epidermis and in the underlying mesoglea (Thomas et al., 1987), with an aboral plexus likely involved in coordinating sensory inputs. Neural cells in the larva, including the nematocytes (Rentzsch et al., 2017), are likely generated principally from hydrozoan-specific multipotent stem cells called i-cells occupying the endodermal region, although neurosensory and secretory cells appear to derive from the aboral ectoderm during gastrulation (Kraus et al., 2020; Thomas et al., 1987).

Axis establishment

As introduced in Section 1, historically important studies (Freeman, 1981a,b) demonstrated that polarity of the fertilized egg presages the larval oral-aboral body axis, and that endoderm derives from the egg animal/future oral pole. In the early 2000s, experimental manipulation of embryos from the hydrozoan *Podocoryne* (Momose & Schmid, 2006) and demonstration of localized Wnt/β-catenin pathway activation in relation to endoderm formation during *Nematostella* embryogenesis

(Wikramanayake et al., 2003) together suggested that cnidarian eggs likely contained axis determinants at the animal (future oral) pole that initiate Wnt/β-catenin signalling. Such determinants could be identified for the first time in *Clytia*, in the form of localized mRNAs for the Frizzled family receptors, CheFz1 and CheFz3 and the Wnt ligand CheWnt3 (Figure 2BC, Figure 3) (Momose et al., 2008; Momose & Houliston, 2007). Experimental reorientation of the oral-aboral axis achieved by low-speed centrifugation of fertilized eggs (Freeman, 1981a), can be explained by the co-displacement of Fz1 mRNA with the zygote nucleus in these experiments (Amiel & Houliston, 2009). Morpholino and mRNA injection experiments showed that Fz1, Wnt3 and Fz3 proteins produced from the localized mRNAs act together to stabilize β-catenin in the oral domain and thus set up distinct programmes of gene expression along the axis (Fig 3A, B). Zygotic gene expression, which starts from mid/late blastula stage, is globally controlled by Wnt3. Two distinct transcriptional programmes activated downstream of Wnt3 can be distinguished in the oral ectoderm versus ingressing presumptive endoderm on the basis of sensitivity to PCP-mediated cellular interactions (Lapébie et al., 2014).

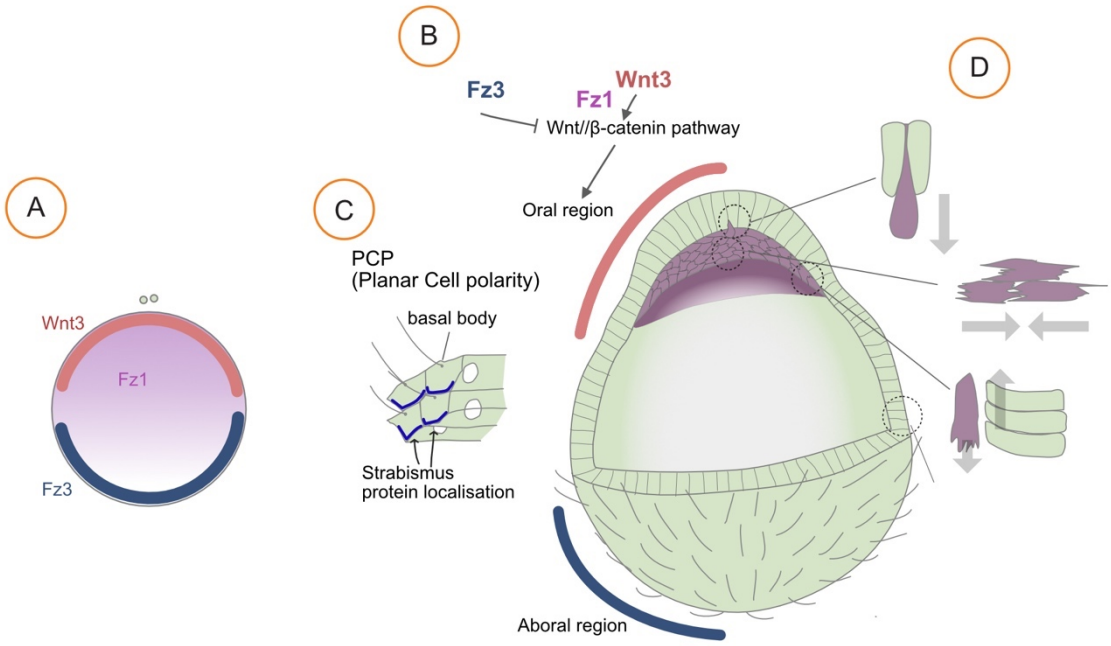

Figure 3: Establishment of oral-aboral polarity in the embryo
A) Schematic of an unfertilized egg showing the localization of Wnt/β-catenin pathway component mRNAs. B) The proteins synthesized from these mRNAs locally activate the pathway and initiate transcriptional programmes that define oral and aboral regional characters, including presumptive endoderm fate at the oral pole. C) In parallel, conserved planar cell polarity (PCP) pathway proteins coordinate tissue polarity coordination in the ciliated ectoderm. (D) Individual ingression of presumptive endoderm cells from the oral pole, and PCP-mediated intercalation of cells in both layers underlie the morphogenetic changes during gastrulation that largely contribute to the elongated form of the planula.

Morphogenesis directed by global polarity

Based on the extensive ability of embryo fragments, and even dissociated cells, to reestablish polarity, Gary Freeman proposed that the embryonic axis was determined by a global, ie non-localized, polarity property (Freeman, 1981b). We can now equate this property with Planar Cell Polarity (PCP), the common alignment of cells within epithelia or other coherent tissues mediated by specific interactions between neighboring cells (Butler & Wallingford, 2017). *Clytia* possesses a full set of proteins for the conserved 'Wnt-PCP" pathway, including Strabismus, Flamingo and Prickle orthologs (Momose et al., 2012). Morpholino-mediated knockdown of *Clytia* Strabismus (*CheStbm*) revealed a requirement for PCP both for cell alignment in the ciliated ectoderm and for planula elongation (Figure 3C). The role in elongation likely involves intercalation between the ectodermal epithelial cells perpendicular to the axis of elongation (Byrum, 2001), but also between ingressing presumptive endoderm cells (Figure 3D) (Kraus et al., 2020; van der Sande et al., 2020).

As first described at the end of the nineteenth century, gastrulation in *Clytia* involves ingression of individual cells from the future oral pole of a single cell-layered blastula (Byrum, 2001; Kraus et al., 2020; Metchnikoff, 1886). As they ingress, the presumptive endoderm cells crawl along the blastocoel wall and against each other, simultaneously narrowing and elongating the embryo along the oral - aboral axis (Y. Kraus et al., 2020). Computational simulations of gastrulation require lateral interactions between ingressing cells to be included, simulating PCP, to account for full embryo elongation (van der Sande et al., 2020). Thus PCP acts in the *Clytia* embryo to generate the main morphological features of the larva: the axially elongated form and oriented cilia beating. It can be considered as a non-localized axial determinant acting in parallel with Wnt/β-catenin signalling to direct gene expression programmes across the embryo (Lapébie et al., 2014).

Modes of embryogenesis between different species of Cnidaria vary widely, with the two body layers forming by ingression in *Clytia*, but by cell sheet invagination uncoupled from embryo elongation in *Nematostella,* and partitioning during the cleavage divisions in *Hydractinia* (Kraus & Markov, 2016; Technau, 2020). Cnidarian embryos are thus a very valuable source of comparative material for addressing how morphogenetic processes function and evolve. In this context it was revealing to find that simulations of gastrulation in *Clytia* could be derived from simulations set up for *Nematostella* by changing parameters representing adhesion between cells, allowing apical cell contraction to occur in all cells rather than being orally restricted, and adding lateral interactions ('PCP') between ingressing cells (van der Sande et al., 2020).

5) Medusa formation and Regeneration

Hydrozoan jellyfish have provided a rich source of material for biologists since the late nineteenth century. Early workers documented for example their development, nervous systems, musculature and

regeneration. Molecular studies to explore this rich heritage have only just begun (Peron et al, 2021; Leclère et al., 2016). Here we will provide an overview of recent findings in each of these areas.

Medusa development

Clytia jellyfish are produced clonally by lateral budding of specialized polyps, the gonozooids (Figure 1C). This process appears highly similar to that described in other hydrozoan species (e.g. Boelsterli, 1977). Jellyfish budding starts with bulging of the gonozooid body wall. A specialized cell layer called the entocodon, formed from a group of cells that delaminate within the early bud, generates smooth and striated muscle layers of the subumbrella and manubrium (Kraus et al., 2015). The remaining epidermis of the bud gives rise to the exumbrella, the tentacle epidermis and the outer layer of the velum of the medusa, while the bud gastrodermis provides the gastrovascular system of the medusa. The precise ontogeny of medusa formation as well as the molecular processes underlying budding, growth and aging of *Clytia* jellyfish are still very largely unexplored.

Nervous system

The nervous system of the *Clytia* jellyfish is still poorly characterized. As in other hydrozoan jellyfish, two nerve rings running around the bell margin play a central role in integrating sensory inputs to coordinate behavioural responses, while subpopulations of neural cells also occupy epidermal and gastrodermal layers of the manubrium and gonads. Analysis of scRNAseq data (Chari et al., 2021) revealed at least 12 mature neural cell types expressing different neuropeptides, whose precise morphologies, locations and roles mainly remain to be determined. A recent pioneering functional study shows that RFamide-expressing neurons in the subumbrella mediate the oriented flexing of the bell that brings prey captured by the tentacle to the mouth (Weissbourd et al., 2021). GCaMP imaging revealed these RFamide-positive neurons to be organized in distinct and largely independent modules under the control of the nerve ring.

Nerve cells and nematocytes likely derive principally from i-cells in the *Clytia* medusa, as shown in the polyps hydrozoans *Hydra* (Hobmayer et al., 2012) and *Hydractinia* (Gahan et al. 2016). In the *Clytia* medusa, i-cell pools are located not only in the gonads (see Section 3) but also at the base of the manubrium and, conspicuously, in the ectoderm at the base of each tentacle bulb (Condamine et al., 2019; Denker et al., 2008b; Leclère et al., 2012). Given the continuous growth of the tentacles and the constant renewal of nematocytes, the *Clytia* jellyfish tentacle bulb is an attractive system to investigate neurosensory cell differentiation. It can be viewed as a cellular conveyor belt, with stem cells close to the bell margin and differentiation into nematocytes progressing along the longitudinal axis of the bulb and into the tentacle (Denker et al., 2008b; Chari et al., 2021) (Figure 4A). Transcriptome comparisons between the base, middle and distal parts of the bulb uncovered sets of genes differentially expressed along the bulb (Condamine et al., 2019), confirming previous in situ

hybridization studies of particular regulatory gene families (Denker et al., 2008b; Hwang et al., 2010; Jager et al., 2011; Leclère et al., 2012; Steinmetz et al., 2012). Regulation of this differentiation likely involves both the Hippo (Coste et al., 2016) and Wnt pathways (Condamine et al., 2019). Wnt related genes are expressed differentially both along the proximo distal axis of the bulb and in relation to its perpendicular oral-aboral axis, likely reflecting complex spatial regulation of neural differentiation within the bulb.

Muscle systems and regeneration

The *Clytia* jellyfish umbrella consists of exumbrella and subumbrella cell layers, separated by a thick ECM structure called the mesoglea (the 'jelly'). The exumbrella comprises flattened, non-contractile polygonal cells. The sub-umbrella is made up of three layers of cells: (i) the endodermal plate of polygonal non contractile epithelial cells, covered by two contractile epithelial layers; (ii) a muscle layer displaying smooth radial muscle fibers oriented radially and responsible for the crumpling of the bell, and between these (iii) a layer of circular striated muscle layer responsible for the pulsing bell contractions used for swimming. *Clytia* striated muscles strongly resemble those of bilaterian animals in structure, but molecular phylogeny and gene expression studies of the proteins comprising the typical periodic actomyosin arrays suggest that this similarity arose through convergent evolution (Steinmetz et al., 2012). The radial smooth muscles have been recently studied for their role in food transfer from the bell margin to the mouth, stimulated by the action of associated networks of RFamide positive neurons (Weissbourd et al., 2021). Intriguingly, reorganisation of these radial muscle fibres upon injury driven by actomyosin-driven wound closure may also have a patterning role in the jellyfish, determining the site where a new manubrium will form (Sinigaglia et al., 2020). These observations on the jellyfish muscle systems open an exciting era of exploration of the molecular basis of their development and function, comparisons of the composition and structure of the contractile fibers with canonical bilaterian muscles, and understanding of their role in regeneration and body patterning (Sinigaglia et al., 2020).

Cnidarians are well known for their extensive regenerative capacities, which have been well characterized in polyp-only species such as *Hydra*, *Nematostella* and *Hydractinia,* but less so in medusae. Building on a body of historical work (Neppi et al. 1979; Schmid, 1974; Schmid et al., 1976; Schmid & Tardent, 1971), recent cellular and molecular analyses have shed a new light on regeneration processes in *Clytia*. Studies of wound healing exploited the optical clarity of the exumbrella monolayered epithelium for high resolution imaging and revealed the extreme rapidity and simplicity of this process, which occurs much faster than for cultured cells or bilaterian embryos (Kamran et al., 2017; Malamy & Shribak, 2018). Both purse-string-like supra-cellular actomyosin contractions and lamellipodia-dependent cell crawling contribute to wound healing (Kamran et al.,

2017; Malamy & Shribak, 2018). The balance between these mechanisms depends on the depth of the wound and the extent of damage to the basement membrane / ECM.

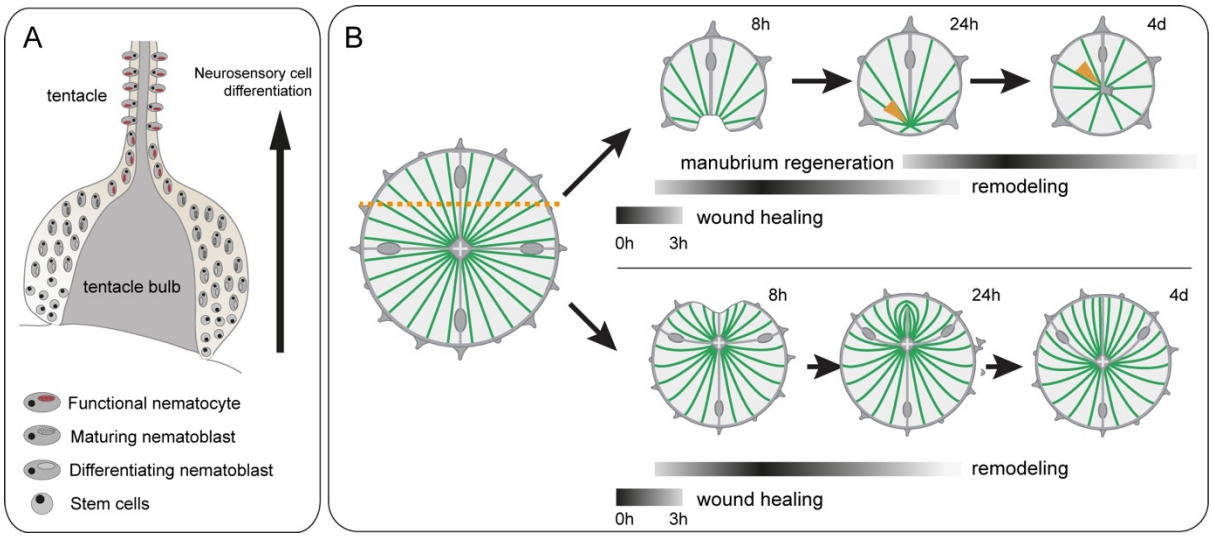

Figure 4: Nematogenesis and regeneration in *Clytia* medusae.
(A) Nematocyte differentiation ordered along the proximo-distal axis of the *Clytia* medusa tentacle bulb (Condamine et al., 2019; Denker et al., 2008b). (B) Successive stages of wound healing, remodeling and regeneration in *Clytia* medusa following bisection (orange dotted line; Sinigaglia et al., 2020). Only the small fragment missing the manubrium (top) will reform one. The muscle fibers (green) merge into a hub (orange arrows), which becomes the regeneration site of a new manubrium 4 days after dissection. In larger fragments bearing a manubrium (bottom), re-circularization also causes the formation of a muscle hub, but this one later disaggregates, and no new manubrium forms.

Remarkably, the *Clytia* medusa can also cope with much more extreme damage. Even small fragments are able to rapidly restore shape, functionality and even missing organs (Sinigaglia et al., 2020). This occurs in three steps: initial wound healing is followed by a purse-string mediated remodeling process to re-establish the circular shape of the medusa. Finally, reformation of missing organs occurs, prioritizing the manubrium to allow feeding to resume (Figure 4B). Manubrium reformation requires both cell proliferation and cell migration from other parts of the medusa, notably the gonads. Grafting experiments revealed that the position of the new manubrium is not directed by a morphogen based system of coordinates within the subumbrella (Volker Schmid et al., 1976; Sinigaglia et al., 2020). It seems rather that patterns of reorganization of the radial smooth muscles of the sub-umbrella during remodelling define the site of the new manubrium. These mechanical contraction cues lead to activation of Wnt signaling at the remodeling site, which promotes manubrium regeneration or not, depending on the organization of the muscle fibers in the regenerating fragment (Figure 4B). A similar patterning function for muscle is also emerging from studies of regeneration in *Hydra* (Livshits et al., 2017, 2021; Maroudas-Sacks et al., 2020). Understanding of how this influence of muscle integrates with the well established role of Wnt in *Hydra* head regeneration awaits future studies (Cazet et al., 2021; Wang et al., 2020). Another important player

during manubrium regeneration in *Clytia* is the radial canal system, which both transports construction material to the blastema site and acts locally to direct manubrium morphogenesis.

Much remains to be understood about the extreme and fascinating regeneration capacity of *Clytia* medusae. Open questions to be addressed notably include i) the role of cell migration in regeneration, ii) the integration of mechanical cues and signaling pathways during regeneration, iii) the link between signaling, ECM, wound healing and mechanical forces, and iv) the nature of the local cues that direct regeneration and morphogenesis.

6) Genomics of the jellyfish

Cnidarians are the closest living relatives of the bilaterians, the major group of bilaterally symmetric animals that includes most familiar species including humans. Cnidaria itself comprises two great clades, Anthozoa, consisting of corals and anemones (including the laboratory model *Nematostella vectensis*), and Medusozoa, most simply understood as those that have a jellyfish-like stage in their life-cycle. The divergence between Anthozoa and Medusozoa is ancient (dos Reis et al., 2015), and both are large and diverse clades. The four medusozoan classes are Hydrozoa (including *Clytia* and *Hydra*) Staurozoa (stalked jellyfish), Scyphozoa (true jellyfish) and Cubozoa (box jellyfish). The last few years have seen the publication of high quality genomes of several Medusozoa, *Nemopilema nomurai* (Kim et al., 2019); *Rhopilema esculentum* (Li et al., 2020; Nong et al., 2020); *Sanderia malayensis* (Nong et al., 2020); *Chrysaora quinquecirrha* (W. Xia et al., 2020; W.-X. Xia et al., 2021); *Aurelia aurita* (Gold et al., 2019; Khalturin et al., 2019); *Morbakka virulenta* (Khalturin et al., 2019). Of all these, except the box jellyfish *Morbakka*, are Scyphozoa. Another box jellyfish, *Alatina alata* and a staurozoan *Calvadosia cruxmelitensis* are available as drafts (Ohdera et al., 2019).

After *Hydra* (Chapman et al., 2010; Hamada et al., 2020), *Clytia* was the second hydrozoan species for which the genome has been sequenced, and the first with planula and medusa stages (Leclère et al. 2019). The 445 Mb assembled genome of *Clytia* is typical for hydrozoans, with moderate GC and repeat contents (35% and 39% respectively). As in other hydrozoans, messenger RNAs in *Clytia* are frequently subject to transplicing, involving addition of short RNA leader sequences to their 5' ends (Derelle et al., 2010). Work on improving the contiguity of the *Clytia hemisphaerica* genome continues; an improved assembly with N50 > 1Mb is now available at Ensembl (link in Table 1). A central result of the published *Clytia* genome analysis was that gene order in *Clytia* and *Hydra* is disrupted relative to the last common ancestor of cnidarians and bilaterians. Comparisons with the new scyphozoan genomes suggest that this disruption is indeed a hydrozoan phenomenon, with better gene order conservation between Anthozoa and Scyphozoa than between Anthozoa and *Clytia* or *Hydra*. These results will need to be confirmed as more contiguous hydrozoan genomes become

available (Zimmermann et al., 2021). In this context, it is interesting that *Clytia* appears to have the same number of chromosomes (15) as the ancestral Cnidarian (Leclère et al., 2019; Zimmermann et al., 2021). Gene order disruption of Hydrozoa does not, therefore, appear to have been an inevitable consequence of genomic restructuring necessary to form another life-cycle stage.

The accessibility of the planula, polyp and medusa in the laboratory environment has enabled detailed study of gene use in the different stages of *Clytia*. The likelihood that the jellyfish is an evolutionary novelty (i.e. 'invented' in the medusozoan lineage), raised the possibility that it might be enriched in lineage specific genes. All studies that have looked at this question, however, have concluded that this is not the case (Gold et al., 2019; Khalturin et al., 2019; Leclère et al., 2019). Instead, genes showing differential expression between life cycle stages are more likely to be taxonomically restricted (i.e. more recently evolved), but they are not preferentially associated with the medusa stage (Khalturin et al., 2019; Leclère et al., 2019). In contrast, a careful analysis of transcription factors showing medusa specific expression showed that several had been lost in *Hydra*, despite being ancestral to Medusozoa and present in the closest medusoid-producing relatives of *Hydra* for which data were available (Leclère et al., 2019). These cases of gene loss *are* thus associated with loss of a life-cycle stage. Analysis of life-cycle stage gene use also suggested that the *Clytia* planula and polyp stages have been secondarily simplified relative to the Anthozoa, as genes with apparent conserved roles in patterning the apical organ and directive axis formation were lost or are not expressed. In this context it is notable that Scyphozoa similarly lacks many of these genes (*Gbx*, *Hox2* etc.). These analyses also suggested that, of all the stages, the medusa expresses the most complex set of transcriptional regulators. Many of these appear to associate with specific cell types, notably within the nervous system (Chiori et al 2009; Leclère et al 2019). A current research focus is understanding this phenomenon within the context of the medusa cell-types and their evolution (Chari et al., 2021).

More widely, the accumulation of genomic and sequence resources for *Clytia* (see Table 1) is providing a wealth of information valuable for the understanding of the evolution of animals and their genomes. Improvements to genome annotation and epigenomic studies in *Clytia* and other jellyfish species will fuel further insights into life cycle evolution and development in medusozoans, and the evolution of cell types in these different stages.

8) Perspectives

This review has illustrated how practical advantages combined with phylogenetic position have enabled *Clytia hemisphaerica* to contribute a number of insights in the fields of cell and developmental biology, and animal genome evolution. Existing *in silico* resources and well

established methods like egg MO/mRNA injection and CRISPR/Cas9-mediated gene knockout already allow efficient functional testing of genes expressed during larval development or specifically in the adult, respectively. The recent emergence of an efficient transposon-mediated transgenesis method opens significant further perspectives to the community, notably widening the scope of gene function exploration in adult (polyp and medusa) stages via the use of inducible promoters. A detailed protocol including workflows to select and maintain strains is being prepared (B. Weissbourd and T. Momose). Transgenesis will also allow researchers to tease out relationships between cell lineages via *in vivo* tracking, ablation experiments, imaging of particular cellular components etc. We thus anticipate that use of transgenesis in *Clytia* will advance hand in hand with the development and wider application of live imaging approaches. Recent work focusing on jellyfish wound healing (Karam et al. 2017), regeneration (Sinigaglia et al. 2020), neurobiology (Weissbourd et al., 2021) and cell type characterization via scRNAseq (Chari et al. 2021) represent valuable steps forward in this direction, opening many new questions. For instance, what are the functions and organisation of the different neural subtypes of the jellyfish in relation to physiology and behaviour such as swimming, diel migrations, spawning and feeding? How do mechanical forces and transcriptional processes cooperate during wound healing and regeneration in *Clytia* jellyfish? The power of genomics, imaging, transgenesis and ablation approaches coupled with the transparency, small size and relative complexity of the jellyfish stage, place *Clytia* in an ideal position to tackle these fundamental questions.

The time is also ripe now for *Clytia* to be used also for studies at the level of the whole organism. One notable application in the context of climate change concern is to dissect biological responses to changing environmental parameters such as temperature and UV irradiation and food availability. Transparent planktonic organisms are especially vulnerable to UV damage, especially when they rise to the ocean surface to spawn. One hypothesized adaptation for UV protection is its absorption by a set of endogenous GFPs, highly expressed in the epidermis of the gonad and tentacle bulbs as well as in the planula ectoderm and egg mitochondria (Fourrage et al., 2014). This idea can now be tested using stable CRISPR/Cas9 Knockout strains lacking different GFP subtypes (Fig.1D). Population genomics approaches to correlate the GFP repertoires of natural populations with their environment would also be informative. Another possible adaptation to UV exposure currently being explored is the differential deployment of alternative DNA repair pathways between life-cycle stages and cell types. This idea stems from analyses of repair responses to Cas9 mediated DNA breaks, which revealed microhomology mediated end-joining (MMEJ) pathway to be dominant over better known double strand break repair pathways such as non-homologous end-joining (NHEJ) or homology-dependent repair pathway (HR) in the cells from the larva that found the polyp colony (Momose et al., 2018). Another aspect of *Clytia* biology clearly impacted by environmental factors is life-cycle transitions. Metamorphosis of hydrozoan planulae is promoted by bacterial biofilms by largely

unknown mechanisms (Guo et al., 2021). The temperature at which the polyp colonies are established then influences the sex of the medusa which bud from it (D. Carré & Carré, 2000). The molecular and cellular basis of this intriguing observation is now open to be addressed.

Clytia also could be a useful model for investigating the physiological relationships between food processing and regulation of starvation and gametogenesis. Study of the impact of food availability on the medusa has recently been leveraged by a proof of concept Whole Animal Multiplexed Single-Cell RNA-Seq ("WHAM-seq") study in which cells from control and starved *Clytia* jellyfish were simultaneously processed (Chari et al., 2021). This provided a rich body of information concerning the cells and genes affected by starvation, and revealed certain gastrodermal cell types to be particularly responsive. These likely include the "mobilising gastrodermal digestive cells" ("MGD cells"; Sinigaglia et al., 2020) that relocate through the gastrovascular canal system during starving and regeneration, hypothesized to mediate redistribution of nutrient resources between the different organs of the medusa. The functional specialisations of the many distinct secretory and absorptive cell types and physiological states of the jellyfish digestive system are now open for investigation.

To conclude, *Clytia* is now fully established as a laboratory animal, offering many opportunities to a community of researchers progressively expanding across many fields, as well as ideal material for educators. As well as in silico resources (Table 1) animal resources can be shared as "cuttings" of polyp colonies established on glass slides (Lechable et al., 2020). *Clytia* wildtype strains are currently available through the Marine Biological Resource Centre (CRBM, request to be submitted from https://www.embrc-france.fr/) We hope that community expansion will continue, allowing further accelerated development of approaches and resources, and fuelling scientific discovery across domains for years to come.

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