

Molecular and cellular basis of life cycle transition provides new insights into ecological adaptation in jellyfish

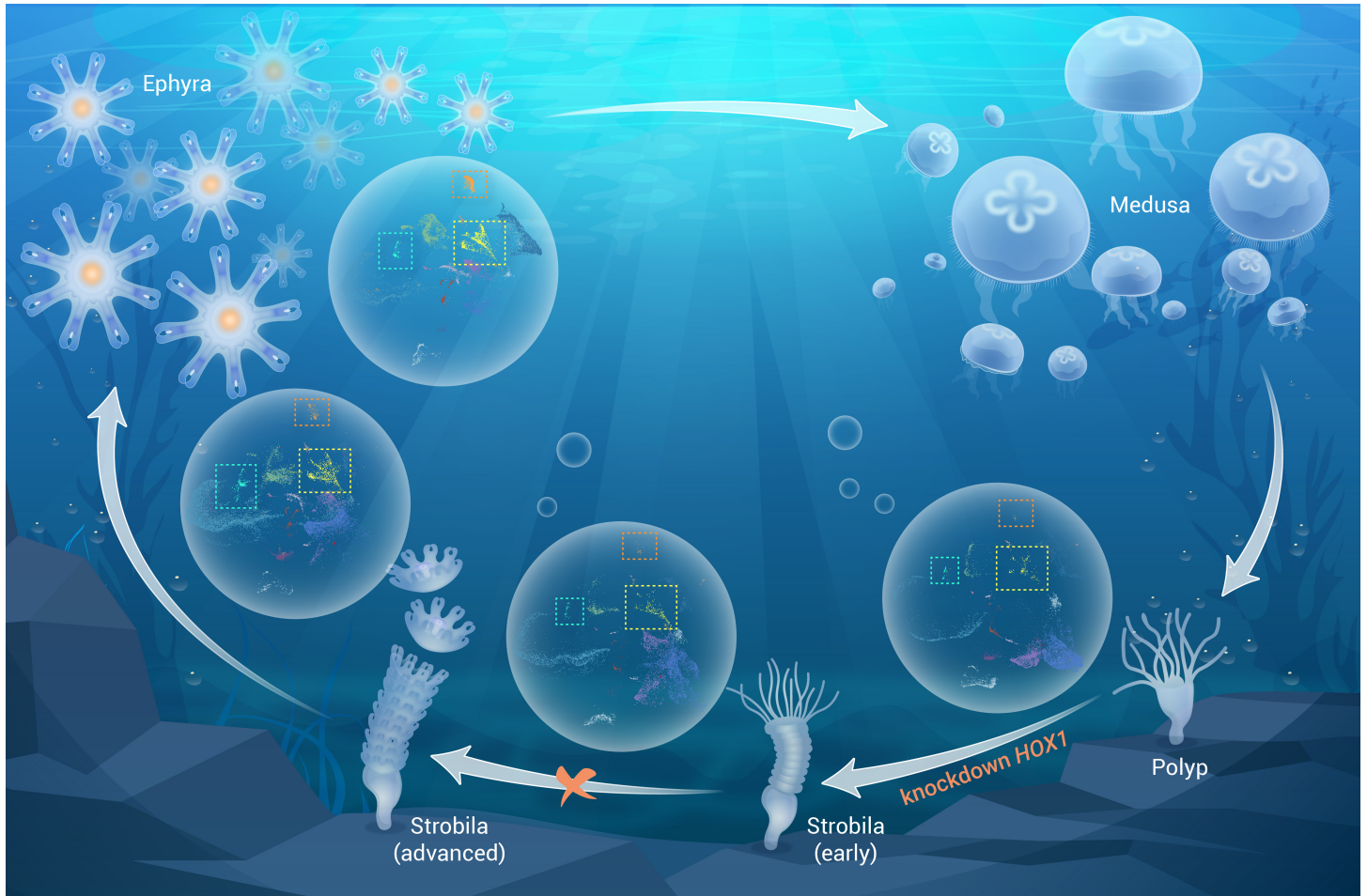
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GRAPHICAL ABSTRACT



PUBLIC SUMMARY

- We present a single-cell atlas for the jellyfish *Aurelia coerulea* during the polyp-to-medusa transition.
- We characterise variations in neural and muscle cells and gene expression patterns during strobilation.
- *HOX1* plays a pivotal role in regulating the formation of striated muscle cells in *A. coerulea*.
- This study provides valuable insights into the cell biology and ecological adaptation of jellyfish.

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Jellyfish are renowned for their complex life cycles and important ecological and evolutionary position. The unique transition from the sessile polyp to motile medusa stages is a key process determining a switch in jellyfish behaviour and regulating the formation of jellyfish blooms. Here, we presented a comprehensive cell atlas spanning four successive life cycle stages during the polyp-to-medusa transition in the scyphozoan jellyfish *Aurelia coerulea*. Moreover, we characterised the variation in cell composition and gene expression patterns during the phase transition, especially in the neuromuscular system. We found several previously unreported cell types that potentially underpin the complex swimming behaviour of jellyfish. Furthermore, we discovered the pivotal role of *HOX1* in modulating the genesis of striated muscles in *A. coerulea*. Collectively, this study provides valuable insights into the cellular and molecular mechanisms underlying the complex life cycle transition and helps to advance our understanding of ecological adaptation in jellyfish.

INTRODUCTION

Life cycle transitions are crucial periods of adaptation that enable organisms to thrive in varied ecological and geographical habitats.^{1,2} Cnidaria, a basal animal phylum with diverse life cycle patterns, offers unique examples to explore the ancestral characteristics of the life cycle regulatory mechanisms (Figure 1A).^{3,4} Cnidarians exhibit diverse life cycle patterns, most of which involve transitions between larval, polyp, and medusa stages.^{3,4} The larval stage is characterised by an ovoid or elongated morphology, complemented by cilia that facilitate movement.^{3,4} The polyp stage is predominantly sessile, featuring radial symmetry and tentacles that are employed for capturing prey.^{3,4} The medusa stage represents a pelagic, free-swimming form, marked by a bell- or umbrella-shaped body that possesses complex physiological attributes, including well-integrated reproductive and digestive systems, as well as specialised muscle tissues that facilitate complex locomotion.^{3,4} Some cnidarians, such as corals and sea anemones, exhibit relatively simple life cycle transitions, with a pelagic larva followed by metamorphosis into a sessile polyp.⁵ In contrast, species of Scyphozoa (subphylum Medusozoa; phylum Cnidaria), the true jellyfish, typically exhibit complex life cycle transitions, including larva-to-polyp and polyp-to-medusa transitions.

The majority of scyphozoans undergo a metamorphic transition from polyp to medusa, termed strobilation (Figure 1B).^{5,7} During this process, single sessile polyps undergo metamorphosis, splitting into multiple motile ephyrae (juvenile jellyfish) within a short period; this leads to an exponential increase in the local medusa population.⁸ Ecologically, the simultaneous release of a multitude of ephyrae can saturate predators and cause resource plundering,⁸ thus enhancing the survival rates of jellyfish and contributing to population blooms. Such blooms subsequently exert numerous deleterious effects on the

fishing industry, tourism, ecological balance, and coastal facilities.⁹ Biologically, the strobilation of jellyfish represents a profound transformation characterised by considerable changes, especially in the neuromuscular system.^{10,11} Initially equipped with a rudimentary contractile apparatus and diffuse nerve network, the polyp stage exhibits basic behaviours like feeding and locomotion.¹² With the transition to the ephyra stage, an intricate neuromuscular system develops, complete with complex musculature, a well-organised motor nerve net, and specialised sensory organs, all of which endow the jellyfish with advanced adaptive capacities such as rhythmic pulsation, navigation, and predator evasion abilities.¹³ This behavioural enhancement, underpinned by neuromuscular changes, not only promotes the survival and proliferation of jellyfish in diverse environments but also raises intriguing questions in evolutionary neurobiology, particularly related to the ability of jellyfish to execute complex behaviours without a centralised brain.^{14,15}

In recent years, escalating incidents of jellyfish blooms and the attractive features of jellyfish for neuroscience have intensified the focus on the regulatory mechanisms of strobilation. Previous studies have identified that environmental (e.g., temperature, light, and salinity)^{16–18} and chemical (e.g., indole compounds, iodine, and 9-cis-retinoic acid)^{19,20} factors can trigger the initiation of strobilation in most scyphozoans. Additionally, molecular regulators such as the retinoid X receptor signalling pathway and CL390 play pivotal roles in the strobilation process of the scyphozoan *Aurelia aurita*.²⁰ Despite these advances, remarkably little is known about the cellular dynamics and molecular machinery involved in strobilation. This gap in knowledge restricts our comprehensive understanding of life cycle transition and impedes the establishment of an effective strategy for controlling jellyfish blooms.

Recently, single-cell sequencing techniques have provided unprecedented insights into the trajectories of cell differentiation, cellular fate determinants, and tissue morphogenesis at the resolution of individual cells.^{21,22} In this study, we used single-cell transcriptomics in conjunction with in situ hybridisation (ISH) to map the first cell atlas of the scyphozoan *Aurelia coerulea*, a cosmopolitan species renowned for its complex life cycle and frequent global blooms.⁹ We also elucidated the dynamic shifts in cell type composition throughout the metamorphosis from polyp to medusa stages and highlighted potential molecular regulators underpinning development of the neuromuscular system. This work provides a valuable resource for understanding the molecular basis of scyphozoan life cycle patterns, as well as novel insights that can be used for the control of jellyfish blooms.

MATERIALS AND METHODS

Culturing and sampling

Polyps of *A. coerulea* originated from the Yantai Sishili Bay of the northern Yellow Sea, China, and were cultured in an incubator at 15 °C. The culture medium was filtered artificial seawater (FASW; 0.22 µm filter) with a salinity

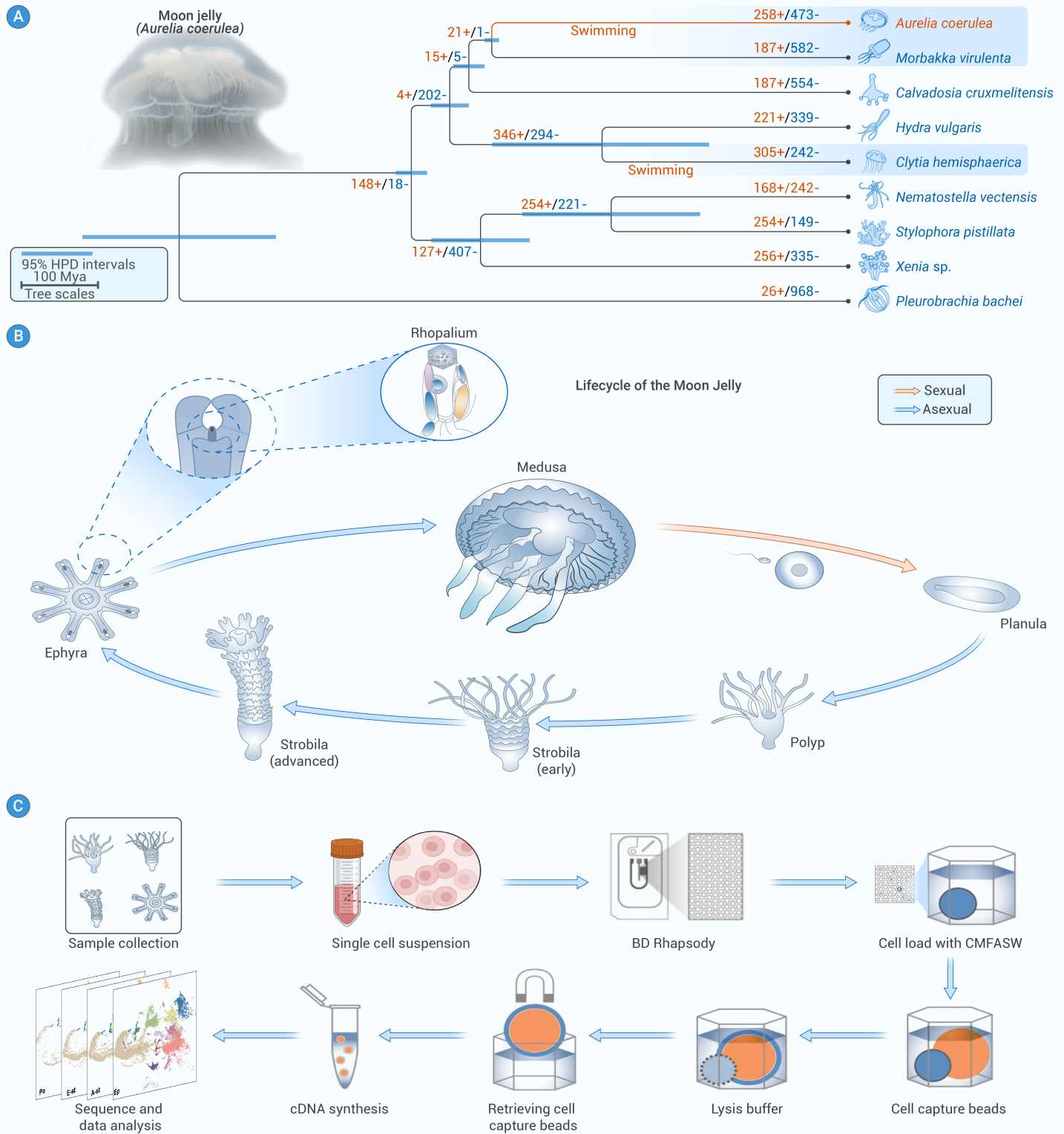


Figure 1. Information on *Aurelia coerulea* (A) Phylogenetic tree of Cnidaria. (B) Life cycle of the moon jellyfish *Aurelia coerulea*. (C) Schematic of the workflow of BD Rhapsody based scRNA-seq for studying *A. coerulea* strobilation.

of 30 ppt. Seawater was changed once per week, and freshly hatched *Artemia* nauplii were provided weekly as prey. To obtain samples at different stages of strobilation, the polyps were incubated at 9 °C to induce strobilation, allowing for the collection of early strobila (14 d), advanced strobila (20 d), and ephyra (25 d) samples.

Preparation of cell suspensions

For single-cell RNA sequencing (scRNA-seq) library preparation, eight

healthy polyps (without signs of asexual reproduction), eight early strobilae, eight advanced strobilae, and ten ephyrae were collected and washed twice in Ca^{2+} -free and Mg^{2+} -free FASW (CMFASW; 25 g/L NaCl, 0.8 g/L KCl, and 0.04 g/L NaHCO_3 in pH 8.5). Immediately thereafter, samples from different life cycle stages were separately transferred to a centrifuge tube containing 2 mL of digestive buffer (3.6 mg/mL dispase 2 [Coolaber, Beijing, China] and 1.25 mg/mL collagenase 3 [ACMEC, Shanghai, China] in CMFASW). Dissociation was carried out at room temperature for 20 min with occasional stirring

and subsequently stopped by adding 8% volume of foetal bovine serum. After dissociation, the single-cell suspension was centrifuged at $500 \times g$ for 5 min at 4 °C, resuspended in pre-chilled CMFASW, and subsequently filtered through a 40 µm cell strainer (FALCON; Corning, NY, USA).

scRNA library preparation, sequencing, and generation of count matrices

A low concentration of Calcein AM (2 mM) and Draq7™ (0.3 mM) was utilized to assess cell viability in this experiment, employing the BD Rhapsody™ system (BD Biosciences, Franklin Lakes, NJ, USA). Only cell suspensions with >90% viability were used for cell capture. The BD Rhapsody™ system was used for single-cell capture and library construction (Figure 1C). The libraries were quantified using a Qubit high-sensitivity DNA assay (Thermo Fisher Scientific, Waltham, MA, USA). Sequencing was performed using the Illumina NovaSeq 6000 platform with 150 bp paired-end runs (Inc., San Diego, CA, USA). Fastq files were processed using the BD Rhapsody™ WTA Analysis Pipeline (version 1.0) on Seven Bridges (<https://www.sevenbridges.com>) following the manufacturer's guidelines. Reads were mapped onto the *Aurelia* genome.²³

Data quality control, integration, and clustering

Unique molecular identifier (UMI) count matrices were imported into R (v.3.6.2) and processed with the R package Seurat (v.4.0.6) (<https://satijalab.org/seurat/>). To remove ambient RNA contamination and putative doublets, low-quality transcriptomes were filtered for each time-course sample as follows: 1) cells with less than 200 expressed genes were discarded; 2) only genes that were expressed in at least three cells in each dataset were considered; and 3) cells were filtered based on total UMI counts, excluding cells with excessively low (<100) or high (>6000) UMI counts. To compare the differences in cell types at different stages of development, quality-filtered datasets of different life cycle stages were merged in Seurat. To generate cell type clusters, the "SCTransform" function with default parameters was used for data standardisation. Following standard downstream processing steps, we performed principal component analysis and uniform manifold approximation and projection (UMAP) in 30 dimensions. We used FindNeighbors (dims 1:30) and FindClusters (resolution 0.1) to generate cell clusters. Analysis of similarity across various cell types was conducted by examining the 2000 genes exhibiting the highest standard deviation, followed by clustering based on Spearman correlation coefficients.

Cross-species comparative analysis

To compare the cellular profiles across different species, expression data matrices were extracted from datasets in EDomics (<http://edomics.qnlm.ac>).²⁴ We screened the scRNA datasets of five model organisms (*Trichoplax adhaerens*, *Stylophora pistillata*, *Hydra vulgaris*, *Clytia hemisphaerica*, and *Danio rerio*) from EDomics and extracted their respective neural cell expression matrices to compare with the nerve cells of the moon jellyfish *A. coerulea*. We used the SAMap²⁵ (mapping single-cell atlases throughout Metazoa unravels cell type evolution) pipeline to generate cell-to-cell similarity matrices between *A. coerulea* and each respective species. Gene homology graphs required by SAMap were constructed using reciprocal BLAST. Default parameters were used for both graph construction and the SAMap algorithm. UMAP visualisations of the target cell type across different species were merged and processed in Seurat (v.4.0.6).

Pseudotime analysis

The R package Monocle2 was used to infer cell differentiation trajectories. Single-cell pseudotime trajectories of epidermal/muscle and neural cell subtype groups during strobilation were constructed using variable genes in Monocle2 with default parameters (<http://cole-trapnell-lab.github.io/monocle-release>). The raw count was first converted to a CellDataSet object using the importCDS function, and dimensional reduction clustering and trajectory inference were then performed with the reduce dimension and order cells functions, respectively, with default parameters. Based on the pseudotime analysis, branched expression analysis modelling (BEAM) was applied to genes at branch points.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis

We used the FindAllmarker (logfc.threshold = 0.25) function in the Seurat R package to obtain differentially expressed genes (DEGs) among different cell clusters or stages. DEGs were then selected and mapped to the GO and KEGG databases for corresponding genome annotation information. Gene set variation analysis (GSVA) and ClusterProfiler were applied to identify GO and KEGG terms between stages and cell clusters.²⁶

RNA interference (RNAi) experiment

In the RNAi experiment, a short interfering RNA (siRNA) targeting *HOX1* was designed by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). The lyophilised siRNA was resuspended in RNase-free water, reaching a final concentration of 20 µM as siRNA stock solution. Given that *Aurelia* continuously resides in seawater, the siRNA soaking transfection method was employed. The detailed methodology is as follows:

Pre-experimental preparation: Healthy polyps of similar size were inoculated in a 24-well culture plate from the rearing tank, with three polyps per well and 2 mL of FASW. The polyps were maintained in a temperature-controlled incubator at 15 °C for 6 d to ensure attachment. During the culture period, feeding (freshly hatched *Artemia* nauplii) was conducted every 2 d, and FASW was changed every 2 d. To investigate the function of *HOX1*, three treatment groups were established: control treatment (CO), siRNA treatment (si*HOX1*), and negative control siRNA treatment (NC) groups. Strobilation was induced using an induction buffer containing 5 µM 5-methoxy-2-methyl indole in FASW.

Preparation of the transfection complex buffer: For CO, 240 µL of 1X riboFECT™ CP Buffer (RiboBio), 4 µL of ddH₂O, 24 µL of riboFECT™ CP Reagent (RiboBio), and 1.886 mL of induction buffer were combined per well. For NC, 240 µL of 1X riboFECT™ CP Buffer (RiboBio), 4 µL of negative control siRNA, 24 µL of riboFECT™ CP Reagent (RiboBio), and 1.886 mL of induction buffer were combined per well. For si*HOX1*, 240 µL of 1X riboFECT™ CP Buffer (RiboBio), 4 µL of 20 µM siRNA stock solution, 24 µL of riboFECT™ CP Reagent (RiboBio), and 1.886 mL of induction buffer were combined per well.

siRNA soaking transfection: The transfection complex buffer was added to the respective treatment groups. All polyps were cultured at 20 °C for 6 d, with the transfection complex being replaced every 3 d. Subsequently, the polyps were collected for morphological observation and reverse transcription quantitative PCR (RT-qPCR) analysis.

RT-qPCR

mRNA was extracted with the Illustra Polyphenol Polysaccharide Plant Total RNA Extraction Kit (ZOMANBIO, Beijing, China), and cDNA was synthesised with the One-Step gDNA Removal and cDNA Synthesis SuperMix kit (TransGen Biotech, Beijing, China), following the manufacturer's instructions. qPCR was performed using SYBR Green Master Mix (Thermo Fisher Scientific) and the Applied Biosystems 7500 real-time PCR system (Thermo Fisher Scientific). All primers (primer sequences are provided in Dataset S1) were diluted to 10 pmol/L. Each analysis was repeated with at least three biological replicates, and all qPCR samples were repeated with three technical replicates. All samples were analysed using beta-actin as a reference gene. Expression was quantified using the $\Delta\Delta C_t$ method.

Whole-mount ISH

Aurelia riboprobes (primer sequences are provided in Dataset S2) generated from templates produced via standard cloning and restriction endonuclease digestion were synthesised using the DIG RNA Labeling Kit (SP6/T7) (Roche, Basel, Switzerland). Colorimetric ISH was performed as described by Fuchs et al.²⁰ with minor modifications. Briefly, *Aurelia* specimens starved for at least 2 d were relaxed in 2% MgCl₂ for 10 min and fixed overnight at 4 °C in 4% paraformaldehyde. Specimens were then dehydrated in a 25/50/75% MeOH series for 20 min to remove undesired pigmentation and stored in 100% MeOH at -20 °C. Samples were rehydrated using a 75/50/25% MeOH series for 10 min each, washed with PBT for 10 min, and bleached in a 3% H₂O₂/PBT solution for 10 min. Specimens were permeabilised for 20 min with 1 µg/mL proteinase K (Sigma-Aldrich, St. Louis, MO, USA) in PBT. Hybridisa-

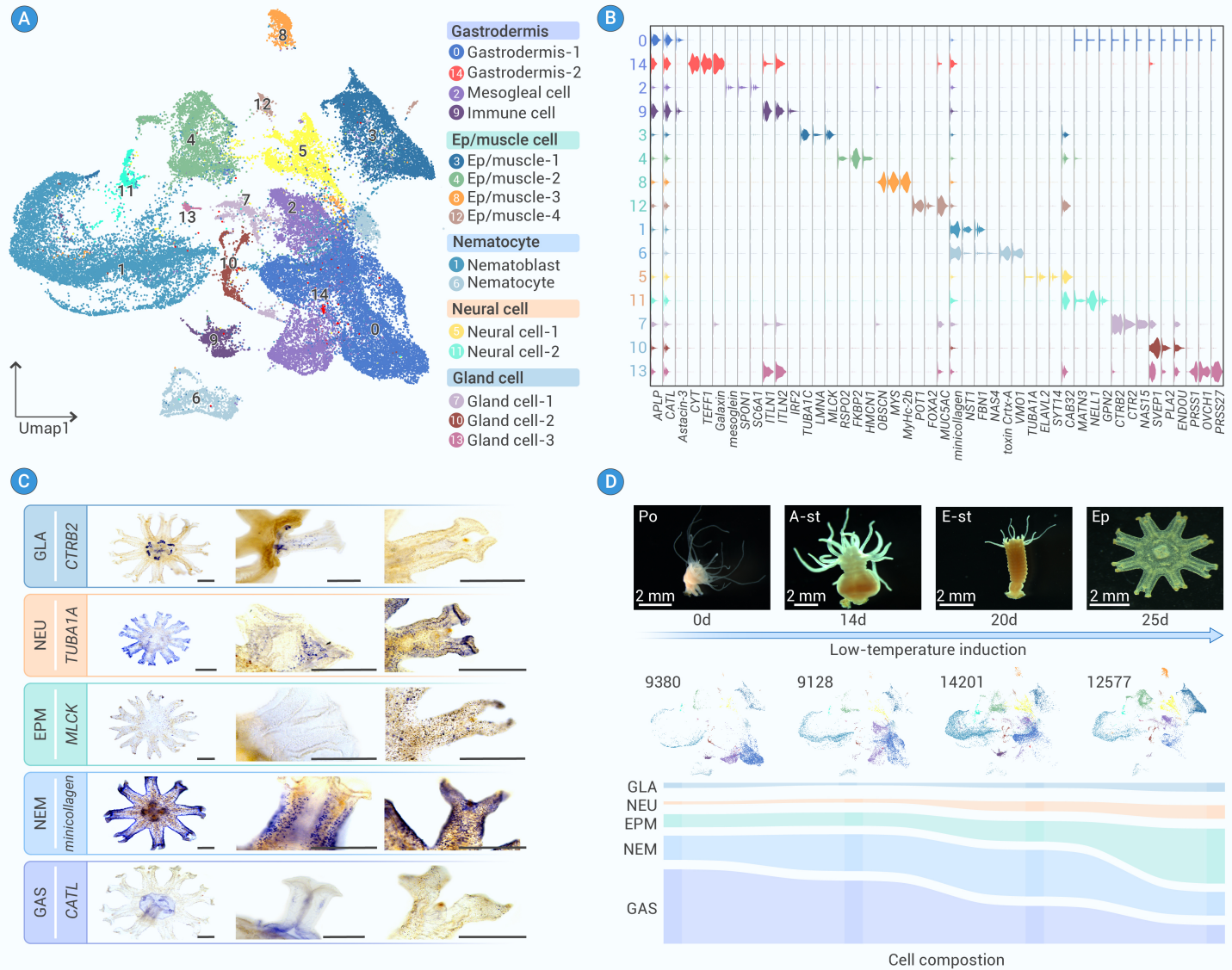


Figure 2. Cell atlas of *Aurelia coerulea* during strobilation (A) UMAP visualisation of the integrated cell atlas from different life cycle stages, colour-coded by 15 clusters. (B) Violin plot showing the marker gene expression profiles of different clusters. (C) ISH for a selection of cluster spatial marker genes in the ephyra stage of *A. coerulea*. Top to bottom: whole ephyra, manubrium, lappets. Scale bars in whole ephyra images are 200 μ m. Scale lengths in images of manubrium and lappets: short, 200 μ m; long, 100 μ m. Blue indicates a positive probe signal. (D) Variation in cell composition at different life cycle stages during strobilation. Top, workflow diagram of sample collection; number at the bottom indicates the time of sample collection during the culture process. Middle, UMAP visualisation of cell atlas each stage, cell type colours are the same in Figure 2A; number on the top left indicates cell numbers. Bottom, river plots showing the variation in cell types at different life cycle stages during strobilation. Abbreviations: Po, polyp; E-st, early strobila; A-st, advanced strobila; Ep, ephyra; GLA, gland cells; NEU, neural cells; EPM, epidermal/muscle cells; NEM, nematocytes; GAS, gastrodermal cells.

tion was carried out at 57 °C for 72 h in hybridisation buffer (50% formamide, 5×SSC, 0.1% Tween-20, 0.1% CHAPS, 1×Denhardt's solution, and 100 μ g/mL heparin in DEPC water) with 0.5 mg/mL torula yeast RNA (Sigma-Aldrich). Samples were incubated with AP-conjugated anti-digoxigenin antibody (Roche) and detected with NBT/BCIP substrate using the DIG Nucleic Acid Detection Kit (Roche). Samples were transferred into 80% glycerol and imaged using a LEICA DMI8 microscope (Leica, Wetzlar, Germany).

RESULTS

Cell atlas of *A. coerulea*

To gain a comprehensive perspective of cellular diversification during the strobilation of *A. coerulea*, we selected four typical life cycle stages for scRNA-seq: the polyp (Po), early strobila (E-st), advanced strobila (A-st), and ephyra (Ep) (Figure 1C). After quality assessment and filtering, a total of 45286 cells expressing 24362 genes with a median of 433 genes/cell and a median of 1103 UMIs/cell with good-quality data were retained and used for downstream analysis (Figure S1). Louvain clustering and UMAP identified 15 clusters of cells from typical life cycle stages during strobilation (Figure 2A). Cell populations were annotated manually by combining data from known *Aurelia*

marker genes or orthologous genes in other taxa (i.e., *Clytia* and *Hydra*)^{27,28}. The cell populations were then clustered into five groups (associated markers are shown in parentheses), including nematocytes (minicollagen, toxin Ctx-A, NAS4, and VMO1), neural cells (ELAVL2, TUBA1A, and SYT14), epidermal/muscle cells (MLCK, MYH2, and POT1), gastrodermal cells (CATL, Astacin-3, and APLP), and gland cells (CTRB2, CTR2, OVCH1, and PRSS1) (Figure 2, Dataset S3). The ISH results showed that 1) cathepsin L (CATL) of the gastrodermis is expressed in cells lining the manubrium in ephyrae; 2) minicollagen is expressed in the internal part of the manubrium, as well as the surrounding lappets; 3) tubulin alpha 1a (TUBA1A) is expressed at the edge of the manubrium and lappets; 4) the genes encoding myosin light chain kinase (MLCK) are scattered sporadically in the lappets; and 5) chymotrypsinogen B2 (CTRB2) is centrally positioned at the gastric filaments and edge of the manubrium (Figure 2C, Figure S2). In particular, the cells in cluster 9, which belong to the gastrodermis population, expressed specific immune-related genes such as interlectin-1 (ITLN1), interlectin-2 (ITLN2), and interferon regulatory factor 2 (IRF2), and were therefore identified as specialised immune cells (Figure 2A–B, Dataset S3). Moreover, cluster 2 specifically exhibited high expression of genes encoding the mesoglea extracellular matrix mesoglein in

jellyfish, suggesting that cells in cluster 2 can be defined as mesogleal cells (Figure 2A–B, Dataset S3).

Additionally, the composition of cell types identified through scRNA-seq was compared across the four different typical life cycle stages, and marked changes were observed in the proportions of cell types during strobilation. The proportions of nematocytes and gland cells remained relatively stable throughout the strobilation process (Figure 2D). The proportion of neural cells increased from approximately 2.4% in the polyp stage to 11.3% in the ephyra stage (Figure 2D). Concurrently, the proportion of epidermal/muscle cells rose from 10.8% in the polyp stage to 46.0% in the ephyra stage. In contrast, the proportion of gastrodermal cells decreased from 62.3% in the polyp stage to 15.6% in the ephyra stage (Figure 2D). Further, similarity analysis revealed stage-specific variation in gene expression patterns among cellular types. Nematocytes and gland cells exhibited high similarities in gene expression profiles during the different stages, whereas neural, epidermal/muscle, and gastrodermal cells showed marked differences among the different stages (Figure S3).

Variation in the neuromuscular system during strobilation

Strobilation is a crucial metamorphic process in scyphozoans, in which the sessile polyp undergoes profound morphological and physiological changes and later develops into the motile ephyra (juvenile medusa). Stage-specific genes were visualised by comparing gene expression patterns in different stages. Of the 24362 genes expressed, only 14280 genes were co-expressed in all four stages (Figure S4A). GO analysis of each developmental stage revealed the enrichment of terms related to development and differentiation of the neuromuscular system during strobilation. Terms associated with muscle development, such as muscle fibre development and muscle organ development, and those associated with neural development, such as nerve development, neuronal action potential, postsynaptic membrane, and central nervous system neuron development, were all enriched during the process of strobilation (Figure S4B), indicating substantial changes in both the muscular and nervous systems.

Remodelling of the neuromuscular tissue during strobilation is crucial for efficient propulsion and precise motor control in jellyfish.²⁹ Analysis of the cellular composition during strobilation revealed a marked increase in the proportion of neural cells and epidermal/muscle cells (Figure 2D), indicating highly dynamic cellular transformation. Furthermore, the gene expression patterns of the neural and epidermal/muscle cell groups differed significantly between the different stages. Only 6657 and 10661 genes were co-expressed in the neural and epidermal/muscle cell groups, respectively. Principal coordinate analysis showed that the transcriptomes of neural and epidermal/muscle cell groups in the polyp, early strobila, and advanced strobila stages were more similar than those in the ephyra stage (Figure S4C–D), indicating that the proportions and functions of epidermal/muscle and neural cells undergo significant changes during strobilation.

Variation in neural cell types during the polyp-to-medusa transition

To comprehensively understand neurobiological changes during the transition from the benthic polyp stage to motile stages, we first compared the functional characteristics of the gene sets of neural cells across the four stages. GO analysis revealed the enrichment of terms such as GABA receptor binding, dopaminergic neuron differentiation, and central nervous system neuron development during strobilation, indicating the development of a more complex nervous system in the medusa stage (Figure S5). Subsequently, we selected the datasets of neural cells and re-clustered 14 neural subpopulations based on specifically expressed markers (Figure 3A–B, Figure S6). Limited by genome annotation and prior knowledge, we were unable to fully annotate all cell types. However, we observed significant changes in the proportions of neural subtypes during strobilation, and most cell types were present across the four life cycle stages. For instance, subtype 2 (highly expressing *Pol-RFamide* and *NCAL*) and subtype 8 (highly expressing *LWamide*) exhibited fixed proportions throughout all four stages of strobilation (Figure 3A–B). Furthermore, subtype 0, which specifically expressed *SOX4*, was identified as a neural stem/progenitor cell subtype.³⁰ Subtype 13, characterised by the specific expression of *OPN4* and *NGB1*, was identified as a photoreceptor cell group (Figure 3A–B).³¹ Neither subtype 0

nor subtype 13 was found in the early strobila stage, but both exhibited fixed proportions in other stages (Figure 3B). Notably, we discovered four newly emerged neural subtypes (subtypes 3, 6, 9, and 12) that appeared at the early strobila, advanced strobila, and ephyra stages (Figure 3B).

Subtype 3 specifically expressed gene *Seg 1359.8* and high levels of *GABRR2*, which is a member of the GABA-A receptor family primarily found in the retinal neurons (Figure 3B–C, Figure S6, Dataset S4).³² GO analysis revealed the enrichment of terms related to neuron differentiation, GABA-A receptor activity, and intracellular signal transduction within subtype 3 (Figure 3C). The ISH results of the marker gene *Seg 1359.8* of subtype 3 demonstrated specific expression in the ocellus (a light-sensing organ) of the rhopalium, suggesting that subtype 3 represents specialised retinal neuronal cells (Figure 3D). Additionally, subtype 3 specifically expressed atrial natriuretic peptide receptor 2 (*ANPR2*), SRSF protein kinase 1 (*SRPK1*), and yippee-like protein 1 (*YPEL1*), which are associated with neuronal development in the central nervous system (Figure 3B–C, Figure S6, Dataset S4).^{33–35} Another newly identified cell type during strobilation, subtype 9, exhibited enrichment in electron carrier activity and calcium-mediated signalling and specifically expressed the marker genes of glycinergic neurons, including *Glyt2*, *CALM*, and *VIAAT*, suggesting its classification as a glycinergic neuron subtype (Figure 3B–C, Figure S6, Dataset S4).³⁶ The newly emerged subtype 12, enriched in terms related to signal transduction, voltage-gated potassium channel activity, and neurotransmitter receptor activity, specifically expressed genes associated with the function of sensory stimuli, including *KCNA2*, *GNAI2*, and *CFAP20*,^{37–39} suggesting its potential as a sensory hair cell subtype (Figure 3B–C, Figure S6, Dataset S4). *ELAVL2* and *Pol-RFamide*, which were highly expressed in the newly identified subtypes 9 and 12, were predominantly expressed in the inner and surrounding regions of the rhopalium when analysed by ISH (Figure 3B–D). However, owing to limitations in the annotation of its specifically expressed genes, subtype 6 could not be characterised.

To evaluate the origins and functions of various neural cell types, we conducted a comparative analysis across species, from basal placozoans (*T. adhaerens*) to chordates (*D. rerio*). The nerve cell group in the scyphozoan jellyfish *A. coerulea* was more closely related to that of the hydrozoan jellyfish *C. hemisphaerica* than to those in other species (Figure S7). Among the newly identified neural cell subtypes, subtypes 3 and 6 were unique to both *C. hemisphaerica* and *A. coerulea*, and subtype 12 was exclusive to *A. coerulea* (Figure S7). Notably, subtype 9, which was dominant in the mobile ephyra stage of *A. coerulea* but absent in the immobile polyp stage, was also present in other motile organisms, including *T. adhaerens*, *C. hemisphaerica*, and *D. rerio*, but not in the sessile species *H. vulgaris* and *S. pistillata* (Figure 3B, Figure S7); thus, subtype 9 may play a critical role in facilitating mobility.

Next, we integrated cell types from *T. adhaerens*, *C. hemisphaerica*, and *D. rerio* comparable to subtype 9 in *A. coerulea* and identified 279 homologous genes with distinct expression patterns (Figure S8A). Functional enrichment analysis highlighted a uniform presence of signal transduction-related processes, notably related to vesicle-mediated transport, intracellular transport, the ciliary basal body, and axoneme processes (Figure S8B). Moreover, genes associated with intracellular transport (e.g., *XPO1* and *VT11A*) and signal transduction (e.g., *PDE6D*, *BBS7*, and *RENK*) were uniformly expressed across these species (Figure S8C), suggesting that these cells played an important function in mediating motor control.

To elucidate the gene regulation of newly emerged neural cell types, the trajectory model Monocle2 was used to delineate neural cell differentiation lineages during strobilation. Cell lineage trajectory analysis revealed the differentiation of neural stem cells (subtype 0) into three newly emerged subtypes (Figure 3E). The putative cell lineage trajectory bifurcated into two major branches. One branch showed that neural stem cells (subtype 0) differentiated into subtype 3 and subtype 12, and the other branch showed that neural stem cells (subtype 0) differentiated into subtype 9 (Figure 3E). The analysis of gene expression patterns along the trajectory of single-cell differentiation showed alterations in the expression profiles of transcription factors (TFs) and regulators implicated in neural differentiation (Figure 3F). Specifically, during the process of neural cell differentiation, TFs such as *SOX4*, *HOXA4*, *BTF3L4*, and *YBX1* exhibited a discernible downward trend in expression from neural stem cells to the newly emerged subtypes, whereas

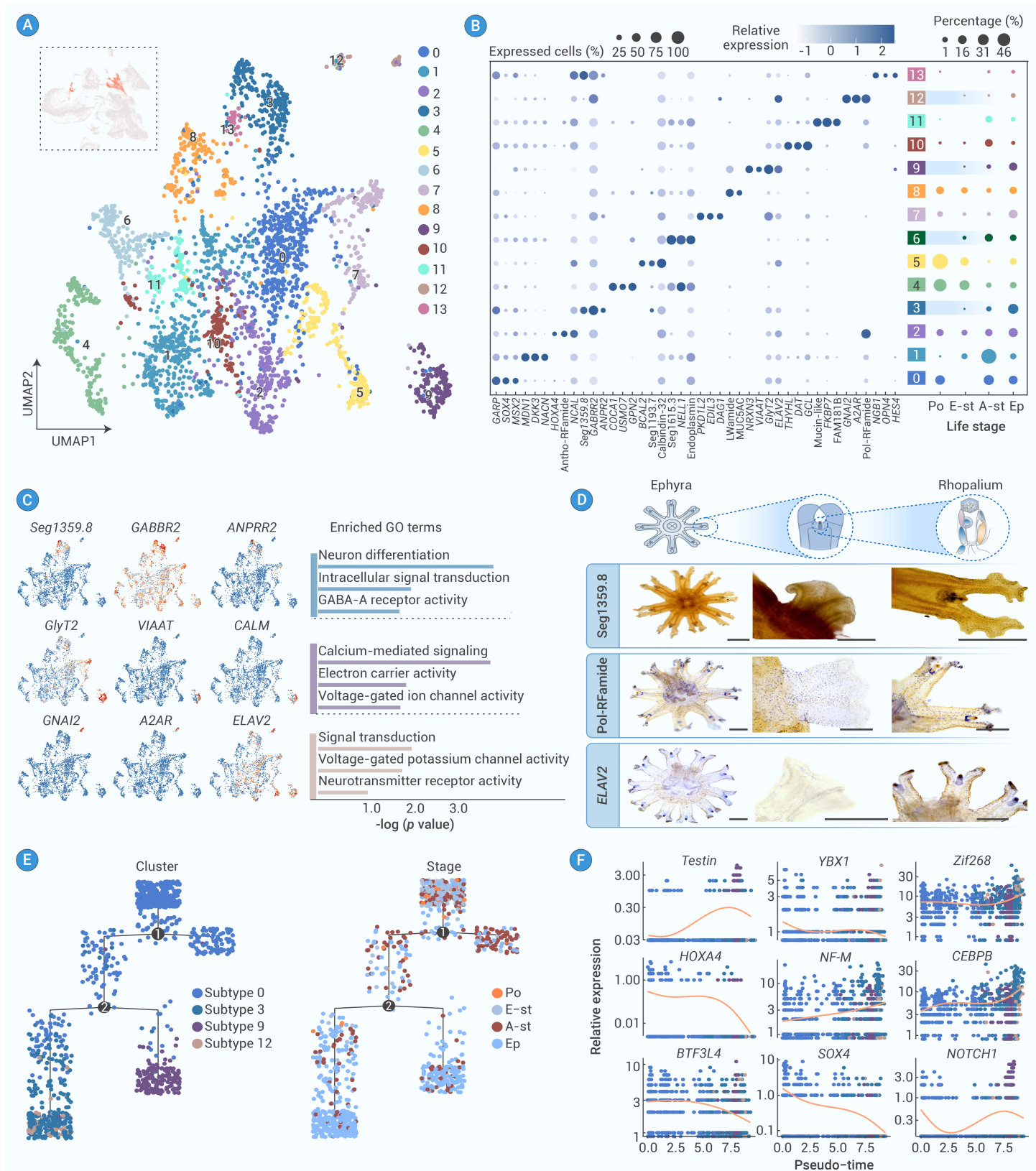


Figure 3. Transcriptional dynamics of neural cells during strobilation (A) UMAP visualisation of neural cell subsets from four stages during strobilation. Cells are coloured according to their cluster identity (ID) from Louvain clustering. Inset: locations of neural clusters in the cell atlas UMAP plot from Figure 2A. (B) Left dot plot shows the relative expression of marker genes (colour) and the proportion of cells (size) in each neural subtype. Right dot plot shows the fraction of neural subtype cells at each developmental stage. (C) Characteristics of newly identified neural cell types during strobilation. Left feature plots show selected marker gene expression of newly identified neural subtypes. Enriched GO terms are shown vertically on the right, and the bar plot shows negative logarithm P values. (D) ISH for a selection of cluster spatial marker genes specifically expressed in the newly identified neural cell types in the ephyra stage of *A. coerulea*. Left to right: whole ephyra, manubrium, lappets. Scale bars in the whole ephyra images are 200 μ m. Scale lengths in images of manubrium and lappets: short, 200 μ m; long, 100 μ m. (E) Pseudotemporal cell ordering of neural stem cells (subtype 0) to the newly identified cell subtypes along differentiation trajectories (left) and corresponding life cycle stages (right). (F) Expression of selected transcription factors involved in neural differentiation, ordered by Monocle2 analysis in pseudotime. Abbreviations: Po, polyp; E-st, early strobila; A-st, advanced strobila; Ep, ephyra.

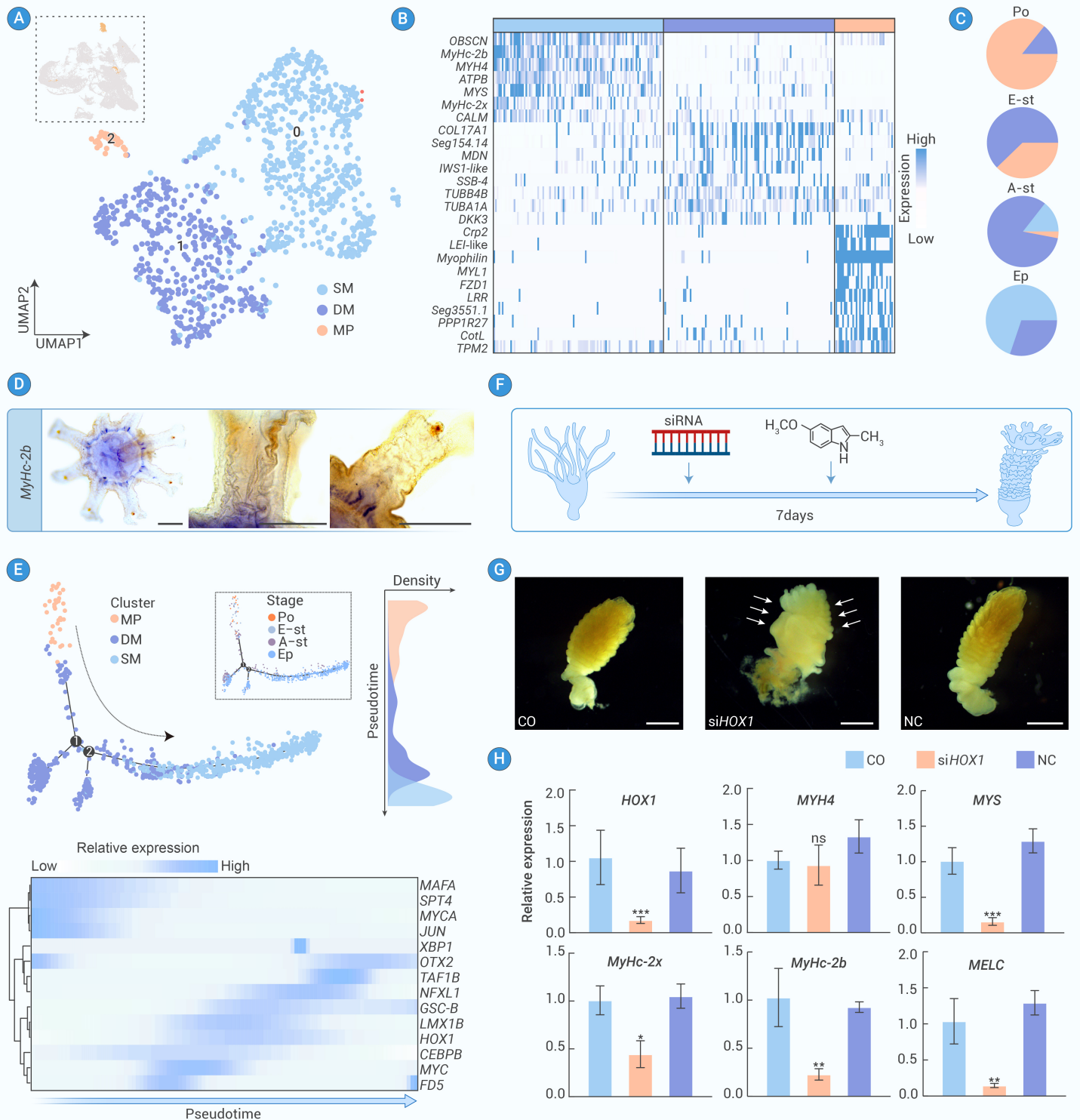


Figure 4. Regulation of striated muscle formation by *HOX1* during strobilation (A) UMAP visualisation of muscle cell subsets from four stages during strobilation. Cells are coloured according to their cluster identity (ID) from Louvain clustering. Inset: locations of cluster 8 on the merged UMAP plot from Figure 2A. The same colours are used in panels (B) and (C). Abbreviations, SM, striated muscle cells; DM, differentiating muscle cells; MP, muscle progenitor cells. (B) Heatmap showing the expression of marker genes in subtype cells. (C) Pie chart showing the cell composition of each muscle subtype per life cycle stage during strobilation. Abbreviations: Po, polyp; E-st, early strobila; A-st, advanced strobila; Ep, ephyra. (D) ISH for specific expression marker *MyHc-2b* of striated muscle in the ephyra stage of *A. coerulea*. Left to right: whole ephyra, manubrium, lappets. Scale bars in the whole ephyra images are 200 μ m. Scale lengths in images of manubrium and lappets: short, 200 μ m; long, 100 μ m. (E) Molecular signature of cell trajectory in subtype cells. Cell trajectory ordering of cell subtype (top left) and life cycle stage (top middle); arrow indicates direction of cell trajectory. River plot (top right) showing changes in the proportion of cell subtypes along differentiation trajectories. Heatmap (bottom) showing expression of selected transcription factors over pseudotime. (F) Schematic representation of the RNAi workflow during strobilation. (G) Phenotypes of different treatment groups 7 d after induction of strobilation; white arrow indicates areas of phenotypic differences. Abbreviations, CO, control group; si*HOX1*, siRNA treatment group; NC, negative control siRNA treatment group. Scale bars, 200 μ m. (H) Bar plot showing the expression of specific genes in striated muscle across different treatment groups. Mean expression $\pm$ SD (n = 3). ns, not significant, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

TFs such as *Zif268*, *NOTCH1*, *NF-M*, *CEBPB*, and *Testin* showed an opposite trend characterised by upregulation (Figure 3F).

Regulation of striated muscle formation by *HOX1* during strobilation

In contrast to the benthic stage, the medusa stage exhibits a more complex musculature for free-swimming, defence, and feeding.⁴⁰ In cluster 8,

we observed the specific high expression of key molecules (e.g., *MyHc-2b*, *MYS*, *MELC*, and *OBSCN*) critical for rapid muscle contraction⁴¹ (Figure 2B, Figure S9). Concurrently, an increase in the cell proportion of cluster 8 was observed during strobilation (Figure 2D). These observations collectively imply that cluster 8 likely represents striated muscle cells integral to the free-swimming functionality. Next, we re-clustered cluster 8 into three subtypes, namely, muscle progenitors (specifically expressing *FZD1*, myophillin, and *TPM2*),⁴² differentiating muscle cells (specifically expressing *DKK3*, *IWS1*-like, and *MDN*),²⁷ and striated muscle cells (specifically expressing *MyHc-2b*, *MYH4*, and *MYS*)⁴¹ (Figure 4A–B, Dataset S5). Notably, striated muscle cells were only present during the advance strobilation and ephyra stages, and they were absent during the sessile polyp and early strobila stages (Figure 4C). The ISH results of *MyHc-2b* showed that striated muscle cells were primarily distributed in the subumbrella (a contractile tissue) of the ephyra (Figure 4D).

Subsequently, putative cell lineage trajectories of different subtypes were reconstructed using Monocle2 software to investigate the potential regulatory mechanisms underlying the formation of striated muscle. The analysis revealed a major trajectory from muscle progenitors to striated muscle cells (Figure 4E). Enrichment analyses based on GO and KEGG datasets revealed the significant enrichment of terms related to motor activity, myosin complex, signalling receptor activity, endocytosis, and tryptophan metabolism during the process of striated muscle differentiation (Figure S10). Additionally, we observed high expression of several TFs, including *OTX2*, *TAF1B*, *LMX1B*, *FD5*, *GSC-B*, and *HOX1*, along the differentiation trajectory, indicating that they might be involved in the genesis of striated muscle (Figure 4E). Specifically, *HOX1* exhibited high specific expression in cluster 8 but was not expressed in the sessile polyp or early strobila stages (Figure S11A–B). Moreover, the expression of genes encoding fast-twitch proteins of striated muscle cells was initiated only after the substantial upregulation of *HOX1* expression (Figure S11C), implying the putative role of *HOX1* in the formation of striated muscle. To investigate the functional impact of *HOX1* on striated muscle cell development, we designed siRNA molecules specifically targeting *HOX1* for knockdown during the strobilation process (Figure 4F).

Following a 7-day incubation, the morphology of polyps in the si*HOX1* (*HOX1* siRNA interference) group exhibited significant differences from that of polyps in the other treated groups. In both the CO and NC (siRNA negative control) groups, polyps formed bodily segments and exhibited early strobilation, displaying an axis-symmetric pattern throughout the entire polyp (Figure 4G). In contrast, polyps of the si*HOX1* group only formed bodily segments on one side, resulting in an asymmetrical body structure, and failed to reach the early strobila stage (Figure 4G). Subsequently, we assessed the expression of target genes. The si*HOX1* group exhibited the significant down-regulation of *HOX1* expression, validating the efficacy of the siRNA treatment (Figure 4H, $P < 0.001$). Furthermore, the expression of genes specifically expressed in striated muscle, such as *MYS*, *MyHc-2x*, *MyHc-2b*, and *MELC*, was significantly downregulated in the si*HOX1* group compared with that in the other groups (Figure 4H, $P < 0.05$), suggesting a pivotal role of *HOX1* in regulating striated muscle formation in jellyfish.

DISCUSSION

In this study, we performed single-cell transcriptomic characterisation across multiple developmental stages of *A. coerulea* and mapped a comprehensive cell atlas of gene expression patterns during strobilation. This work represents an important addition to the growing number of single-cell atlases across the animal tree of life. Furthermore, this atlas provides hundreds of marker genes for more than a dozen specific cell types, advancing our understanding of the diversity of cell types in jellyfish. For example, we identified, for the first time, cell types exhibiting molecular signatures associated with immune function (e.g., *ITLN1*, *ITLN2*, and *IRF2*),⁴³ suggesting the emergence of specialised immune cells in jellyfish. We also identified a distinct cell type that specifically expressed mesoglein, a protein that provides structural support and elasticity to the mesoglea,⁴⁴ highlighting its significant role in maintaining the structural stability of the jellyfish body. Further, cross-stage comparison of the cell atlas revealed stage-specific variations in gene expression patterns among cellular types, illuminating the molecular intricacies of the metamorphic process and providing new insights into the regula-

tory mechanisms driving polyp-to-medusa transitions.

In jellyfish, the presence of a complex network of neurons addresses the need for a centralised brain, but researchers remain mystified as to how this network of neurons manages to control complex behaviours.^{14,45} In this study, we identified 14 distinct nerve cell types with varying compositions, structures, and gene expression patterns across different life forms. These findings suggest complex and dynamic remodelling of the neural system in response to ecological and behavioural changes in jellyfish during the transition from a benthic to a mobile lifestyle. Of particular interest, we identified previously unreported cell types emerging during strobilation, including subtypes 3 (retinal neuron), 9 (glycinergic neurons), and 12 (sensory hair cells), the proportions of which increased as strobilation progressed (Figure 3). Retinal neurons play a crucial role in visual perception, enabling organisms to detect changes in light and navigate their environment, thereby facilitating essential behaviours.^{31,46} Glycinergic neurons, through the coordinated actions of GlyT2 and VIAAT proteins, play a vital role in the nervous system by maintaining the balance of excitatory and inhibitory signalling.³⁶ Glycinergic neurons regulate rhythmic motor patterns, and related sensory processing has been reported in various organisms, such as amphioxus,⁴⁷ ascidians,⁴⁸ *Xenopus* tadpoles,⁴⁹ and zebrafish.⁵⁰ Moreover, a homolog of A2AR, specifically expressed in the sensory hair cells of newly identified subtype 12, plays a vital role in regulating complex locomotive activities in *Drosophila*.^{51,52} Notably, ISH of markers for newly emerged neural cell types revealed their localisation in the rhopalium. The rhopalium, a specialised sensory organ rich in diverse cell types and receptors, is widely recognised for its role in regulating the rhythmic pulsing, swimming, and orientation of jellyfish.^{53,54} The prevalence of newly identified cell types in the rhopalium suggests their critical role in coordinating and integrating the information exchange between different cell types, enabling complex behaviours in jellyfish. Furthermore, TFs (e.g., *Testin*, *NOTCH1*, *Zif268*, and *SOX4*) that are downregulated during the differentiation of neural stem cells to the newly identified neural cell types may play important regulatory roles in the genesis of these new cell types. Future studies should be dedicated to exploring their potential functionalities.

Cnidarians are widely regarded as some of the first organisms in animal evolution possessing a nervous system, offering a genetically tractable model for elucidating the genetic underpinnings of nervous system evolution and development.⁵⁵ The multi-stage *A. coerulea* cell atlas allows for a comparative analysis of neural cell types across various cnidarian species. In this study, we report that a specific neural cell type expressing the neuropeptide LWamide (identified as subtype 8) in the polyp stage of *A. coerulea* is conserved across the polyp stages of other cnidarians, including the stony corals *Stylophora pistillata*⁴⁵ and *Hydra vulgaris*,²⁶ as well as the sea anemone *Nematostella vectensis*.⁵⁶ Additionally, neural cell types expressing the neuropeptide RFamide are specific to the medusa stage of *A. coerulea* and *C. hemisphaerica*²⁷ but are notably absent in species lacking this stage. In the medusa stage, neurons expressing RFamide play roles in the folding behaviour of the umbrella, which thereby facilitates active predation and swimming.¹⁴ Notably, our cross-species analysis revealed that neural cell subtype 9 was only found in mobile organisms and was absent in immobile organisms (Figure S7), underscoring the role of these cells in motor control. Moreover, in the mobile ephyra stage of *A. coerulea*, genes such as *GLYT2* and *VIAAT*, markers of glycinergic neurons crucial for glycine neurotransmitter regulation,^{36,47} were specifically expressed in subtype 9, further emphasising the intricate genetic networks regulating cnidarian motor functions. To date, specific glycinergic neurons that co-express *GLYT2* and *VIAAT* have only been reported in chordates and vertebrates.⁴⁷ To the best of our knowledge, this is the first discovery of specialised glycinergic neurons in the phylum Cnidaria. Collectively, these findings indicate a dual evolutionary pathway of neural cell types within the cnidarian lineage. On the one hand, they appear to fulfil fundamental, conserved functions that may have originated in the early phases of nervous system evolution. On the other hand, variations in neural cell types appear specific to certain life cycle stages or species, which may signify adaptive evolution driven by ecological demands or life cycle characteristics.

Jellyfish, representing one of the earliest organisms to evolve striated muscle cells, acquired active swimming abilities and predatory advantages

crucial for their marine ecosystem dominance.¹¹ Previous studies have suggested that *Aurelia* polyps contain smooth, non-striated muscles in the tentacles, oral disc, and body column, whereas medusae exhibit both smooth and striated muscles.^{57,58} Our study consistently showed that striated muscle cells only emerged in the advanced strobila and ephyra stages (Figure 4). In bilaterians, striated muscles are derived from mesoderm cells.¹¹ However, the diploblastic nature of jellyfish, which lack a mesoderm, raises questions regarding the formation of striated muscle.^{23,59} The process of strobilation provides an excellent opportunity to explore the formation mechanism of striated muscle in jellyfish. Previous studies have implicated several myogenic TFs, such as *twist* and *mef2*, in jellyfish striated muscle formation;¹¹ however, our results did not suggest a correlation between their expression and the cell lineage trajectories of striated muscle formation. Instead, *HOX1* displayed striated muscle- and stage-specific expression, which is in alignment with the findings of Konstantin.⁶⁰ The knockdown of *HOX1* significantly inhibited the expression of specifically expressed genes in striated muscle cells, suggesting that *HOX1* plays a role in regulating the formation of striated muscle cells in *A. coerulea*. HOX genes, which belong to the homeobox gene family, are responsible for the delineation of embryonic body regions along the anterior–posterior axis in animals.⁶¹ HOX proteins specify the characteristics of “position”, ensuring that the correct structures are formed in the correct places in the body.⁶² In this study, the knockdown of *HOX1* resulted in an asymmetrical body structure and the malformation of bodily segments in *Aurelia* polyps during strobilation (Figure 4G). Notably, knockdown of *HOX1* also resulted in failure to transition from the polyp stage to the ephyra stage. This finding may contribute to the development of strobilation antagonists that could be used to control jellyfish blooms.

CONCLUSION

This study represents the first single-cell-resolution transcriptomic characterisation of a scyphozoan jellyfish, which belongs to phylum Cnidaria and has a complex life cycle. This cell atlas provides a valuable resource for further understanding the cellular diversity and molecular regulation mechanisms involved in the benthic–motile transition process and neuromuscular system development. Moreover, this study expands our knowledge of the cell biology of scyphozoan jellyfish and may help control jellyfish blooms in future.

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AUTHOR CONTRIBUTIONS

Z.-J.D conceptualized the study and provided the funding. Y.-X.L., S.-J.P., L.-J.W., K.S., P.-Z.X., T.-T.S., H.-W.Z., L.-J.Y., and L.W. performed the experiments. Y.-X.L., Z.-J.D., S.-J.P., K.C., K.H., Z.-Y.Y., F.-H.W., J.-Y.L., and Y.-Q.M. performed the bioinformatics analysis. Y.-X.L., S.-J.P., Y.-L.L., L.-J.W., and Z.-J.D. wrote the manuscript. Z.-J.D., Q.L., K.C., and J.-M.Z. reviewed and edited the manuscript. All authors read and approved the final manuscript.

DECLARATION OF INTERESTS

Qiang Lin is an Editorial Board member of The Innovation Geoscience and was blinded from reviewing or making final decisions on the manuscript. Peer review was handled independently of this member and their research group. The other authors declare no conflicts of interest.

DATA AND CODE AVAILABILITY

Sequencing data have been deposited in GEO under the accession number PRJNA884642. The codes used to perform the analyses and generate the results are available in: <https://github.com/Changhao051/Aurelia-coerulea-cell-atlas>

SUPPLEMENTAL INFORMATION

It can be found online at <https://doi.org/10.59717/j.xinn-geo.2024.100063>

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