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Day 1 - July 22, 2024, Monday

Session 1: Modeling of Development (Chair: Emma Farley)

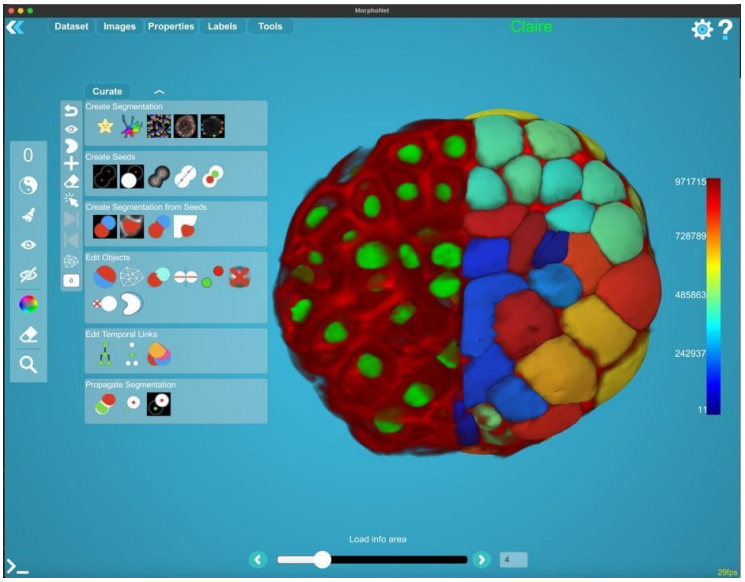
MorphoNet 2.0 : Efficient bio-curation of large 3D and 3D+t imaging datasets

Benjamin Gallean[1,2,3], Tao Laurent[1], Kilian Biasuz[2], Ange Clement[1], Noura Faraj[1], Patrick Lemaire[2] & Emmanuel Faure[1]

[1] LIRMM, Univ Montpellier, CNRS, Montpellier, France.
[2] Centre de Recherche de Biologie cellulaire de Montpellier, CRBM, Université de Montpellier, CNRS, Montpellier, France
[3]: Montpellier Resources Imagerie, Biocampus, Université de Montpellier, CNRS, INSERM, Montpellier France

We will present during this talk, a new standalone application, MorphoNet 2.0, running on all major platforms. It has been developed to provide a novel conceptual solution to a longstanding issue in the field of image analysis: the efficient and intuitive 3D and 3D + time data curation.

Progress in image acquisition led to an exponential growth of 3D fluorescence intensity images and temporal series. While efficient tools (e.g *CellPose)* have been developed to segment these massive datasets, these tools are not error-free. A major challenge is to correct these residual segmentation errors, which can strongly affect data interpretation and also limit our ability to generate highquality ground truth training sets for deep learning segmentation tools. Correcting these errors efficiently and in 3D, however, remains a major challenge limiting progress in the field.



Available software solutions, including ImageJ and Napari, offer nice interaction for 2D image annotation by clicking on a pixel field directly on the surface of the image. This is, however, extremely difficult to generalize to 3D images because they are dense cubes with a large number of superimposed voxels.

We have developed a completely new paradigm for 3D (and 3D+t) data curation. We create for each segmented object in the 3D dataset a dual surface mesh, which, coupled to our object

scattering function, allows to efficiently select the objects to be edited. The duality between the 3D segmentation image and its meshed version then allows to run the curation pipeline on the segmented voxel 3D image, followed by the computation of a new mesh for the edited segmentation. This dual concept takes advantage of the power of the visualization of and interaction with meshes, while running the image processing on the actual segmented image, with reference to the raw intensity image.

The target researcher for this application are biologists, no programming skills and very basic knowledge of bio-image processing are required to perform 3D curation.

Website: https://morphonet.org

Polarization and directionality of cell migration: enhanced navigation of embryonic micro-environments through cell force integration.

Yelena Bernadskaya[1,2], Haicen Yue[3], Calina Copos[4], Selena Gupta[1,2], Alex Mogilner[1,2], Lionel Christiaen[1,5].

[1]Department of Biology, New York University, [2]Courant Institute of Mathematics, New York University, [3]Department of Physics, University of Vermont, [4]Department of Mathematics, Northeastern University, [5]SARS Center, University of Bergen

Cell migration exists on a wide spectrum, ranging from cells undergoing individual migration to coordinated cohorts of cells travelling together and sharing information across multiple cells. The propensity for collective cell migration during development suggests that there may be advantages of cells traveling in groups. The *Ciona* cardiopharyngeal progenitors provide the simplest model of collective cell migration, with cohesive bilateral cell pairs polarized along the leader-trailer migration path while moving between the ventral epidermis and trunk endoderm. Combining computational modeling, confocal microscopy, and molecular perturbations, we identify cardiopharyngeal progenitors as the simplest cell collective maintaining supracellular polarity with differential distributions of protrusive forces, cell-matrix adhesion, and myosin-based retraction forces along the leader-trailer axis. 4D simulations and experimental observations suggest that cell-cell communication helps establish a hierarchy to align collective polarity with the direction of migration, as observed with three or more cells *in silico* and *in vivo*. Our approach reveals emerging properties of the migrating collective: cell pairs are more persistent, migrating longer distances, and with higher accuracy. Simulations suggest that cell pairs can overcome mechanical resistance of the trunk endoderm more effectively when they are polarized collectively. We propose that polarized supracellular organization of cardiopharyngeal progenitors confers emergent physical properties that determine mechanical interactions with their environment during morphogenesis.

Digital ascidian embryos: natural variation and the logical rules of animal embryogenesis

Patrick Lemaire, CRBM, Campus CNRS, 1919 route de Mende, F-34070 Montpellier, France

Ascidians are marine invertebrates which belong to the vertebrate sister group. While adult ascidians show remarkable regenerative capacities, their embryos seem to be living on a different planet: they develop without growth or apoptosis, with a quasi-invariant cell lineage, conserved since the emergence of the group around 400 MY ago (Lemaire, 2011). Ascidian genomes, however, evolve particularly rapidly.

To understand how distinct ascidian species can form very similar embryos despite the divergence of their genomes, we are combining experimental, mathematical and physical approaches (e.g., Guignard, Fiuza et al., 2020). During the talk, I will present our ongoing collaborative efforts with the Faure lab at LIRMM (Montpellier, F), the Malandain lab at INRIA, (Sophia Antipolis, F), Mani lab at Northwestern University (Evanston, USA) to quantify natural variation within and between species during ascidian embryonic development, and our expectation of what natural variation in ascidian embryonic development could tell us more generally about the logic of animal embryonic development.

Session 2: Asexual Reproduction and Regeneration (Chair: Ayelet Voskoboynik)

Chasing the bud: single-cell RNAseq atlas and chromosome-level genome of Botryllus schlosseri

Marie Lebel1, Olivier De Thier2, Sharon Rabiteau1, Tiphaine Deshayes1, Philippe Dru1, Jean-Francois Flot2, Alexandre Alie1, Stefano Tiozzo1

1CNRS, Laboratoire de Biologie du Développement de Villefranche Sur-mer (LBDV), Sorbonne Université, Paris, France.

2Evolutionary Biology \& Ecology, C.P. 160/12, Université libre de Bruxelles (ULB), Avenue F.D. Roosevelt 50, B-1050 Brussels, Belgium.

The colonial tunicate Botryllus schlosseri propagates asexually through a developmental process known as palleal budding. This process involves the continuously rebuilding of the entire body from stereotyped regions of the peribranchial epithelia and the overlying epidermis. While the anatomy of palleal budding has been extensively described, the cells involved in bud initiation and their transcriptomic signatures remain elusive. To investigate the cellular and molecular origin of palleal budding, as well as its differentiation dynamics, we generated a single-cell RNA sequencing atlas covering the entire B. schlosseri budding cycle and we provided usable haploid and diploid genomes assembled at the chromosome level.

The initial clustering analyses of seven separate and integrated developmental stages led to the identification of respectively 37 and 40 cell clusters. By means of in situ hybridization and previously published studies, we were able to identify and annotate different types of blood cells, germline, body wall and cardiac muscles, epidermal and neural cells, gut epithelia and different endostyle cells. The obtained atlas enabled us to target specific clusters, which express known budding markers such as Nk4 and Vimentin. By applying finer sub-clustering and differential expression analyses we were able to identify relatively small populations of cells putatively at the origin of the budding process. Through RNA velocity and pseudotime analyses, we are now able to infer the cell trajectories and extrapolate information on the dynamics of these cells.

The datasets obtained provide unprecedented insights into the mechanisms underlying agametic development in the model B. schlosseri and potentially offer useful tools for deciphering the fundamental principles governing budding across diverse tunicate species.

The Chordate Origins of Heart Regeneration

Keaton J. Schuster1*, and Lionel Christiaen1,2,3*

1 Center for Developmental Genetics, Department of Biology, New York University, New York, NY, USA

2 Center for Genomics and Systems Biology, Department of Biology, New York University, New York, NY, USA

3 Michael Sars Centre, University of Bergen, Bergen, Norway

*Corresponding Authors

The human heart is infamous for not healing after infarction in adults, prompting biomedical interest in species that can regenerate damaged hearts. In such animals as zebrafish and neonatal mice, cardiac repair relies on remaining heart tissue supporting cardiomyocyte proliferation. Natural de novo cardiogenesis in post-embryonic stages thus remains elusive. Here we show that the tunicate Ciona, an ascidian among the closest living relatives to the vertebrates, can survive complete chemogenetic ablation of the heart and loss of cardiac function, and recover both cardiac tissue and contractility. As in vertebrates, Ciona heart regeneration relies on Bone Morphogenetic Protein (BMP) signaling-dependent proliferation of cardiomyocytes, providing insights into the evolutionary origins of regenerative cardiogenesis in chordates. Remarkably, prospective lineage tracing by photoconversion of the fluorescent protein Kaede suggested that new cardiomyocytes can emerge from endodermal lineages in post-metamorphic animals, providing an unprecedented case of regenerative de novo cardiogenesis. Finally, while embryos cannot compensate for early losses of the cardiogenic lineage, forming heartless juveniles, developing animals gain their regenerative ability during metamorphosis, uncovering a fundamental transition between deterministic embryogenesis and a regeneration-competent or “regulative” post-embryonic development.

NEW STEM CELL NICHE FOR DEVELOPMENT AND REGENERATION IN *Botryllus schlosseri*

Virginia Vanni1,2, Federico Caicci1, Anna Peronato1, Graziano Martello1, Davide Asnicar3, Fabio Gasparini1, Federico La Torre1, Simone Domenichi1, Benyamin Rosental4, Loriano Ballarin1, Lucia Manni1

1Dipartimento di Biologia, Universitá di Padova, Padova, Italy

2Living Systems Institute, University of Exeter, Exeter, United Kingdom

3Aquatic Bioscience, Huntsman Marine Science Centre, St Andrews, New Brunswick, Canada

4Department of Microbiology, Immunology and Genetics. Regenerative Medicine and Stem Cell Research Center. Ben-Gurion University of the Negev.

Botryllus schlosseri is a colonial tunicate displaying a regular replacement of adult individuals by new generations of clonal zooids, as part of its natural life cycle and colony expansion. In adult individuals, stem cell niches have been identified, they are transient structures, cyclically replaced. We performed a high-resolution analysis, defining the anatomy of the known B. schlosseri stem cell niches for the first time, evidencing their organization as confined compartments where candidate stem cells are stored, proliferate and differentiate. B. schlosseri also displays whole-body regeneration, a process that occurs even when adult individuals, bearing the stem cell niches, are removed from the colony. Having defined the structure of previously identified stem cell niches, we were able to recognize similar organizations in the ampullae, i.e., the blind peripheral endings of the circulatory system. Through in-vivo approaches we could observe that circulatory candidate stem cells home into ampullae and participate in somatic and germline development. Such cells proliferate in the ampullae and express orthologous of the vertebrate pluripotency markers Sox2, Myc and Pou3. Finally, we demonstrated that the presence of ampullae is necessary to sustain individual's growth, further suggesting that these structures could act as stem cell reservoir in the absence of adult individuals and during regeneration. Overall, this work highlights new similarities in pluripotency control between colonial tunicates and vertebrates, and evidence of a new stem cell niche where stem and progenitor cells contributing to regeneration are stored in B. schlosseri, and possibly in other colonial ascidian with similar regeneration capabilities.

Insights into the cellular and molecular mechanisms of the highly regenerative tunicate *Polycarpa mytiligera*

Tal Gordon1,5, Tal Zaquin2, Noam Hendin3, Omri Wurtzel3, Lucia Manni4, Ayelet Voskoboynik1, Noa Shenkar5

1Institute for Stem Cell Biology and Regenerative Medicine, and Hopkins Marine Station, Stanford University School of Medicine, Stanford, California, USA

2Department of Marine Biology, The Leon H. Charney School of Marine Sciences, University of Haifa, Haifa, Israel

3School of Neurobiology, Biochemistry & Biophysics, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel

4 Department of Biology, University of Padova, 35121, Padova, Italy

5School of Zoology, George S. Wise Faculty of Life Sciences, Tel-Aviv University, Tel-Aviv 6997801, Israel

Regeneration is widespread in the animal kingdom, and a variety of model systems are employed to better understand the principles and genetic programs underlying this process. Ascidians are remarkable for their regenerative abilities, and while the majority of regenerative studies focused on well-known model species, our recent work suggested a new model: the solitary ascidian Polycarpa mytiligera.

In vivo experimental observations revealed this species extraordinary ability to regenerate all body parts following their removal, including the central nervous system (CNS).

Our current study further describes P. mytiligera's impressive regenerative potential and presents the morphological, cellular, and transcriptomic dynamics that lead to entire CNS regeneration. Our results revealed the expression of key neuro developmental markers that are not otherwise present in the adult CNS. Removal of the entire CNS resulted in high cell proliferation in the regenerated area. Transcriptome analysis revealed enhanced stem- cell related gene activity, with high expression of P53 and piRNA pathways preceding the activation of Notch, Wnt, and Nanos pathways.

Our new findings provide an in-depth characterization of P. mytiligera's regeneration process, presenting insights into the cellular and molecular aspects of CNS regeneration, further emphasizing the importance of this new model system in the study of the evolution of chordate regeneration.

Maintenance of putative stem/progenitor cells during extended experimental dormancy in a colonial tunicate

Laurel S. Hiebert*, Tal Scully, Tony De Tomaso

University of California Santa Barbara

Some organisms are able to regress into a morphologically-simple dormant state to survive extreme environmental conditions. Exiting dormancy requires the ability to regenerate the original body plan, a process referred to as whole-body regeneration (WBR). Consequently, WBR is dependent on the survival of somatic stem cells during extreme conditions, and the organisms' ability to detect and respond to environmental changes in order to protect them. Botrylloides diegensis is a colonial tunicate that consists of a group of bodies, called zooids, each with a chordate body plan (complete with heart, nervous, and digestive systems) that are connected by a common vascular network. During the induction of dormancy, the bodies die and are resorbed, leaving only a condensed vasculature congested with blood cells. Remarkably, this highly reduced animal form can survive in non-optimal conditions for months, maintaining the ability to regenerate all adult structures when conditions improve. Thus, the stem cells are protected, but also poised to initiate WBR if given the right stimulus. Here we found we could experimentally induce and terminate dormancy in the lab for extended periods simply using changes in temperature. We utilized this system to investigate the molecular mechanisms underlying the maintenance of pluripotency during dormancy. Comparative transcriptome profiling of dormant and non-dormant stages showed upregulation of pluripotency markers, such as Piwi, and genes related to regeneration, such as Notch signaling components. Single cell sequencing of circulatory cells extracted from dormant and non-dormant colonies resulted in the identification of distinct populations of putative progenitor/stem cells based on expression profiles.

Unraveling the Secrets of Chordate Whole-Body Regeneration using single-cell and ATAC sequencing

Berivan Temiz, Michael Meier and Megan J. Wilson.

Department of Anatomy, University of Otago, Dunedin, New Zealand.

Botrylloides diegensis, a marine chordate, displays a remarkable ability for whole-body regeneration (WBR) by regenerating an entire body system from its vascular network. Our research encompasses gene and pathway characterisation, transcriptome profiling, and haematological analyses during various stages of regeneration. Recently, we have used single-cell RNA sequencing (sc-RNA-seq) of mature colonies and multiple WBR stages and ATAC-sequencing to identify gene regulatory elements to gain new insights into this chordate model of WBR.

Notably, we observed the emergence of large transient cell populations exclusively during the early stages of WBR. Although lacking distinct highly expressed markers, sub-clustering revealed shared molecular signatures with committed cell clusters, suggesting orchestrated differentiation processes. We identified SoxC as a pivotal stem cell marker, exhibiting robust expression within aggregates of stem-like cells, regeneration vesicle-forming cells, and cells initiating organogenesis. Our cell trajectory analyses consistently depict a trajectory from SoxC+ cell populations through transient states towards more specialized cell lineages. Our findings collectively highlight the remarkable plasticity inherent in B. diegensis WBR.

Evolution of Cell Types in Tunicate Blood

Tal Scully1, Laurel Hiebert2, C. J. Pickett3, Henry Rodriguez Valbuena4, Nicolas A Gort-Freitas1, Anthony De Tomaso4, Bradley Davidson3, Allon M Klein1

1 Department of Systems Biology; Harvard University, Boston, MA, USA

2 Department of Ecology, Evolution, and Marine Biology; University of California Santa Barbara, Santa Barbara, CA, USA

3 Department of Biology; Swarthmore College, Swarthmore, PA, USA

4 Department of Department of Molecular, Cellular, Developmental Biology; University of California Santa Barbara, Santa Barbara, CA, USA

Novel cellular functions are a major source of evolutionary novelty, but the developmental and genetic mechanisms driving their emergence remain poorly understood. Single-cell RNA sequencing (scRNA-seq) offers an unbiased, systematic approach to study cell functional diversification at a genome-scale. It can map out developmental hierarchies and reveal how genes are redeployed to generate diversity during differentiation.

Tunicate blood represents an instructive model for studying cell type diversification. Tunicates inhabit a wide range of marine environments, display diverse lifestyles, have a rich array of blood cells, have compact genomes, and can be ethically and inexpensively collected. Because tunicates are closely related to vertebrates, knowledge regarding the genetic basis of vertebrate immune function can be used to interpret functional diversification in tunicate blood.

We collected scRNA-seq data of blood from 11 tunicate species and have begun to map out their developmental hierarchy and evolutionary diversification. Tunicate blood is more complex than vertebrate blood in its cell type composition, and scRNA-Seq shows it to be more considerably complex than was evident from morphological analysis. In Ciona robusta, we used in situ hybridization to associate classically-defined cell morphotypes with transcriptional clusters. We observe a proliferative hierarchy in the circulating blood, with clear conservation between some of the species examined. Many gene expression programs identified in vertebrates are fragmented and remixed across cell types in tunicates. We will report on our progress towards searching for mechanisms and examples of cell type diversification in this data.

The dynamics of parental allelic imbalance at single-cell resolution in hybrid prote-vertebrate lineages

Laurence Lemaire1, Michael Levine2, Chen Cao3

1Saint Louis University, 2Princeton University, 3The University of Texas at Dallas

The inheritance of both maternal and paternal copies of the zygotic genome is essential for normal animal development. Yet, we are not simply the sum of our parents' genes. Classical studies have identified a handful of examples of allelic imbalanced events including imprinted genetic loci, olfactory receptors, and X-inactivation. Allelic imbalance has been suggested to influence key developmental processes during embryogenesis, and also influence genetic predilections to disease. Emerging RNA sequencing technologies raise the possibility that conditional allelic imbalance may be prevalent in animal genomes, but the underlying mechanisms are poorly understood. Here we obtained the first high-resolution map of allelic imbalance for a complete animal embryo during development, using hybrid embryos of two divergent species of the ascidian, Ciona: C. intestinalis and C. savignyi. The resulting comprehensive single cell transcriptome lineages of hybrid embryos identified global allelic heterogeneity in single cells, which is masked by conventional bulk measurement of average gene expression across cells. Notably, we observed allelic imbalance in the migrating trunk ventral cells, which may contribute to heart cell migration. This finding provides insights into how transient regulatory states interface with the dynamic cellular processes underlying morphogenesis. Considering the close relationship between ascidians and vertebrates, uncovering the regulatory patterns of allelic imbalance in Ciona could provide valuable insights into how such imbalances contribute to inherited diseases in humans.

The integration of in situ hybridization spatial and developmental lineage information with single-cell RNA sequencing datasets

Yishen Miao, Abhinav Bharat, Bela Machado, Matthew Tang, and William C Smith

Until the advent of spatial sequencing, single-cell RNA sequencing (scRNAseq) did not preserve the spatial information of cells in the sample tissue. However, it may be possible to infer the location of a cell in the tissue if there are known differentially-expressed genes. The neural plate of the basal chordate Ciona robusta forms a clearly delineated grid during the gastrula and neurula stages. Previous studies have produced an abundance of neural plate in situ hybridization data with single cell resolution. If a link between the blastomeres and larval neurons can be established, potential new markers for a specific neuron in the larval central nervous system can be identified. In this study, we compiled all available neural plate in situ images on the Aniseed database. We used the Ciona developmental ontology to infer the blastomere identity for expression patterns lacking single-blastomere annotations. We then manually curated all records and produced marker gene matrices for late gastrula and neurula stages. We were able to map the in situ matrices to clusters in the scRNAseq dataset based on the similarity of the in situ vectors and the single-cell vectors. Finally, while the lineages of most neural plate blastomeres is known, this has not been integrated with the Ciona larval-stage connectome. To address this issue, we calculated the gene expression similarities of cells between developmental stages. We are in the process of establishing a lineage of cells from the known blastomeres on the neural plate to the larval cell to link to the connectome.

Investigating temperature stress in the ascidian *Ciona intestinalis* by single-cell genomics

Jonas Brandenburg, Chiara Castelletti, Lionel Christiaen

Michael Sars Centre, University in Bergen, Bergen, Norway

Multicellular organisms need to yield consistent developmental outcomes in the context of a variable environment. Therefore, patterns of gene expression are tightly regulated. Deviations from the optimal developmental temperature can be buffered in a certain permissive range, leading to a temporal scaling of developmental speed, but will become detrimental outside of this range. As the effect of thermal stress is often cell type-specific, investigations of the effects of such environmental perturbations need to be performed at a scale and resolution matching this non-uniform response by using single-cell genomics.

Here, we leverage a norwegian population of *Ciona intestinalis* to investigate thermal effects at the single-cell level. We generated a *de novo* genome for this population using long-read sequencing and profiled over 100.000 cells of embryos developing at 15˚C. This continuous atlas revealed the transcriptomic and cellular dynamics of *Ciona* development and was further used as a reference for the investigation of temperature-sensitive developmental dynamics in *Ciona*.

Based on a scRNA-seq dataset (10 000 cells) of *Ciona* embryos grown at various permissive temperatures (12 to 18˚C) and using cell-type abundance, developmental progression and differential gene expression, we identify muscle and notochord cells as most sensitive to temperature stress. Additional sequencing of *Ciona* embryos grown at permissive and non-permissive temperatures will enhance our insights in developmental scaling and cell type-specific responses to thermal stress in this organism.

Study the evolutionary biology of cardiovascular system in olfactores

Max Makem Pekouankouang1, Jonas Brandenburg1 and Lionel Christiaen1

1 Michael Sars Centre, University of Bergen, Bergen, Norway

Tunicates have been widely recognized as an important clade for to study the evolutionary origins of vertebrate organ systems, as well as fundamental principles of cell and developmental biology. Tunicates combine a unique phylogenetic position, and a simple and stereotyped embryogenesis, which ends with the hatching of a tadpole-like larvae typical of the chordate phylum. However, several important organ systems do not fully form and become functional before the completion of metamorphosis. These include the conserved and essential digestive, reproductive and circulatory systems. The heart, for example, acquires its blood pumping shape and ability, with contractile cardiomyocytes, at juvenile stages; and the cellular details underpinning the formation of the vasculature, its connection with the heart and the onset of circulation remain elusive, highlighting the need for further investigation. To gain comprehensive insights into the cellular and molecular underpinnings of post-embryonic development in Ciona intestinalis, we use animals collected from the Norwegian coast to obtain an extensive time series of whole animal single cell RNA-seq (scRNA-seq) covering 20 times points between the newly hatched larval stage until the maturation of young adults 50 days later. We obtained >160,000 single cell transcriptomes covering the main tissues and cell types composing essential organ systems, identifying known as well as novel markers genes. Focusing on the circulatory system, we pursue an in-depth characterization of the cellular lineages and gene regulatory networks underlying hematopoiesis, vasculo- and angiogenesis, as well as heart organogenesis. This study paves the way to further our understanding of post-embryonic development in Ciona, and the evolutionary origins of mesendodermal organ systems in chordates.

Cellular Evolution of the Chordate heart: A Tunicate Perspective

Wei Wang

Fang Zongxi Center for Marine Evo-Devo

College of Marine Life Sciences

Ocean University of China, Qingdao, China

The heart is vital for sustaining animal life and has evolved in parallel with evolution and environmental adaptation. However, a panoramic description of the cellular changes of the heart on large evolutionary scales, as well as the underlying genomic mechanisms, is currently missing. In this study, we harness the power of single-cell spatial transcriptomics and comparative genomics to provide a comprehensive understanding of the cellular profiles of adult hearts throughout chordate evolution. We demonstrate the emergence of distinct cell types as the heart anatomical structures acquire increasing complexity, starting from the simplest tubular tunicate heart, accompanied by the incorporation of novel functional and regulatory genes. Our study has provided the resources and analytical tools necessary to address the important questions concerning the origin of the multichambered heart and the underlying genetic driving force.

Session 4: Ecology (Chair: Noa Shenkar)

Effects of biological filtration by ascidians on microplastic composition in the water column

Eden Harel1, Ines Zucker2,3†, Noa Shenkar1,4†

1School of Zoology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, 69978, Israel.

2School of Mechanical Engineering, Faculty of Engineering, Tel Aviv University, Tel Aviv 69978, Israel.

3Porter School of the Environment and Earth Sciences, Faculty of Exact Sciences, Tel Aviv University, Tel Aviv 69978, Israel.

4The Steinhardt Museum of Natural History and National Research Center, Tel Aviv University, Tel Aviv, 69978, Israel.

Plastic pollution, a widespread environmental challenge, significantly impacts marine ecosystems. The degradation of plastic under environmental conditions results in the generation of microplastic (MP; <5 mm) fragments, frequently ingested by marine life, including filter-feeders such as ascidians (Chordata, Ascidiacea). These organisms are integral to benthic-pelagic coupling, in transporting MP from the water column through the marine food web.

Here, we explored the effect of filtration and digestion by the solitary ascidian Styela plicata on the composition of MP in the water column and on the sinking rates of fecal matter, focusing on differences between bioplastics (polylactic acid, PLA) and conventional plastics (polystyrene, PS). The ascidians efficiently removed 2-5 µm particles within two hours of filtration. We show that following digestion and secretion process, PS concentrations increased while PLA concentration remained stable. Some particles were egested into the water column repackaged inside fecal pellets, which significantly increased the pellets' drag force and sinking velocity. Raman spectral analysis of digested MP revealed distinct spectrum alterations due to coating by organic substances. These findings highlight the role of ascidians — and other filter-feeders— in modifying the structure of MP in their environment. Research into such modifications is crucial for understanding the MP cycle and its consequences for the marine environments.

The Regulatory Mechanisms of Ascidian Larval Settlement and Metamorphosis: Insights for Developing Novel Antifouling Strategies

Li-Kun Yang a,#, Qi-Shu Qin a, Tong-Ye Han a, Bo Dong a,b,c,*

a Fang Zongxi Centre, MoE Key Laboratory of Marine Genetics and Breeding, College of Marine Life Sciences, Ocean University of China, Qingdao 266003, China; b Laoshan Laboratory, Qingdao 266237, China; c Institute of Evolution & Marine Biodiversity, Ocean University of China, Qingdao 266003, China.

Presenting author

* Corresponding author: bodong@ouc.edu.cn

Abstract: The biofouling problem caused by adhesive ascidians is one of the most severe factors leading to substantial economic losses in shellfish aquaculture. However, the current biological approaches to prevent ascidian biofouling are not effective due to a lack of understanding of the molecular mechanisms that mediate ascidian larvae to settle on aquaculture facilities and then undergo metamorphosis to facilitate aggregation. In the present study, we focus on the chemosensory and neuroregulatory systems of ascidian larvae and attempt to delineate the significant molecular signaling pathway(s) (external cues—chemosensation--neuroregulation) involved in mediating larval settlement and metamorphosis. Parallelly, a systematic screening of active substances from marine natural products is being carried out, which aims to search for effective anti-adhesion/metamorphosis substances targeting chemosensory and neuroregulatory pathways and having the potential to be developed as antifouling agents in the future. Our present work provides the basis for elucidating the regulatory mechanisms of ascidian larval settlement-metamorphosis and offers insights into developing novel antifouling strategies for ascidian biofouling prevention in shellfish aquaculture.

Systems eco-devo: towards using high-content image-based morphometrics to quantify the impacts of changing temperatures on developmental dynamics at single cell resolution.

Chiara Castelletti, Jonas Maurice Brandenburg, Lionel Christiaen

Michael Sars Centre

University of Bergen, 5008 Bergen, Norway

Embryonic development is canalized to produce a narrow range of phenotypes despite environmental or genetic variation. Recent increases in the amplitudes of perturbations, owing to climate change, are exposing organisms to new and unpredictable environments. Marine invertebrates are directly exposed to fluctuating temperatures, which can result in significant phenotypic variation. Understanding the complexity of temperature tolerance and stress response will enable us to predict climate change's impact on developing organisms. In this study, we aim to model the correlation between temperature and developmental dynamics and provide a quantitative estimation of the impact of climate change on early life stages. Because of its highly stereotypical development, the tunicate Ciona intestinalis provides an appealing model for evaluating how environmental cues shape development. Here, we tested early embryonic development of Ciona at different temperatures. Hatching rates were used to measure fitness and determine the lower and higher temperature bounds and optimum temperature range, as well as scaling of developmental tempo within the permissive range. Temperature dependent morphological variations were assessed with single cell resolution from neurula stage onward. From each cell we extracted quantitative parameters that describe shape, size and location within the embryo. By integrating this information, we aim to create a high-resolution phenotypic map of thermal tolerance based on a quantitative description of cellular phenotypes. We hope the map will become a standard tool to track temperature dependent developmental dynamics and to identify asynchrony and susceptibility to challenging temperatures at tissue/cell resolution.

"Bend, but don't break: Styela plicata's mechanical response to wave stress"

Raz Platin1 & Noa Shenkar1,2

1 School of Zoology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel-Aviv, Israel.

2 The Steinhardt Museum of Natural History, Israel National Center for Biodiversity Studies, Tel Aviv University, Tel-Aviv, Israel

Tunicates are the only animals that produce cellulose, an important component of their tunic providing it with strength and support. Styela plicata is commonly found in sheltered habitats such as marinas and ports around the world. It is known for its resilience to environmental stressors and its ability to form substantial populations in disturbed environments. Its thick and robust tunic likely plays a significant role in providing physical defense against predators and anchoring the animal to the substrate. As an invasive species, one of the spatial barriers of S. plicata is their ability to disperse outside sheltered environments. Here we investigated the influence of water turbulence and wave action on the mechanical and chemical properties of S. plicata tunic. We collected 8-10 individuals from two adjacent sites: Akko Marina, characterized by low wave action, and "The Horse's Beach", subject to high wave action. We analyzed the cellulose percentage, plasticity, and elasticity of the tunic between individuals from both sites to determine any differences. Additionally, we are currently conducting experiments to examine the effect of four controlled water flow regimes on the tunic properties of young specimens (1-2 cm in size, n=15-20) over a two-month period. Experimental results will shed light on the physiological traits that facilitate the establishment of solitary ascidians outside sheltered marine habitats. Such insights are essential for a better understanding of the potential range expansion and ecological impact of S. plicata and similar invasive ascidian species thriving in ports worldwide.

Predicting the future with solitary ascidians- an holistic approach for experimenting global change effects on benthic fauna

Noa Shenkar*, Ahmad Ayoub, Doron Bereza, Raz Platin, Amit Unger

*shenkarn@tauex.tau.ac.il

School of Zoology, George S. Wise Faculty of Life Science, And The Steinhardt Museum of Natural History, Israel National Center for Biodiversity Studies, Tel-Aviv University, Tel Aviv 69978 Israel

Predicting the response of ascidians to global change is essential for understanding their potential impact on marine biodiversity, and for developing appropriate conservation and management planning that will promote marine environmental protection. Our overarching goal is to elucidate the combined effects of key elements of global change- increased ocean temperatures, salinity fluctuations, ocean acidification, and increased international shipping, on ascidian physiology and distribution. While some factors may promote the establishment of certain species, other factors may hinder their spread, and their interaction is often overlooked. In addition, free-swimming early life stages may differ in their ecological requirements from those of sessile adults, further complicating our ability to predict their response. The current project focus on: 1) Investigating the physiological and sub-lethal effects of key parameters of global change on both adult and early life stages of native and introduced species using a factorial exposure design; 2) Comparing the physiological responses of solitary ascidians to a cascade of changing environmental conditions during simulated voyages based on major shipping networks; and 3) Predicting future distribution of ascidians based on environmental sensitivities and vectors of transfer. Our novel approach to incorporating acquired data into spatial distribution models under global change scenarios, combined with higher order network analysis of global shipping, provide an holistic perspective that will enhance scientific understanding of the impact of global change on ascidian physiology and distribution, and promote the development of efficient tools to minimize the associated negative effects arising from unwanted competitive advantage of ascidians in changing oceans.

Day 2 - July 23, 2024, Tuesday

Session 1: Gene regulation and regulatory networks (Chairs: Bob Zeller and Bradley Davidson)

Single base-pair changes within heart enhancers dramatically increase binding affinity and disrupt heart development

Authors: Granton A Jindal, Alexis T Bantle, Joe J Solvason, Jessica L Grudzien, Agnieszka D'Antonio-Chronowska, Fabian Lim, Sophia H Le, Benjamin P Song, Michelle F Ragsac, Adam Klie, Reid O Larsen, Kelly A Frazer and Emma K Farley

Transcriptional enhancers direct precise gene expression patterns during development and harbor the majority of variants associated with phenotypic diversity, evolutionary adaptations and disease. Pinpointing which enhancer variants contribute to changes in gene expression and phenotypes is a major challenge. Here we find that suboptimal or low-affinity binding sites are necessary for precise gene expression during heart development. Strikingly, in Ciona we find that single base-pair changes can optimize the affinity of ETS binding sites, causing gain-of-function gene expression, cell migration defects and phenotypes as severe as extra beating hearts. Mouse and human developmental heart enhancers also contain low-affinity binding sites that are likely important for encoding precise gene expression patterns. Indeed, in human iPSC-derived cardiomyocytes, a single base-pair change within a GATA4 enhancer increases ETS binding affinity and causes gain-of-function enhancer activity. Our work illustrates a potential vulnerability in genomes created by the prevalent use of low affinity sites, namely that single base-pair affinity-optimizing variants within enhancers can lead to gain-of-function gene expression and changes in cellular identity and organismal level phenotypes.

An Arduino-based platform for optogenetics experiments in transgenic ascidian embryos

Robert W. Zeller

Department of Biology

San Diego State University, San Diego, CA 92182

Optogenetics is a powerful approach to manipulate and monitor biological systems with spatiotemporal precision. The method combines genetic engineering with optical techniques to allow the manipulation of light-sensitive proteins in-situ. In other systems, optogenetics has been used to study ion channels, neuronal excitability and gene expression. Here I describe a culturing platform we developed for performing optogenetics in ascidian embryos. The system is modular and consists of an Arduino-based controller coupled with an array of bright LEDs which are arranged above the dishes in which transgenic embryos are grown. The Arduino controller has the ability to time the duration of illumination and the start and stop points of the illumination. Illumination can be provided constantly, or via pulse-width modulation, where both duty cycle and period are programmable, depending on the nature of the experiment and light-sensitive molecules. The LED arrays can be swapped out for different wavelengths, depending on the protein of interest, and multiple control modules can be used to provide a variety of experimental conditions simultaneously. Here we will be report on the development and testing of the system and provide examples of using optogenetics to regulate gene expression and function in transgenic ascidian embryos.

Session 2: Adult Development, Aging, and Physiology (Chair: Lucia Manni)

Characterizing Germline Stem Cells And Their Function In Gonadal Regeneration Throughout The Asexual Cycle Of A Colonial Tunicate

Tom Levy1,2; Chiara Anselmi1,2; Katherine J. Ishizuka1,2; Erin McGeever3; Tal Gordon1,2; Angela Detweiler3; Rahul Sinha2; Benjamin F. Ohene-Gambill2; Shelly Huynh3; Maurizio Morri3; Karla J. Palmeri1,2; Yotam Voskoboynik4; Benyamin Rosental5; Virginia Vanni6; Lucia Manni6; Norma Neff3; Irving L. Weissman1,2; Ayelet Voskoboynik1,2,7

1 Hopkins Marine Station, Stanford University, Pacific Grove, CA, USA

2 Institute for Stem Cell Biology and Regenerative Medicine, Stanford University School of Medicine, Stanford, CA, USA

3 Chan Zuckerberg Biohub, San Francisco, CA, USA

4 Department of Bioinformatics and System Biology, Jacobs School of Engineering, University of California San Diego, San Diego, CA, USA

5 The Shraga Segal Department of Microbiology, Immunology, and Genetics, Faculty of Health Sciences. Center for Regenerative Medicine and Stem Cells. Ben Gurion University of the Negev, Beer Sheva, Israel

6 Dipartimento di Biologia, Università degli Studi di Padova, 35131 Padova, Italy

7 Department of Biology, Stanford University, Stanford, CA, USA

Botryllus schlosseri is a colonial tunicate which undergoes a weekly stem-cell-mediated whole body regeneration processs. While various types of stem cells regenerate different tissues, only germline stem cells (GSCs) will establish the gonads and pass their genes to the next generation via sexual reproduction. To isolate GSCs and explore their fitness, testes were dissociated from various developmental stages, cells were stained with non-species-specific stem cell markers and analyzed using flow cytometry. Based on morphological parameters, different stages of cells along the spermatogenesis process were separated. While spermatozoa and spermatid stages were easy to identify based on morphology, GSCs were impossible to tell apart. Therefore, cells were index-sorted followed by single-cell RNA sequencing using the SMART-Seq III pipeline. Analysis of the cell clusters revealed expression of homolog genes to germline cell markers, that were proved, by in-situ hybridization, to be specific to different cell types in Botryllus testes. By employing index-sorting, we could associate the transcriptome of potential GSCs with their positions on the FACS plots and sort those specific cells for functional assays. Subsequently, these cells were sorted and transplanted into reproductive colonies. A week after transplantation, the cells were tracked in-vivo and discovered within the testes of the recipients. Some of the labeled transplanted cells underwent differentiation into sperm, indicating that the transplanted cell population was enriched with GSCs. The isolation of these cells marks a pivotal step in exploring the factors that govern gonadal regeneration and how the fitness of germline stem cells changes as an animal ages.

Molecular characterization of tunicate coronal organ mechanosensory cells

Gwynna K. Fuller1, Ye-Jin Park1, Yanyan Qi1, Sriikhar Vedurupaka2, Hongjie Li1, Alberto Stolfi2, Andrew K. Groves1

1Baylor College of Medicine, Houston, TX, USA

2School of Biological Sciences, Georgia Institute of Technology, Atlanta, GA, USA

The vertebrate inner ear is a complex sensory organ that detects balance and sound information using specialized mechanosensory cells called hair cells. Hair cells possess an apical bundle made of actin-rich stereovilli and a single kinocilium that extend from the cell body. In response to mechanical stimuli, hair cells depolarize and release neurotransmitters to the afferent neurons that innervate them. While the development and function of vertebrate hair cells are well studied, the evolutionary origin of this cell type in chordates remains unknown. Tunicates, the closest living relatives to vertebrates, are sessile filter-feeders as adults and use the coronal organ, a row of mechanosensory “hair cell-like cells”, to detect particle and water flow through their oral siphons. We have begun characterizing these mechanosensory cells using single-nucleus RNA-seq (snRNA-seq) and hybridization chain reaction (HCR). From our snRNA-seq data, we have identified a putative hair cell-like cell population which expresses homologs of vertebrate hair cell-associated genes, including human deafness genes like Otof and Cdh23. Additionally, these cells express genes associated with vertebrate hair cell mechanotransduction and synaptic activity. Our preliminary results indicate that the tunicate coronal organ sensory cells are molecularly homologous to vertebrate hair cells. Our future experiments will focus on functional and developmental testing to support this conclusion.

Learning and memory in the solitary ascidian Polycarpa mytiligera

Bar Gabso, Noa Shenkar, Yossi Yovel

School of Zoology, George S. Wise Faculty of Life Sciences, Tel-Aviv University and Museum of Natural History, Tel Aviv 69978 ISRAEL

The ability to learn and remember is crucial for survival across species, influencing behaviors and evolutionary adaptations. This ability spans a wide range of organisms, including both vertebrates and invertebrates. Despite their close evolutionary relationship to vertebrates, learning and memory capabilities of adult solitary ascidians have remained unexplored. Our study examines non-associative learning, particularly habituation, in the solitary ascidian Polycarpa mytiligera.

We conducted an experiment with 18 individuals, divided into a training group that learned habituation over six days and a control group. Both groups were assessed following seven days, with long-term memory tests at one, two, and three weeks post-training.

Results show that P. mytiligera is capable of habituation, a fundamental form of learning where a response diminishes upon repeated exposure to a stimulus. Our experiments reveal that the contraction of the oral siphon habituates to the repeated delivery of a weak tactile stimulus.

Further, we establish the presence of long-term memory (LTM) in P. mytiligera, expanding the known cognitive capabilities of ascidians. This evidence opens new paths for investigating the molecular and cellular mechanisms of learning and memory in a basal chordate model system.

The demonstrated habituation and long-term memory in P. mytiligera not only promote our understanding of ascidian neurobiology but also offers a new comparative perspective for the study of learning and memory mechanisms across the animal kingdom.

A change in cis-regulatory logic underlying obligate versus facultative muscle multinucleation in chordates

Christopher J. Johnson1, Zheng Zhang2,3, Haifeng Zhang3, Renjie Shang2,3, Katarzyna M. Piekarz1, Pengpeng Bi2,3, Alberto Stolfi1

1. School of Biological Sciences, Georgia Institute of Technology, Atlanta, GA, USA

2. Department of Genetics, University of Georgia, Athens, GA, USA

3. Center for Molecular Medicine, University of Georgia

Vertebrates and tunicates are sister groups that share a common fusogenic factor, Myomaker (Mymk), that drives myoblast fusion and muscle multinucleation. Yet they are divergent in when and where they express Mymk. In vertebrates, all developing skeletal muscles express Mymk and are obligately multinucleated. In tunicates, Mymk is only expressed in post-metamorphic multinucleated muscles, but is absent from mononucleated larval muscles. In this study, we demonstrate that cis-regulatory sequence differences in the promoter region of Mymk underlie the different spatiotemporal patterns of its transcriptional activation in tunicates and vertebrates. While in vertebrates Myogenic Regulatory Factors (MRFs) like MyoD1 alone are required and sufficient for Mymk transcription in all skeletal muscles, we show that transcription of Mymk in post-metamorphic muscles of the tunicate Ciona requires the combinatorial activity of MRF/MyoD and Early B-Cell Factor (Ebf). This macroevolutionary difference appears to be encoded in cis, likely due to the presence of a putative Ebf binding site adjacent to predicted MRF binding sites in the Ciona Mymk promoter. We further discuss how Mymk and myoblast fusion might have been regulated in the last common ancestor of tunicates and vertebrates, for which we propose two models.

Spatially-resolved single-cell atlas of ascidian endostyle provides insight into the origin of vertebrate pharyngeal organs

Bo Dong*

Fang Zongxi Center for Marine EvoDevo, Ocean University of China

Qingdao, China. bodong@ouc.edu.cn

The pharyngeal endoderm, an innovation of deuterostome ancestors, contributes to pharyngeal development by influencing the patterning and differentiation of pharyngeal structures in vertebrates; however, the evolutionary origin of the pharyngeal organs in vertebrates is largely unknown. The endostyle, a distinct pharyngeal organ exclusively present in basal chordates, represents a good model for understanding pharyngeal organ origins. Using Stereo-seq and single-cell RNA-seq, we constructed the first spatially resolved single-cell atlas for the endostyle of the ascidian Styela clava. We determined the cell composition of the hemolymphoid region, which illuminates a mixed ancestral structure for the blood and lymphoid system. In addition, we discovered a cluster of hair cell-like cells in zone 3, which has transcriptomic similarity with the hair cells of the vertebrate acoustico-lateralis system. These findings reshape our understanding of the pharynx of the basal chordate and provide insights into the evolutionary origin of multiplexed pharyngeal organs.

Exploring fine-scale behavioral changes in response to stimuli in tunicates

Sissel Norland 1¤*, Oleg Tolstenkov 1¤, Ingrid Cavazos-Contreras 1, Tarek Ebida 1, Marios Chatzigeorgiou 1

1 Michael Sars centre, University of Bergen, Bergen, Norway

¤ These authors equally contributed to this work

*Presenting author: sissel.norland@uib.no

Understanding animal behavior requires quantifying their movements using image processing algorithms. Recently, machine vision and neural networks have improved animal tracking and pose estimation-based kinematic features extraction from videos. Therefore, these techniques are oriented toward the detection of animal behavior at a fine scale while overcoming the enormous time and effort of manually reviewing behavioral traits. As close relatives of vertebrates, Chordata provide valuable insights into the evolution of their nervous system, thus, understanding the function of their nervous system and behavioral repertoire provides insight into the conservation and diversity of locomotor patterns and their evolutionary locomotor origin.

We developed a pipeline using DeepLabCut (1) video tracking combined with post-tracking analyses to detect and quantify a wide range of behavioral repertoires. We extend the behavioral work on adult Oikopleura dioica (2) and larval Ciona intestinalis (3, 4) and apply it to adult Ciona intestinalis. Our overall aims are a better understanding of what constitutes “baseline” behavior in Ciona intestinalis and Oikopleura dioica and what types of behavioral changes are observed when animals are exposed to neuromodulators or mechanical stimuli. To achieve this, animals were recorded during exposure to different antagonists for neuromodulations, while responses to mechanical stimuli were explored with poking, water stream or vibrations. Our results help us to better understand how tunicates perceive and respond to diverse types of stimuli.

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Session 3: Early Embryogenesis (Chairs: Bob Zeller and Billie Swalla)

Orchestrating cell fate: Cyclin-dependent Kinase 1 (CDK1) promotes FGF receptor storage across cell division

Tran (Tracy) H. Huynh1,2, AJ K. Feldman1, Mateus B. Nascimento1, Danelle Devenport2, Brad Davidson3, Christina D. Cota1

1Department of Biology, Colby College, Waterville, ME 04901

1Department of Molecular Biology, Princeton University, Princeton, NJ 08544

3Department of Biology, Swarthmore College, Swarthmore, PA 19018

Cellular trafficking of receptors and associated membrane proteins is crucial for regulating cell signaling. It has long been thought that membrane trafficking shuts down during cell division, thus the specific mechanisms that govern membrane trafficking during mitosis remain poorly characterized. In Ciona robusta, active cyclin-dependent kinase 1 (CDK1) suppresses the degradation of Fibroblast Growth Factor receptors (FGFRs) allowing these receptors to be stored and redistributed during asymmetric pre-cardiac founder cell division (Cota et al., 2021). However, the precise cellular mechanism underlying CDK1-dependent suppression of degradation is unclear. Here we report evidence that CDK1 inhibits mitotic degradation of FGFRs via phosphorylation of the Rab7-GTPase, Rab7a, which is required for the fusion of late endosomes to lysosomes. phospho-mimetic Rab7a expression plasmids. Through genetic perturbation of a putative conserved CDK1 phosphorylation site in Rab7a in transgenic Ciona founder cells, we have found that Rab7 phosphorylation impacts both mitotic FGFR degradation and cardiopharyngeal cell (Trunk Ventral Cell, TVC) fate induction. Both CDK1 and Rab7a are highly conserved proteins whose roles in regulating mitotic entry and late endosome to lysosome transport, respectively, are preserved across species. As such, this mechanism of CDK1-dependent mitotic receptor storage that we have identified in Ciona may translate to other cell types, providing a new model for how cells carry growth factor signaling components through cell divisions.

Tug-of-war between cytoplasmic and cortical forces determines the planar shape of the ascidian 4-cell stage embryo

Silvia Caballero-Mancebo1, Daniel González1, Alex McDougall1, Hervé Turlier2, Rémi Dumollard1

1Ascidian BioCell Group, Sorbonne Université, CNRS, Laboratoire de Biologie du Développement de Villefranche-sur-mer (LBDV), CNRS UMR7009, 06230 Villefranche-sur-Mer, France.

2Center for Interdisciplinary Research in Biology (CIRB), Collège de France, CNRS, INSERM. Université PSL, 75005 Paris, France

The precise arrangement of blastomeres during the initial stages of embryogenesis is crucial to establish the blueprint for embryo morphogenesis. The geometry of these cellular configurations varies between different species and depends on physical and molecular parameters such as adhesion forces between cells, cell shape, and cell division orientation. In the ascidian 2-cell stage embryo the two mitotic spindles must be coplanar and parallel to the anterior-posterior axis to generate a square 4-cell stage embryo. Such configuration deviates from the lowest energy configuration of 4 packed cells, which takes the shape of a tetrahedron. Here, we use the Phallusia mammillata model system to characterize the molecular and geometrical cues underpinning the stereotyped positioning of the two spindles at the 2-cell stage that generate a square-shaped 4-cell stage ascidian embryo.

3D analysis of the migration of centrosomes at the 2-cell stage reveals that initially centrosomes are not mirrored between blastomeres but become progressively parallel to the anterior-posterior axis and co-planar during interphase due to pulling forces exerted on astral and centrosomal microtubules. By using a combination of live imaging and mechanochemical manipulations we show that cortical pulling is dispensable for spindle positioning even though cell shape has a critical role in spindle orientation. Our observations suggest that cytoplasmic pulling regulates centrosome and spindle positioning. We are currently analyzing how cell shape can instruct spindle positioning via coupling of cortical and cytoplasmic pulling forces to determine the stereotypical shape of the 4-cell stage ascidian embryo.

Signaling Mechanisms Orchestrating Asymmetric Divisions in the Ciona robusta b6.5 Neuromesodermal Lineage

Cathryn Haas*, Michael Veeman

Division of Biology

Kansas State University Manhattan, KS 66502

The Ciona b6.5 lineage gives rise to posterior dorsal neural tube, tail tip secondary muscle and dorsal midline tail epidermis (DTME). Unlike most Ciona lineages, there are major gaps in understanding the progression of cell fate decisions in the descendants of b6.5. This lineage is of particular interest, however, because of potential homology to the NeuroMesodermal Progenitors (NMPs) that give rise to posterior spinal cord and paraxial mesoderm in vertebrate embryos. Our lab's scRNAseq atlas identified distinct gene expression patterns at mid-gastrula between the b6.5 descendants b8.18/20 (DMTE), b8.19 (neural) and b8.17 (neuromesodermal). We have used an MsxB lineage-specific reporter plasmid to confirm that b8.17 does contribute to both tail tip muscle and cells that are morphologically contiguous with the posterior neural tube. In doing so, we found that b8.19 divides to give two neural daughters of similar size whereas b8.17 divides asymmetrically to give a fate-restricted muscle precursor (b9.34) that is much larger than its sibling. The previous cell cycle in which epidermal vs neural/neuromesodermal fates are segregated exhibits a different mode of asymmetry involving the differential segregation of mitochondria without major differences in sibling cell volume. Our data points towards both FGF and Wnt signaling being involved in Ciona b-line neuromesodermal cell fate specification. Our findings highlight key similarities and differences between posterior neuromesodermal cell types in tunicates and vertebrates.

Deciphering the biophysical principles of invariant cleavage pattern in a compact-type blastula

Authors: Daniel Gonzalez Suarez1, Alex McDougall2

Author's affiliation:

1Laboratoire de Biologie du Développement de Villefranche – Sorbonne University

2Laboratoire de Biologie du Développement de Villefranche – CNRS

Abstract:

In holoblastic embryos, two main blastula's shapes can be distinguished: compact or hollow blastula. It is interesting to note that embryos that display an invariant cleavage pattern display compact-type embryos (nematodes, spiralians, ascidians) which is not the case of hollow-type embryos (jellyfish, sea urchin, etc.). The structure that makes these two types different is the blastocoel. The blastocoel seems to form mechanically with cell divisions in many of species. Compact blastulae tend to show an invariant cleavage pattern, meaning the division pattern is stereotypical. On the contrary, hollow blastulae show high variability in division patterns.

My goal is to find the biomechanical and cell division features that lead to the emergence of a compact blastula. I will use the ascidian Phallusia mammillata, which forms a compact blastula, to study the features that prevent blastocoel formation. A small number of features associated with early ascidian developmental may be linked with those embryos displaying a compact-type blastula, such as cell cycle asynchrony or cell deformation.

A loss of one of these features in the ascidian embryo leads to the formation of a variable cleavage pattern and the emergence of a hollow blastula. I also intend to analyze an embryo that forms a hollow blastula naturally, and for this I will use the jellyfish Clytia hemisphaerica embryos. One of my goals is to try to determine what mechanisms lead to the emergence of either the hollow-type embryo or the compact-type embryo, and how these types affect the variability of cell patterning.

Cell cycle regulation by Myc and the Cdk inhibitor in the Ciona embryo

Ryuji Ashita, Manami Ariyoshi, Hatsuki Doi, Shigeki Fujiwara

Department of Chemistry and Biotechnology, Kochi University, Kochi 780-8520, Japan

Possible involvement of Myc and the Cdk inhibitor in strict regulation of the number of cell divisions during Ciona robusta embryogenesis was assessed. The muscle and notochord-lineage cells exit the cell cycle before neurulation. Forced expression of Myc in these lineages produced smaller cells, suggesting extra cell division(s). These cells were labeled with EdU during neurulation. Disruption of the Cdk inhibitor, Cdkn1.b, by the CRISPR/Cas9 system caused supernumerary notochord cells. RNAi inhibition of Cdkn1.b did not increase the number of cells in the notochord and muscle. However, forced expression of Myc, in combination with RNAi inhibition of Cdkn1.b resulted in smaller muscle cells. These results suggest that the expression and/or function of Myc should be suppressed for cell cycle exit in these tissues. In addition, Myc may contribute to repress Cdkn1.b.

Inhibition of Cdkn1.b by RNAi or morpholino oligos caused impaired convergent extension of the notochord. Forced expression of Myc also perturbed the convergent extension of the notochord. Forced expression of a nuclear localization signal-containing Cdkn1.b in the notochord reduced the number of notochord cells, without affecting convergent extension. In contrast, expression of a nuclear exporting signal-containing Cdkn1.b resulted in abnormal convergent extension, without disturbing cell division. A mutant form of Cdkn1.b, containing point mutations in the Cyclin-binding and Cdk-binding motifs, neither affected the cell division nor morphogenesis in the notochord. These results suggest that nuclear Cdkn1.b contributes to the cell cycle exit, while cytoplasmic Cdkn1.b is involved in morphogenetic movement of the notochord.

Molecular and functional analysis of sperm protein s-Themis in self-incompatibility.

Takako Saito 1, Kana Hanazaki 1, Keita Kinjo1, Noritaka Hashii 2, Kogiku Shiba 3, Kazuo Inaba 3, Hitoshi Sawada 4

1, Department of Agriculture, Graduate School of Integrated Science and Technology, Shizuoka University, Japan.

2, Division of Biological Chemistry and Biologicals, National Institute of Health Sciences, Japan

3, Shimoda Marine Research Center, University of Tsukuba, Japan

4, Graduate School of Sciences, Nagoya University, Japan

Fertilization is a fundamental biological event in living organisms to generate new life with a mixed genetic background. To achieve successful fertilization, sperm and egg undergo a complex sequence of processes. Ascidians are hermaphrodites and inbreeding depression is a serious problem. Therefore, *C. intestinalis* type A possess a self-incompatibility (SI) system to avoid self-fertilization. We identified the genes responsible for SI: sperm-side polycystin 1-like receptors *s-Themis-A/B/B2* and egg-side fibrinogen-like ligands on the vitelline coat (VC) *v-Themis-A/B/B2*. Since *s/v-Themis* genes are polymorphic, their genetic variability could control identification. In fact, fertilization is blocked when SI allelic gene pairs are matched. After self/nonself-recognition on the VC, only self-recognized sperm are rejected because self-recognition triggers an increase in intracellular Ca2+ of sperm, which causes sperm quiescence.

Although the sperm side *s-Themis* genes are expressed in the testis, these proteins were not detected in the sperm extract by mass spectrometry because of the feature and characteristics of s-Themis proteins. We therefore produced specific antibodies and demonstrated localization analysis. Finally s-Themis proteins are detected by LC/MS/MS following immunoprecipitation. Immunocytochemistry indicated that s-Themis proteins are localized to the tip of sperm head, the region surrounding the mitochondria and flagellum. s-Themis-B and -B2 are cation channel proteins and Ca2+ plays a crucial role in the regulation of flagellar motility. Our current research focuses on how s-Themis-B/B2 contribute to the increase in intracellular Ca2+, which triggers self-recognition.

Session 4: Late embryogenesis (Chair: Alberto Stolfi)

Stress granule-related genes during embryogenesis of an invertebrate chordate

Laura Drago1, Alessandro Pennati2, Ute Rothbächer2, Ryuji Ashita3, Seika Hashimoto3, Ryota Saito3, Shigeki Fujiwara3, Loriano Ballarin1

1 Department of Biology, University of Padova, Via Ugo Bassi 58/B, 35131, Padova, Italy

2 Institute of Zoology, University of Innsbruck, Technikerstrasse 25, 6020 Innsbruck, Austria

3 Department of Chemistry and Biotechnology, University of Kochi, 2 Chome-5-1 Akebonocho, Kochi, 780-8072, Japan

Control of global protein synthesis through the assembly of stress granules (SGs) represents a strategy adopted by eukaryotic cells to face various stress conditions. TIAR, TTP and G3BP are key components of SGs, allowing the regulation of mRNA stability and thus controlling not only stress responses but also cell proliferation and differentiation. In the present work we aimed to investigate the role of tiar, ttp and g3bp during embryogenesis of the solitary ascidian Ciona robusta, both in physiological and stress conditions. We carried out CRISPR/Cas9 knockout to evaluate the effects on normal embryonic development, and gene reporter assays to study time and tissue specificity of gene expression, together with whole mount ISH and qRT-PCR. To induce acute stress conditions, we used iron and cadmium as “essential” and “non-essential” metals, respectively. Our results highlight, for the first time, the importance of tiar, ttp and g3bp in the control of development of mesendodermal tissue derivatives during embryogenesis of an invertebrate chordate.

Developmental buffering under thermal stress: How development can be altered by maternal environment?

Atsuko Sato*

Department of Biology, Ochanomizu University

Otsuka, Bunkyo-ku, 112-8610 Japan.

Graduate School of Life Sciences, Tohoku University

6-3, Aramaki Aza Aoba, Aoba-ku, Sendai 980-8578, Japan

Organismal development is robust under fluctuating environmental stress by developmental buffering. Sister species of the sea squirt Ciona robusta (type A) and Ciona intestinalis (type B) are adapted to different sea water temperature, therefore provide an excellent model to study molecular basis of developmental buffering under thermal stress. My previous study showed that developmental buffering level is maternally inherited. Using this as a criteria, I have investigated molecular basis of developmental buffering. However, important questions still remain: (a) How buffering level can be modified and (b) which developmental network can be affected by environmental stress. In order to address these questions, conducted transcriptome analyses of multiple single eggs from crosses using eggs of the different species to compare the effects of maternal thermal stress on heterogeneity in egg provisioning, and followed the effects across generations. In contrast to 'bet-hedging' hypothesis, we found overall decreases of heterogeneity of egg maternal mRNAs associated with maternal thermal stress in the F1 generation. By examining individual genes, we found no consistent overall effect of thermal stress on heterogeneity of expression in genes involved in developmental buffering, suggesting that developmental buffering level is determined by the genotype but not solely by maternal environment. On the other hand, heterogeneity of expression in signaling molecules was directly affected by thermal stress. Further investigation in alteration of signaling pathways by maternal environment will shed new lights into the evolution of chordate body plan.

Characterizing biomechanics in a developing chordate nervous system

Andreas Midlang*, Carl Jones, Marios Chatzigeorgiou

Michael Sars Centre, University of Bergen, Norway

Nervous systems across the animal kingdom are characterized by remarkable diversity in form and function. Recognizing the crucial role of mechanics and mechanobiology in embryonic development, our research focuses on delineating the contribution of mechanical forces to nervous system morphogenesis during embryonic development. Ciona intestinalis, serves as our model organism for this mechano-developmental study.

Leveraging Genetically Encoded Tension Sensors (GETSs) we map in vivo the mechanical tension changes that occur throughout nervous system development at Focal Adhesions (FAs) and the Nuclear Envelope (NE). In addition, we use subcellular transgenic reporters to perform brain-wide cellular morphodynamics profiling and correlate these to our GETSs data. Using pharmacological perturbations, we perform loss of function studies to investigate the importance of the cytoskeleton, mechanosensitive channels and Hippo signaling in the context of mechanical regulation of nervous system development.

We report that anatomically and functionally distinct regions of the nervous system exhibit disparate patterns of mechanical forces at FAs and NEs across development. In addition, we report the presence of mechanical tension gradients along the anterior-posterior axis of the brain. These findings suggest that mechanochemical cues coregulate patterning during nervous system morphogenesis. Cellular morphodynamic analysis reveal that even a relatively simple nervous system can exhibit a surprising complexity of morphotype. Pharmacological perturbations indicate a tight coupling between cellular morphodynamics and mechanical forces.

This study advances our understanding of chordate nervous system development and opens new research lines into the biomechanical regulation of embryonic development.

Cell cycle-driven transcriptome maturation confers multilineage competence to cardiopharyngeal progenitors

Ariel Kuan2,*, Yelena Bernadskaya2,*, Andreas Tjärnberg2*, Jonas Brandenburg1, Ping Zheng3, Keira Wiechecki2, Nicole Kaplan2, Margaux Failla1,2, Basile Gravez2, Maria Bikou2, Oliver Madilian2, Wei Wang2,3,6,*, and Lionel Christiaen1,2,6,$

1 Michael Sars Centre, University of Bergen, Bergen, Norway

2 Department of Biology, New York University, New York, NY, USA

3 Fang Centre, Ocean University of China, Qingdao, China

6 Corresponding authors: Lionel.Christiaen@uib.no (L.C.); ww8898@ouc.edu.cn (W.W.)

* These authors contributed equally to this manuscript

$ presenting author

During embryonic development, dozens to hundreds of distinct cell types emerge through divisions of pluripotent stem cells and multipotent progenitors whose fates are progressively canalized toward defined cell identities. In vertebrates and tunicates, the cardiopharyngeal lineage produces a variety of heart cells and non-cardiac muscles through a stereotyped series of cell divisions and fate decisions. Specifically, multipotent cardiopharyngeal progenitors, aka trunk ventral cells (TVCs), emerge from Mesp+ naive mesodermal progenitors and migrate as polarized pairs of cells before dividing asymmetrically along the mediolateral axis to produce small medial first heart precursors and large lateral Tbx1/10+ second cardiopharyngeal progenitors (aka STVCs). Here, we combined perturbations of cell cycle progression, multiplexed single cell RNA sequencing, CRISPR/Cas9-mediated mutagenesis, quantitative image analysis and reporter gene expression assays to (1) characterize transcriptome maturation in multipotent cardiopharyngeal progenitors; (2) study the role of the "mature state" for subsequent oriented divisions and Tbx1/10 activation, and (3) uncover the role of feed-forward circuits and cell cycle progression in controlling the acquisition of multilineage competence in cardiopharyngeal progenitors.

Day 4 - July 25, 2024, Thursday

Session 1: Cell biology (Chair: Bradley Davidson)

Dynamic separation of cell-cell contacts induces RhoA and Myosin activity to drive zippering and neural tube closure in a simple chordate

Hidehiko Hashimoto1, Takeo Horie1, Edwin Munro 2

1 Graduate School of Frontier Biosciences, Osaka University, Japan

2 Department of Molecular Genetics and Cell Biology, University of Chicago, USA

Dynamic patterns of force production at cell-cell contacts control multicellular movements in many types of morphogenesis. These patterns are governed by mechanochemical feedback between neighboring cells, but how cells interact to produce dynamic patterns of force production remain poorly understood. We are addressing this question in the context of zippering and neural tube closure in the basal chordate, Ciona robusta. We previously showed that zippering and neural tube closure are driven by RhoA-dependent activation of Myosin II along the neural/epidermal (Ne/Epi) boundary just ahead of the advancing zipper. Here we show that local activation of RhoA and Myosin II is governed by local separation of junctional contacts. During normal zippering, local accumulation of RhoA and Myosin II activity correlates with local separation of Ne/Epi contacts at vertices where more than 3 junctions meet. Acute treatment with EGTA to reduce calcium-dependent adhesion, or direct application of force, induces local separation of cell-cell contacts along Ne/Epi boundary. These separations initiate at vertices, and they are followed by rapid local accumulation of active RhoA and Myosin II. Both adherens and tight junction components are reduced along the Ne/Epi contacts, suggesting that these contacts are primed to separate under force. Our results suggest that three factors synergize to control local separation of Ne/Epi contacts ahead of the advancing zipper: (1) reduced adhesion along Ne/Epi contacts, (2) contractile forces acting along junctions perpendicular to Ne/Epi contacts to pull vertices apart and (3) forces generated by RhoA/Myosin II activity on separated contacts just ahead of the zipper. We propose that these factors constitute a novel form of mechanochemical feedback that couples force generation and separation along to Ne/Epi boundary to drive zipper progression.

Ciona Embryo Exhibits Axial Rotation Regulating Two Components, Twisting and Bending

Yuki S. Kogure*1; Satoru Okuda*2; Kotaro Oka*1,3; Kohji Hotta*1

*1 Department of Biosciences and Informatics, Faculty of Science and Technology, Keio University

*2 Nano Life Science Institute, Kanazawa University

*3 School of Frontier Engineering, Kitasato University

In some chordates, there is a high similarity to morphology and genetic profile during organogenesis period. One of them is a dynamic body-shape change called “axial rotation.” For example, mouse and rat embryos initially form a dorsally curved U-shape and rotate their axis 180 degrees with twisting to become a ventrally curved shape (Fujinaga et al., 1995; Faisst et al., 2002). Axial rotation is also conserved at least some birds and reptiles exhibited 90-degree rotation (reviewed in Poelmann et al., 1987). Despite its significance, its detailed mechanisms are still unknown. In recent studies, in vitro synthetic mouse embryos (Amadei et al., 2022; Tarazi et al., 2022) exhibited lethality before axial rotation (corresponding to E8.5), elucidating its mechanism has become increasingly important.

We discovered ascidian Ciona intestinalis type A also exhibits axial rotation. Its tail twisted and changed tail bending orientation from ventral to dorsal through lateral. To quantify the twisting and bending factors separately, we created the 2D surface map along the longitudinal axis and measured the twisting angle using epidermal cell lines at each notochord cell position. The angle increased clockwise during the 15–18 hpf tailbud stages. The twisting orientation and angle were maintained even if the trunk and tail tip were removed from embryos.

In Ciona, it is known that the dechorionation disturbs the left-sided Nodal expression and changes to bilateral and randomizes some organs’ left-right asymmetry (Yoshida and Saiga, 2008; Kourakis et al., 2021). Interestingly, dechorionation did not affect clockwise twisting but the Nodal-signaling inhibitor SB431542 randomized twisting orientation. In contrast, bending orientation was randomized in both conditions. These findings imply that axial rotation consists of two components, twisting and bending, likely regulated by different Nodal-signaling pathways. We also explored upstream regulators of these pathways. Our study will help to understand the mechanism of the evolutionary conserved early morphogenic event among chordates dissecting at a trans-scale level.

Lhx3/4 initiates a cardiopharyngeal-specific transcriptional program in response to widespread FGF signaling

C. J. Pickett1, Hannah N. Gruner1, Bradley Davidson1

1 Department of Biology, Swarthmore College, Swarthmore, PA, USA

Individual signaling pathways, such as fibroblast growth factors (FGFs), can regulate numerous inductive events. According to current paradigms, signal-dependent transcription factors, such as FGF/MapK-activated Ets family factors, partner with lineage-determining factors to achieve regulatory specificity. However, many aspects of this model have not been rigorously investigated. One key question relates to whether lineage-determining factors dictate lineage-specific responses to inductive signals or facilitate these responses in collaboration with other inputs. We utilize Ciona robusta to investigate mechanisms generating lineage-specific induction. Previous studies in C. robusta have shown that cardiopharyngeal progenitor cells are specified through the combined activity of FGF-activated Ets1/2.b and an inferred ATTA-binding transcriptional cofactor. Here we show that the homeobox transcription factor Lhx3/4 serves as the lineage-determining transcription factor that dictates cardiopharyngeal-specific transcription in response to pleiotropic FGF signaling. Targeted knockdown of Lhx3/4 leads to loss of cardiopharyngeal gene expression. Strikingly, ectopic expression of Lhx3/4 in a neuroectodermal lineage subject to FGF-dependent specification leads to ectopic cardiopharyngeal gene expression in this lineage. Furthermore, ectopic Lhx3/4 expression disrupts neural plate morphogenesis, generating aberrant cell behaviors associated with execution of incompatible morphogenetic programs. Based on these findings we propose that combinatorial regulation by signal-dependent and lineage-determinant factors represents a generalizable, previously uncategorized regulatory subcircuit we term cofactor-dependent induction. Integration of this subcircuit into theoretical models will facilitate accurate predictions regarding the impact of gene regulatory network rewiring on evolutionary diversification and disease ontogeny.

Geometry establishment in ascidian embryos: dissecting the cell-cell contact network topology critical for embryo shape

Carolina Camelo, Carl-Philipp Heisenberg

Institute of Science and Technology Austria (ISTA), Am Campus 1, 3400 Klosterneuburg, Austria

carolina.camelo@ist.ac.at

Embryo shape is achieved through orchestrated cell behaviours, giving rise to correctly sized cells and tissues whose shapes are tightly linked to cell fate and function. Understanding how embryos take shape is thus essential to uncover the key design principles of development. Although, biochemical and biophysical inputs, such as cell-cell adhesion, spindle positioning and tissue tension, have been proposed to determine embryo shape, how the dynamic interplay of these cues regulates the emergence of cell-cell contact patterns giving rise to embryonic shapes, is only poorly understood.

To understand how the topology of cell-cell contact networks determines embryo shape, we characterized how cell-cell contacts are formed in cleavage-stage Phallusia mammillata embryos. We showed that adherens, tight and gap junctions are present in early-stage Phallusia embryos. Concomitantly, Cadherins, as well as the scaffold protein Scribble, p-myosin and actin are enriched at cell-cell contacts already in 2 cell-stage embryos. These results suggest that cell-cell contacts might be functional early-stage Phallusia embryos.

Moreover, we developed an approach to reconstitute embryonic shapes by re-establishing Ca2+-dependent cell-cell adhesion in previously dissociated embryos. Using this system, we analyzed whether there is an adhesion-specific code at cleavage-stage embryos and how embryo geometries arise from previously dissociated blastomeres.

This work provides initial insights into the process by which adhesion complexes and the actomyosin cortex are built up at cell-cell contacts, thereby setting the stage for a comprehensive analysis of the role of cell-cell contact networks in defining the ascidian cleavage pattern and, thus, embryo shape.

Understanding Evolution and Development of Aquatic Organismal Transparency

Kohji Hotta*, Shunsuke O. Miyasaka1, Kotaro Oka1,2 and Takumi T. Shito1

1 Department of Bioscience and Informatics, Faculty of Science and Technology, Keio University, Yokohama, 223-8522, Japan

2 School of Frontier Engineering, Kitasato University, Sagamihara, Japan

Organismal transparency is an ecologically important trait that can provide camouflage advantages to diverse organisms. Such transparent organisms are quite common—especially in oceans. Transparency requires low absorption and scattering of light in organisms at multi-scale levels. However, it is still not fully understood how such organisms achieve these requirements. Understanding this process requires multiple approaches from various fields and methods. Here, we offer recent insights on this topic from the viewpoints of evolution, developmental cell biology, and methods to evaluate transparency. We further suggest that tunicates are an ideal model animal for studying in vivo organismal transparency.

Actomyosin contractility drives the redistribution of molecules for tubulogenesis

Yuji Mizotani*, Edwin Munro

Department of Molecular Genetics and Cell Biology

University of Chicago, Chicago, IL 60637

De novo lumen formation during tubulogenesis requires the redistribution of molecules required for apical domain specification and lumen growth. But how mechanical forces contribute to these processes is not clearly understood. Ascidian notochord provides simple model of tubulogenesis in which apical lumens initiate at cell-cell contacts, then grow and fuse to form a single tube. Here, we combine chemical and genetical manipulations to identify distinct roles for basal and lateral actomyosin in redistributing molecules that underlie lumen initiation and growth. We show that during apical polarization, lateral actomyosin is required to concentrate apical determinants PAR3, PAR6, and aPKC, at the center of cell-cell contacts to initiate lumen growth. Basal contractility is then required for overall lumen growth, while lateral contractility restricts lumen opening along the cell-cell contacts. Using 3D multispectral live imaging, we identify a basal contractility cycle in which an equatorial actomyosin ring periodically assembles on the basal surface, contracts and moves inward through the cytoplasm to the apical lumen surface. Strikingly, this contractility cycle drives the detachment and basal-to-apical translocation of basal membranes. Selective inhibition of basal myosin activity blocks the periodic inward movements of actomyosin rings, the associated movements of basal membranes, and lumen growth. We propose that this basal contractility cycle represents a novel mechanism for translocation of material for lumen growth. Together these findings reveal new mechanistic insights into how basal and lateral contractility can synergize to direct large-scale redistribution of molecules required for lumen initiation and growth.

Session 2: Metamorphosis and post-metamorphic juvenile development (Chairs: Bradley Davidson and Billie Swalla)

Neck cell behaviors and differentiation controlled by Pax2/5/8, Phox2 and FGF signaling.

Eduardo D. Gigante and Alberto Stolfi

School of Biological Sciences, College of Sciences

Georgia Institute of Technology, Atlanta, GA 30332

During metamorphosis, the transition between larval and adult phases, larval neurons are largely replaced by adult-specific ones. This is thought to require the establishment of quiescent neural progenitors during the larval phase. The regulatory mechanisms underlying this neural replacement remain largely unknown. Using tissue-specific CRISPR/Cas9-mediated mutagenesis in the tunicate species Ciona robusta, we show that orthologs of conserved hindbrain and branchiomeric neuron regulatory factors Pax2/5/8 and Phox2 are required to specify the “neck”, a compartment of cells set aside in the larva to give rise to cranial motor neuron-like neurons in the adult. Surprisingly, we find that neck-derived adult ciliomotor neurons begin to differentiate in the larva, contrary to the long-held assumption that the adult nervous system is formed only after settlement and the death of larval neurons during metamorphosis. Moreover, we find evidence for complex functions for Phox2-mediated regulation of cell proliferation and neuronal differentiation. Finally, we show that manipulating FGF signaling during the larval phase alters the patterning of the neck and its derivatives. Suppression of FGF converts Neck cells into larval neurons that fail to survive metamorphosis, while prolonged FGF signaling promotes an adult neural stem cell-like fate instead. Taken together, we provide the first insight into Neck-specific gene regulatory networks and unique cell behaviors not yet characterized. This work is supported by NIH grants R01GM143326 and F32GM150234.

Complex transkingdom interactions and host specificity underlie gut immune homeostasis in *Ciona robusta*.

Dishaw, L.J.[1*], O. Natarajan[1], S. Gibboney[1], and A. Liberti[2]

[1] University of South Florida, Morsani College of Medicine Pediatrics, Children's Research Institute, Saint Petersburg, Florida, 33701 USA.

[2] Biology and Evolution of Marine Organisms (BEOM), Stazione Zoologica Anton Dohrn, 80122 Naples, Italy

*correspondence, ldishaw@usf.edu

The fundamental processes that govern the establishment and stabilization of gut microbiomes possess long phylogenetic histories involving conserved ecological principles interwoven into the functionality of epithelial barriers and innate immunity. As a more basic chordate system that lacks adaptive immunity and can be reared germ-free (GF), *Ciona robusta* (formerly *C. intestinalis* subtype A) offers a unique and intriguing opportunity to study host and environmental interfaces under controlled conditions using animals that develop rapidly into thousands of transparent juveniles with exceptionally prominent guts. To facilitate our studies on how the immune system helps legislate transkingdom interactions, we have characterized structural, genetic, and functional properties of the bi-functional V-type Ig chitin-binding proteins (VCBPs), which are abundantly secreted into the gut lumen of *Ciona* and other early chordates. These secreted effectors can become tethered to chitin-rich mucus of the gastrointestinal compartments, bind, and opsonize bacteria. They can also bind fungi and their spores via their C-terminus chitin-binding domain. Preliminary co-culture data suggests that VCBPs can shape interactions between bacteria and fungi, thus impacting local ecologies. Diverse selective forces can drive bacterial strain variation, and this includes binding by secreted host effectors. In addition, the presence of integrated prophages in bacterial genomes of the gut microbiome can influence bacterial behaviors and drive strain variation; these variants can be recognized by the host in a process that demonstrates a surprising specificity, impacting the outcome of gut colonization. Thus, the *Ciona* gut continues to surprise with its seemingly simple organization yet emergent complexity. It can model how the structure of mucosal surfaces influences susceptibility and onset of DSS-induced colitis, revealing novel insight into how early chordates maintain gut-immune homeostasis with streamlined defense mechanisms.

Thyroid hormones conserved role in timing of Ciona metamorphosis

Andrea Mariossi1,2,#, Michael S. Levine1,2,#

1 Lewis-Sigler Institute for Integrative Genomics, Princeton University, Princeton, NJ, USA

2 Department of Molecular Biology, Princeton University, Princeton, NJ, USA

correspondence: mariossi@princeton.edu, msl2@princeton.edu

The timing of metamorphosis is a central life history trait influenced by a complex interplay of developmental progression, environmental cues, and coordination between multiple pathways. In anurans, thyroid hormones (THs) orchestrate this process, acting as the primary morphogen. Here, we leverage the model chordate, Ciona, to dissect the evolutionary underpinnings of TH signaling. Like vertebrates, Ciona has thyroid pathway components and expression profiles which are regulated by the thyroid hormone receptor (TR). We observed changes in the expression of enzymes responsible for TH degradation and activation, leading to their accumulation and peak levels within the larval endoderm, mesenchyme, and muscles immediately preceding metamorphosis. Most importantly, exogenous TH signaling is sufficient to trigger and accelerate metamorphosis. Photoreceptor-intrinsic control of TR levels and activity in the sensory vesicle appears to be a key initiator of metamorphosis. We discuss the possibility of an ancient role of TR in photoreceptor differentiation and metamorphosis timing, suggesting a mechanism by which Ciona larvae adapt to twilight conditions coupled with TH-dependent organ remodeling. This study further establishes Ciona as a valuable model for metamorphosis research, shedding light on its evolutionary significance.

Transition of photoreceptor opsins during life cycle of Ciona

Takehiro G. Kusakabe1*, Xin Zeng2,3, Fuki Gyoja1, Daisuke Shimizu1, Ayana Maruo1, Nanako Okawa1,4, Kaori Den1, Tatsuki Mizojiri1, Yutaka Suzuki3, Keiichi Kojima5, Takahiro Yamashita6, Yoshinori Shichida6, Kenta Nakai2

1. Institute for Integrative Neurobiology, Department of Biology, Konan University, Kobe 658-8501, Japan

2. Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan

3. Graduate School of Frontier Sciences, The University of Tokyo, Kashiwa 277-8568, Japan

4. Graduate School of Frontier Biosciences, Osaka University, Suita 565-0871, Japan

5. School of Pharmaceutical Science, Okayama University, Okayama 700-8530, Japan

6. Graduate School of Science, Kyoto University, Kyoto 606-8502, Japan

The life history of ascidians consists of distinct larval and adult stages. The adult ascidians are sessile animals, bearing an extremely modified version of the chordate body plan with a simple nervous system. In contrast, the body plan of the free-swimming, tadpole-like larvae shares basic features with the body plan of vertebrates. Swimming behavior of the tadpole larva is controlled by photoreception and gravity sensing of the ocellus and otolith, respectively. The ocellus contains ciliary photoreceptor cells, similar to those of the retina and the pineal organ of vertebrates. The ocellus photoreceptor cells in Ciona larvae uses a vertebrate-type visual pigment opsin (Ci-opsin1) as the photoreceptor molecule. Three types of light-responsive behaviors have been described in the adult ascidians: siphon retraction, phototropism and gamete release. To address molecular and cellular mechanisms of photoreception in adult ascidians, we investigated opsin gene expression in the neural complex of Ciona intestinalis type A (Ciona robusta) by spatial transcriptomic analysis. Ci-opsin2, a paralog of Ci-opsin1, was specifically expressed in the cerebral ganglion. Absorption spectrum of Ci-opsin2 was blue-shifted compared with that of Ci-opsin1. In fish, the absorption wavelength of rhodopsin is generally shifted toward shorter wavelengths in species that live in deeper water. While ascidian larvae respond to light in the layer near the sea surface, adults stick to the seafloor and live deeper habitats than larvae. Thus Ci-opsin1 and Ci-opsin2 may have evolved to adapt to the environment that inhabit larval and adult ascidians, respectively.

Tunicate larval adhesion: cells, species differences and analyses of their secretions

Ute Rothbächer*

Department of Zoology and Center for Molecular Biosciences Innsbruck (CMBI)

University Innsbruck, Austria

Ascidian larvae attach via their sensory adhesive papillae for both, substrate and habitat selection towards metamorphosis and permanent settlement of the adult. We recently described in detail the different papillar cell types in Ciona, notably the adhesive producing collocytes. In collaboration, we showed how papillar cell types are specified developmentally and also how inner and outer glue secretive cell types arise. We have furthermore shown their substrate preferences on defined chemistries and selected common features of the adhesive secretions between species. More data on the adhesive producing cells will be presented and on their underwater glues that remain poorly described. Notably, several differences exist between species, such as in Phallusia. High resolution imaging (HPF-TEM) and molecular analyses are presented to understand the papillar composition and functioning of adhesive cells. For Phallusia mammillata we have, furthermore, generated a long-read genome assembly that better resolves the entity of very long proteins often found in extracellular secretions.

Session 3: Immune system and histocompatibility (Chair: Loriano Ballarin)

Origin and diversification of gut-derived organs in chordates

Arun R Chavan1, Parastou Yaghoubi1, Naomi Philip1, Kejue Jia2, Kun-Lung Li3, Jr-Kai Yu3, Jacob M Musser2, Ruslan Medzhitov1

1 Department of Immunobiology, Yale School of Medicine, New Haven, CT, USA

2 Department of Molecular, Cellular, and Developmental Biology, Yale University, New Haven, CT, USA

3 Institute of Cellular and Organismal Biology, Academia Sinica, Taiwan

The gut, despite its structural diversity across animal lineages, performs a set of stereotypical functions for digestion of food and barrier defense. In some animal lineages, e.g., some mollusks and vertebrates, these functions are performed by elaborate specialized organs derived from the gut tube. Their phylogenetic distribution suggests that the ancestral gut was a multifunctional tubular organ that carried out all digestive functions; while these functions were subsequently allocated to organs specialized for secretory (e.g., liver and pancreas in vertebrates) and absorptive (intestine) functions in a lineage-specific manner. Here, we aim to understand the evolutionary origin of vertebrate digestive organs by means of a comparative analysis of the digestive systems of chordates. We present single-cell transcriptomic data from gut tissues of a cephalochordate, amphioxus (Branchiostoma floridae), and a tunicate, Ciona robusta, as representatives of two closest lineages to vertebrates. In the gut of both of these species, we identify cell types homologous to those from the vertebrate digestive system, including the pancreatic exocrine cells, enteroendocrine cells, absorptive enterocytes, hepatocytes, as well as putative tissue-resident macrophages. We will present our ongoing efforts to trace the evolutionary history of the liver and the pancreas in vertebrates in light of these findings, and discuss their implications for the origin and diversification of animal tissues.

First Evidence of Mast-Cell Like Cells in a Colonial Ascidian

Nicolò Brunelli*, Francesca Cima

Department of Biology

University of Padua, Italy, Pd 35121

Vertebrate mast cells play an important role in the immune system, being the first cells to initiate the inflammatory response. The origin of these highly specialised cells belonging to the innate immunity represents an intriguing problem inside chordates and tunicates represent the best candidates due to their close relationship with vertebrates. The colonial ascidian Botrylloides leachii has been chosen because it is easily reared in laboratory and a single colony can be subdivided in subclones, which are genetically identical. A particular cell type circulates in the haemolymph, namely 'granular cell', which is a distinct immunocyte from both phagocytic and cytotoxic lines. Like mast cells, granular cells were labeled by CD117 (c-kit) antibody on their plasmalemma and exhibited a high content of heparin in their granules revealed by fluorescence of berberine sulphate, Csaba's reaction, and metachromatic reaction of toluidine blue. Immunopositivity to anti-heparin and anti-histamine antibodies at both light and electron microscopy displayed an arrangement of heparin and histamine at the periphery and in the centre of the granules, respectively, corresponding to that inside the granules of mast cells. Histoenzymatic assays showed the presence of mast-cell enzymes such as β-glucuronidase, arylsulphatase, chloroacetyl esterase and proteases. These cells degranulated after exposure to bacteria, compound 48/80 or heterologous plasma. During 60-min exposure of colonies to bacteria, they massively crowd into the visceral sinus and then moved across the epithelium of the digestive tract, where they degranulated. The release of TNF-alpha, the most important inflammatory cytokine, is also associated to this event.

Testing the role of the kinetic proofreading model in allorecognition specificity in Botryllus schlosseri.

Bouallegui, Y1; De Tomaso, A.W.1

1Department of Molecular, Cellular and Developmental Biology, University of California, Santa Barbara, CA

Allorecognition in Botryllus schlosseri occurs at the tips of an extracorporeal vasculature (ampullae) when individuals grow into each other, which results in either a vascular fusion, and an inflammatory rejection response that block fusion. Fusion or rejection is determined by a single, highly polymorphic fuhc locus (fusion/histocompatibility), and two individuals will fuse if they share one or both fuhc alleles, and reject if they share neither. The fuhc locus encodes both putative ligands and receptors in this reaction. We have recently found a new family of proteins that are encoded next to each fester gene, which we are calling the fester co-receptor (FcoR) proteins. FcoR's encode canonical signaling domains called ITIMs and hemITAMs. Receptors with hemITAMs control activation (by activating kinases) and those with ITIMs inhibit activation (by activating phosphatases) in almost all immune reaction in mammals, with outcome due to integration of the two pathways. Ampullae express all festers, FcoR and every signal transduction molecule and downstream transcription factor utilized by mammalian ITAM and ITIM signaling. The current study investigates the potential involvement of a model called kinetic proofreading that is predicted to control TCR based ligand discrimination, where a full activation of the ITAM signal transduction pathway is due to the TCR binding half-life to the pMHC complex, which in turn is defined by the affinity to the pMHC ligand, and only strong signals can fully activate the pathway. To investigate this, we are utilizing the natural variability in the rejection reaction, which between certain genotypes can be fast and robust (< 12 hrs following contact), or slow and weak (ca. 72 h following contact). We are currently characterizing the global changes in phosphorylation patterns of ampullae cell lysates following contact of ampullae and correlating those with the outcome of the allorecognition response (fusion, weak rejection, strong rejection). The kinetic proofreading model predicts that the magnitude of the phosphorylation response to fusion and rejection phenotypes will correlate to the strength of rejection: specifically, that the stronger rejection phenotype will show the highest changes in phosphorylation, and there will be a decrease in intensity in the weaker rejection, and no phosphorylation will be seen in the fusion. Our goal is to isolate candidate phosphorylated intermediates via protein sequence identification from cut bands in westerns and identify the genes using our reference genome and proteome. Current results will be presented.

The fuhc histocompatibility locus is a greenbeard that mediates germline progenitor transfer between adults and juveniles

Anthony W De Tomaso

Molecular, Cellular and Developmental Biology, University of California, Santa Barbara, CA, USA.

Allorecognition is the ability to discriminate between self and non-self between individuals within the same species, and examples are found throughout the metazoa, from sponges to humans. Yet, despite the complexity of this recognition event, there is no conservation of candidate genes between models in which this has been studied. In contrast, one characteristic that is conserved is that all allorecognition systems maintain an enormous amount of genetic variation, which is due to strong selection for diversity at these loci. What is the selective pressure responsible for creating and maintaining highly polymorphic allorecognition systems?

Botryllus schlosseri is a colonial ascidian that has a well-studied allorecognition system that is controlled by a single highly polymorphic locus known as fuhc (fusion/histocompatibility). In contrast to other models, allorecognition plays a well understood role in Botryllus, as it limits the transfer of parasitic germline stem cells between individuals, an event that occurs following fusion of compatible individuals. If this is the only role of allorecognition, there are a number of puzzling aspects of this system which are difficult to explain, including the single-match rules of acceptance, co-dominant expression of the fuhc locus, and what appears to be a fuhc mediated larval settlement behavior and its effect on population structure. Here we provide evidence that evolution of the fuhc may also include selection due to an altruistic interaction between juveniles and adults. We show that germline chimerism in natural populations is normal, with up to 70% of individuals harboring one or more testis of another individual, and this was true in East and West coast US populations over two breeding seasons. We have repeated these studies in the lab, and results suggest the presence of an altruistic interaction between juveniles and adults that underlie frequency dependent selection of the fuhc locus. Thus, polymorphism provides a metric of relatedness within a population, and shared alleles of the fuhc locus are greenbeards which mediate what is essentially a real estate trade-off between established adults and motile larvae.

Evolution of a histocompatibility locus in basal chordates

Henry Rodriguez-Valbuena1,a, Jorge Salcedo1, Carina Chen1, Stefano Tiozzo2, Anthony W De Tomaso1,b

1Molecular, Cellular and Developmental Biology, University of California, Santa Barbara, CA, USA.

2 CNRS, Laboratoire de Biologie du Développement de Villefranche Sur-mer (LBDV), Sorbonne Université, Paris, France.

Multiple species across the tree of life can distinguish related and unrelated tissues and organs within the same species. Botryllus is a colonial tunicate where this allorecognition phenomenon is observed as two phenotypes: fusion and rejection. Allorecognition in Botryllus is controlled by a single highly polymorphic locus known as fuhc (fusion/histocompatibility), where six allorecognition genes have been isolated. However, it has not been established how these genes have evolved in tunicate species. We used transcriptomic and genomic data to understand the evolution of the fuhc locus. We found that this locus was assembled in a stepwise manner, where allorecognition genes appeared as non-polymorphic genes and subsequently acquired polymorphism. These results offer a unique opportunity to observe how a histocompatibility locus was assembly though evolution, which can not be observed at the same resolution level with the MHC complex of vertebrates. On the other hand, previous studies have shown that allorecognition in Botryllus is controlled by two receptors (fester and uncle fester). Using transcriptomic, genomic, and PCR data, we established that these receptors belong to a gene family with at least 40 fester members in Botryllus. These receptors exhibited different molecular mechanisms to generate variability. Moreover, we identified a new family of receptors with tyrosine-based motifs in the cytoplasmic regions, which were called fester-coreceptors. We found that each fester gene is linked to a fester-coreceptor gene in the ascidian genomes, exhibiting similar mechanisms of variability. These findings illustrate that allorecognition in Botryllus is controlled by multiple immune receptors, which share characteristics with allorecognition receptors of vertebrates.

BHF, the Botryllus Gene that Predicts Fusion Rejection Outcome via Allelic Polymorphism, Aligning with Mendelian Inheritance of This Trait

Voskoboynik A

Department of Biology, Hopkins Marine Station, Stanford University

ayeletv@stanford.edu

The major histocompatibility complex (MHC), a set of cell surface molecules encoded by a large gene family, was discovered through its role in the rejection of transplants. It has been shown that MHC controls a major part of the immune system in all vertebrates and determines self from non-self. In Botryllus schlosseri, a marine organism closely related to vertebrates, the decision to reject or fuse is governed by a different mechanism through a single polymorphic histocompatibility gene. A candidate gene called FuHC was first identified as the Botryllus histocompatibility gene (De Tomaso et al., 2005), but it was later found to be inconsistent with the expected genetic outcomes. Following the sequence and assembly of the Botryllus genome, a gene called BHF, which is located very close to FuHC, was then found to be a better candidate for the Botryllus histocompatibility gene (Voskoboynik et al. 2013). BHF mRNA sequence perfectly predicts fusion rejection outcomes, matches heterozygosity/homozygosity lines and self-BHF recognition inhibits cytotoxicity, whereas non self BHF recognition induces killing (Voskoboynik et al. 2013; Rosental et al. 2018). It does not have any homologues in vertebrates based on its nucleic acid sequence, amino acid sequence, or predicted structure. It also does not have any motifs to indicate that it is a membrane-bound or secreted protein by classical criteria. However, BHF is expressed on the cell surface, which suggests that it may have a unique mode of action. Here I'll review the pathway that led to the discovery of BHF and its connection to FuHC, and will discuss the current knowledge surrounding these genes.

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Session 4: Neural development and function (Chair: Alberto Stolfi)

Neural crest lineage in the proto-vertebrate model Ciona

Lauren G. Todorov, Kouhei Oonuma, Takehiro G. Kusakabe, Michael S. Levine, and Laurence A. Lemaire

Neural crest cells are multipotent progenitors arising from the neural plate border and producing ectomesenchymal derivatives such as pigment cells, most of the peripheral nervous system, and craniofacial bones. These cells are unique to vertebrates with no equivalent in invertebrates. However, their evolutionary origin remains elusive. To explore this question, we use the tunicate Ciona since this invertebrate chordate is among the closest living relatives to vertebrates. A previous study showed that one of the potential closest cell types to neural crest derivatives is the pigment cells of the central nervous system of Ciona larvae, which originate from the neural plate border. Here, we show that the pigment cell lineage also produces neural progenitor cells that differentiate into the nervous system of juveniles during metamorphosis. Neural progenitors are another major derivative of neural crest in vertebrates suggesting that the last common ancestor of tunicates and vertebrates contained a multipotent progenitor population at the neural plate border. It would therefore appear that a key property of neural crest, multipotentiality, preceded the emergence of vertebrates.

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Single-cell gene expression profiles of the developing cell-autonomously oscillating motor neuron MN2

Marina Takahashi*1, Madoka Utsumi*1, Takumi Shito*1, Nozomu Totsuka*1, Yuki Kogure*1, Kotaro Oka*1*2, Kohji Hotta*1

*1 Department of Science and Technology, Keio University, Japan

*2 School of Frontier Engineering, Kitasato University, Japan

Neuronal circuits controlling animal motor rhythms have been extensively studied (Song et al., 2016). Although various ion channels are known to regulate neural firing (Goaillard & Marder, 2021), the full mechanism of rhythm generation has not yet been elucidated. The central nervous system of Ciona larvae consists of only 170 neurons, making it the simplest model organism in neuroscience. Ciona larvae have five pairs of motor neurons, of which MN2 shows autonomous Ca2+ oscillation from the tailbud stage (Akahoshi et al., 2017) and continues the oscillation to later stages, changing its interval (Utsumi et al., 2023). MN2 also exhibits calcium oscillations when isolated (Akahoshi et al., 2021). To elucidate the molecular mechanism that generates the autonomous oscillation of MN2, we aimed to reveal the gene expression profiles of MN2s at the single-cell level. MN2 was isolated from tailbud embryos and the oscillation was observed for more than 1 hour. The interval changes of the oscillation in the isolated MN2 were similar to that of MN2 in individuals, suggesting that the change is intrinsic to MN2. To compare gene expression profiles in MN2s at different developmental stages, MN2s from early, middle, and late tailbud embryos were collected and analyzed by RNA-seq. Differentially Expressed Gene (DEG) analysis revealed multiple channels are differentially expressed during these stages. Further comparison with the spatio-temporal specificity in the Aniseed database and published scRNA-seq data (Cao et al., 2019) narrowed down the candidate genes. We also report the further progress of our experiments to date.

Regulation of Motor Neuron Development in Ciona robusta

Sydney Popsuj*, Alberto Stolfi

Georgia Institute of Technology

The Ciona Larval Motor Ganglion has been proposed to be homologous to the vertebrate rhombospinal region (hindbrain and/or spinal cord) and holds a great deal of promise as a neurodevelopmental model. The “core” Motor Ganglion is composed of only 8 bilaterally symmetric pairs of neurons: 6 pairs of interneurons and 2 pairs of motor neurons. Understanding the regulatory networks and developmental trajectories of these core Motor Ganglion neurons is of great interest to the study of chordate evolution and gene regulation in neurodevelopment. Of note, the 2 motor neuron types, despite both being cholinergic, form drastically different neuromuscular synapses, with motor neuron 1 (MN1) forming large, frondose endplates hypothetically required for symmetric, alternating left-right tail contractions during swimming, and Motor Neuron 2 (MN2) forming en passant graded synapses associated with asymmetric tail movements necessary for hatching initially, then later for swimming turns and steering. Recently, we have reported that Motor Neuron 2 uses a conserved Neural Agrin/LRP interaction dependent on alternative splicing of Agrin mRNAs by Nova, similar to vertebrates. However, to date, little to no functional work has identified any genes specific only to MN1. I have identified the Wnt inhibitor Dkk3, and the tight junction protein Claudin.j as unique markers for Motor Neuron 1 downstream of the MN1-specific regulatory factor Nk6. I am also investigating their functions using CRISPR-Cas9 mediated knockouts to piece together how they impact MN1 development with specific interest surrounding the establishment and maturation of synapses.

Investigation of neuron-glia interactions using optogenetics in Ciona swimming larvae

Nanako Okawa1,2*, Haruka Motomura2, Misaki Okahata1,2, Atsushi Kuhara2, Takehiro G. Kusakabe2

(1) Graduate School of Frontier Biosciences, Osaka University, Osaka, Japan

(2) Institute for Integrative Neurobiology, Konan University, Kobe, Japan;

The human brain contains about 100 billion neurons and as many glial cells. Recent studies suggest that glial cells play important roles in complex brain functions. However, it is still unclear how the glial cells are involved in higher brain functions. In previous studies, we reported that active Ca2+ transients in glial ependymal cells of the nerve cord of Ciona larvae were associated with swimming behavior, suggesting that the glial cells were involved in the control of neurons and muscle activity. In this study, we analyzed the relationship between neuronal activation or inhibition and glial cell activity using optogenetics in combination with calcium imaging. Photoactivatable proteins, ChrimsonR or halorhodopsin, were expressed in cholinergic neurons, excitatory neurons involved in tail movement. Cholinergic neurons in the motor ganglion were periodically activated or inhibited by light irradiation and the activity of the glial cells in the nerve cord were monitored by calcium imaging. Ca2+ concentration in the glial cells were increased upon activation of cholinergic neurons. By contrast, the activities of the glial cells were suppressed when cholinergic neurons were inhibited. Moreover, single-cell transcriptomic analysis revealed that acetylcholine receptors and some other neurotransmitter receptors were expressed in the glial cells of the nerve cord. These results suggest that the glial ependymal cells receive information from various types of neurons. The glial cells of Ciona larva may exhibit properties of both oligodendrocytes and astrocytes of vertebrates in addition to those of ependymal cells.

Measurement of neuronal activities and swimming behavior during larval development in *C. intestinalis* type A

Hina Mizutani*1, Nozomu M. Totsuka*1, Madoka K. Utsumi*1, Kotaro Oka*1,2, Kohji Hotta*1

*1 Department of Science and Technology, Keio University, Japan

*2 School of Frontier Engineering, Kitasato University, Japan

Larvae of the Ciona intestinalis type A indicate negative phototaxis and gravitaxis (Tsuda et al., 2003) . Each taxis behavior is regulated by the inputs from two types of sensory neurons, which are an otolith and ocellus in the brain vesicle, and the changes of those taxis behaviors depend on the developmental stage (Tsuda et al., 2003) . However, what change of a neuronal activity can induce the transformation of the swimming behavior has been unclarified. In this research, we acquired some videos of the larval swimming behavior with far-red light (850 nm) responding to a light stimulus in different developmental stages, then the data was analyzed to track the trajectory of the swimming behavior by ImageJ and DeepLabCut (Utsumi et al., 2023) . As a result, different behaviors were captured in respond to changing visible light according to the developmental stages as previously reported (Bostwick et al., 2020) . We also constructed a system to visualize neuronal activities of sensory neurons. For imaging the neuronal activities related to phototaxis, we utilized a genetically encoded near-infrared (NIR) calcium ion (Ca2+) indicator, NIR-GECO2, which has an excitation and emission in the near-infrared region that may not be perceived by larvae. This time, we will introduce the current advancements in our research related to the visualization of neuronal activity and analysis of the swimming behavior.

Spatial transcriptomic analysis of the Ciona adult brain

Ayana Maruo1*, Xin Zeng2,3, Fuki Gyoja1, Nanako Okawa1,4, Ken-ichi Mizutani5, Yutaka Suzuki3, Kenta Nakai2, Takehiro G. Kusakabe1

1. Institute for Integrative Neurobiology, Department of Biology, Konan University, Kobe 658-8501, Japan

2. Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan

3. Graduate School of Frontier Sciences, The University of Tokyo, Kashiwa 277-8568, Japan

4. Graduate School of Frontier Biosciences, Osaka University, Suita 565-0871, Japan

5. Graduate School of Pharmaceutical Science, Kobe Gakuin University, Kobe 650-8586, Japan

Swimming larvae of ascidians metamorphose into sessile adults, and the adult brain is formed after metamorphosis using glial ependymal cells of the larval brain as progenitor cells. The adult brain is a simple structure consisting of two parts: a cerebral ganglion and a neural gland. The cerebral ganglion is the main body of the central nervous system consisting of a neuron-rich cortex and a neuropile-rich medulla. The neural gland, on the other hand, consists of an intricate epithelium with an anterior neural gland duct that connects to an opening called the ciliated funnel. To date, however, little is known about the function, cellular composition, and developmental mechanisms of the adult brain. In this study, to lay the foundation for elucidating the structure and physiological functions of the adult ascidian brain and its developmental mechanisms, spatial transcriptome analysis of the adult brain of the ascidian Ciona intestinalis type A was performed. Major structures of the adult brain, such as cerebral ganglion, neural gland, and ciliated funnel, were clearly recognized by gene expression. Genes conspicuously expressed in each tissue were related to predicted function or structure of these tissues. To overcome technical limitation of the spatial resolution of the 10xVisium system, we adopted a computational method and successfully created super-resolved gene expression maps. The present study obtained comprehensive spatial gene expression data of the ascidian adult brain and revealed functional regionality of the cerebral ganglion. Thus, our results provide new insights into structure and function of the adult brain of Ciona.

Investigating the role of neuronal oscillators in phototactic behavior in *Ciona* larvae

Janeva Chung, Erin Newman-Smith, Matthew J. Kourakis, Yishen Miao, Cezar Borba, Eamon Bashiri, Katarina Westermark, William C. Smith

Department of Molecular, Cellular, and Developmental Biology, and Neuroscience Research Institute, University of California, Santa Barbara

The primitive chordate *Ciona robusta* is an emerging model for neural circuit analysis. At the larval stage, the *Ciona* CNS shows extensive homology with those of vertebrates. However, the *Ciona* larval CNS contains only 177 neurons. *Ciona* has the only chordate CNS with a fully characterized connectome, making it an ideal model for understanding vertebrate neural circuitry and behavior. *Ciona* larvae display a number of behaviors, including negative phototaxis. To initiate negative phototaxis, the larvae first performs a short spontaneous rhythmic casting swim to discern the direction of light. When a directional cue is detected, an extended negative phototactic swim is evoked. Previously, our group identified a single slow-oscillating inhibitory neuron called *corr-assBVIN78* that projects to the midbrain, where it targets key interneurons of the phototaxis circuit known as the *photoreceptor relay neurons (*prRNs). Ablation of cor-assBVIN78 in larvae results in extended phototaxis-like swims, even in the absence of phototactic cues. These results indicate that cor-assBVIN78 provides tonic, but temporally oscillating inhibition to the prRNs, and that some or all of the prRNs are intrinsically active. Our group is now investigating the properties of the prRNs, as well as a second group that targets the prRNs, the coronet cells. Preliminary data has shown that the prRNs and coronet cells have spontaneous and rhythmic spiking activity. We are currently investigating the roles of several neuropeptides that are uniquely expressed in the coronet cells.

Day 5 - July 26, 2024, Friday

Session 1: Systematics and taxonomy (Chairs: Billie Swalla and Tony De Tomaso)

Comparative anatomy of endostyles in Appendicularia: cladistic interpretation and functional implications

Le, Mai-Lee Van: mai-lee.van.le@hu-berlin.de; Humboldt-Universität zu Berlin Vergleichende Elektronenmikroskopie, Philippstraße 13, 10115 Berlin

Stach, Thomas1: thomas.stach@hu-berlin.de; Humboldt-Universität zu Berlin Vergleichende Elektronenmikroskopie, Philippstraße 13, 10115 Berlin

The endostyle is a glandular organ secreting mucus for capturing food particles. It is an apomorphy of Chordata and homolog to the vertebrate thyroid gland. The primitive form of chordate endostyles, as seen in ascidians and amphioxus, is a longitudinal groove composed of several rows of functionally specialized cells. The endostyle of appendicularians is reduced to a shorter structure consisting of few cells. However, appendicularian endostyles are mainly known from the model organism Oikopleura dioica and no study using modern microscopic techniques or cladistic phylogenetic reasoning is available. Here, we present a comparative analysis of ten appendicularian species from all three families. Five oikopleurids (Oikopleura vanhoeffeni, O. dioica, Bathochordaeus stygius, Megalocercus huxleyi, Folia mediterranea), two fritillariids (Fritillaria pellucida, F. borealis), and two kowalevskiids (Kowalevskia tenuis, K. oceanica) were analyzed using serial light microscopy and 3D-reconstruction in combination with transmission electron microscopy. The diversity of endostyle structures within Appendicularia was higher than expected. Endostyle morphology ranged from a relatively long groove with two rows of glandular cells separated by ciliated cells in M. huxleyi to more compact organs with only a single row of glandular cells in other oikopleurid species and fritillariids to complete reduction and partial functional replacement by ciliated pharyngeal projections in the two kowalevskiid species. We suggest hypotheses of homology of individual cell types and discuss phylogenetic implications in comparison to recently published molecular phylogenies. Functional considerations based on our structural findings remain currently speculative, but result in testable hypotheses.

Furthering Ascidian Taxonomy through the use of Molecular Markers

Nicholas Gulnick*1, Marie Nydam2, Susanna López-Legentil3, Patrick Erwin3, Lauren Stefaniak1

1Department of Marine Science, Coastal Carolina University, 100 Chanticleer Dr E, Conway, SC, USA

2SOKA University of America, 1 University Drive, Aliso Viejo, CA, USA

3Department of Biology & Marine Biology, Center for Marine Science, University of North Carolina Wilmington, 5600 Marvin K. Moss Lane, Wilmington, NC, USA

Ascidians are our closest invertebrate relatives and comprise nearly 3,000 species separated into three orders: Aplousobranchia (largest), Stolidobranchia, and Phlebobranchia (smallest). Ascidians can be classified as either solitary or colonial organisms. Species delimitation using morphological characters alone has had varied results. Molecular markers can help mitigate some of the issues presented by strictly using morphological observations, including resolving the status of cryptic species, and accessing the expert knowledge required to identify a species. By incorporating molecular markers and pairing them with morphological observations, more species may be correctly identified by the scientific community. Our project focuses on the ascidian families Ascidiidae, Pyuridae, and Styelidae. In it we compare the utility of the molecular markers mitochondrial cytochrome oxidase 1 (mtCO1) and 18S rRNA, both commonly used to barcode marine invertebrates, in terms of successfully delimitated species within families and ease of amplification and sequencing. This presentation will focus on samples collected from a variety of habitats in Belize during July 2022 and July 2023.

The Perophoridae (Chordata, Ascidiacea) of Belize: identification by molecular and morphological taxonomy combined.

Lauren Stefaniak1*, Tiffani McNeil1, Marie Nydam2, Susanna López-Legentil3, Patrick Erwin3

1Department of Marine Science, Coastal Carolina University, 100 Chanticleer Dr E, Conway, SC, USA

2SOKA University of America, 1 University Drive, Aliso Viejo, CA, USA

3Department of Biology & Marine Biology, Center for Marine Science, University of North Carolina Wilmington, 5600 Marvin K. Moss Lane, Wilmington, NC, USA

Ascidians (also known as sea squirts) are a group of marine benthic invertebrates in the Phylum Chordata. Proper identification of ascidians is important because many ascidian species have been introduced beyond their native ranges and can become invasive, posing a threat to marine environments and aquaculture. However, identifying ascidians using morphological techniques alone can be difficult. Many related species have very similar morphological characters, and there are few taxonomic experts. DNA barcoding uses molecular markers to aid in proper identification and increases accessibility to taxonomic information. Indeed, several morphologically cryptic ascidian species have been found using molecular-based phylogenetics. The Family Perophoridae consists of social ascidians in two genera: Ecteinascidia (29 species) and Perophora (22 species). There are a limited number of morphological characters available for this family, and for some species pairs, the variation in their defining characteristics overlaps, making conclusive morphological identification impossible. In this study, we identify members of the family Perophoridae from Belize using the mitochondrial COI gene and the ribosomal 18S rRNA as molecular markers to validate morphological identifications and generate DNA barcodes for use in future research.

Phylogenomics and systematics of botryllid ascidians, and implications for the evolution of allorecognition

Marie L. Nydam1*, Alan R. Lemmon2, Emily M. Lemmon3, Kevin Ziegler3, C. Sarah Cohen4, Lilian A. Palomino-Alvarez5,6, Carmela Gissi7,8,9

1Life Sciences Concentration, Soka University of America, Aliso Viejo, CA, USA

2 Department of Scientific Computing, Florida State University, Tallahassee, FL, USA

3 Department of Biological Science, Florida State University, Tallahassee, FL, USA

4 Biology Department and Estuarine and Ocean Science Center, San Francisco State University, Tiburon, CA, USA

5Posgrado en Ciencias del Mar y Limnología, Universidad Nacional Autónoma de México, Ciudad de México, México

6Unidad Multidisciplinaria de Docencia e Investigación Sisal (UMDI-Sisal), Facultad de Ciencias Nacional Autónoma de México, Ciudad de México, México

7Department of Biosciences, Biotechnologies and Environment, University of Bari “Aldo Moro”, Bari, Italy

8IBIOM, Institute of Biomembranes, Bioenergetics and Molecular Biotechnologies, Consiglio Nazionale delle Ricerche, Bari, Italy

9CoNISMa, Consorzio Nazionale Interuniversitario per le Scienze del Mare, Roma, Italy

Allorecognition, the ability of an organism to distinguish kin from non-kin, or self from non-self, has been studied extensively in a group of invertebrate chordates, the colonial ascidians called botryllids (Subphylum Tunicata, Class Ascidiacea, Family Styelidae). When two conspecific botryllid colonies come in contact, there are two potential outcomes to an allorecognition reaction: fusion or rejection. The rejection outcome of allorecognition varies by species, and has been classified by type (referred to as R-Type). R-Type is defined according to how far the fusion process progresses before the rejection begins, since the rejection reaction appears as an interference of the fusion process. Here, we map the evolution of R-Types onto an extended and robust phylogeny of the botryllids. In this study, we have reconstructed the largest phylogenomic tree of botryllids, including 97 samples and more than 40 different species, and mapped on it nine of the 13 species for which the R-Type is known. Based on the R-Type known in a single outgroup species (Symplegma reptans), we infer that at least R-Type B and E-like could be ancestral to the Botrylloides/Botryllus group. Notably, all R-Type A species are clustered together and certainly evolved later than other R-Types. Our phylogenomic tree has been built on 177 nuclear loci and nearly all clades are well supported. Moreover, our phylogenetic analyses also take into account the resultsof species delimitation analyses based on the mitochondrial COI gene and of careful morphological analyses of the samples. The implementation of this integrated taxonomic approach, combining morphological as well as nuclear and mitochondrial data, has allowed the description of six new species, and the identification of a number of putative unnamed taxa. Thus, our results also demonstrate the existence of an unexplored hidden diversity within botryllids.

Didemnids along the Turkish Coastlines: Their Symbionts and Bioactive Secondary Metabolites

Arzu Karahan*

Middle East Technical University, Institute of Marine Sciences,

33731, Erdemli-Mersin, TURKIYE

Antimicrobial agents are essential for treating infectious diseases, but many microorganisms have developed resistance due to excessive use. Many drugs have lost or are losing effectiveness. Oceans, covering 71% of the Earth's surface and containing 50-80% of global biodiversity, offer immense chemical diversity. There is a growing global interest in Marine Bioactive Secondary Metabolites (MBSM) to combat antibiotic resistance and cancer. Marine organisms, evolutionarily older than terrestrial ones, contain many MBSMs, both self-produced and from symbionts, waiting to be discovered. Every year, hundreds of new compounds, particularly from marine invertebrates, are isolated and identified. However, Turkiye, with its 8333 km coastline, has lagged in obtaining natural products from marine organisms and transforming them into technology. Out of 1080 bioactive compounds identified from ascidians for clinical and pre-clinical processes, 375 belong to the Didemnidae family, with over 200 of these in the genus Didemnum. Limited taxonomic studies have been conducted on these organisms along the Turkish coasts. Considering the species diversity of the Didemnidae family, it is likely that unidentified species exist along our coasts. Some metabolites are produced by symbionts and vary regionally. The main objective of our research is to discover new MBSMs targeting medical and other technological fields from Didemnidae along the coast from Artvin to Hatay. Another aim is to identify the organism's symbionts using metabarcoding methods and reveal their relationship with MBSMs. So far, samples have been collected from all around the Turkish coastlines, DNA barcoded, species delimitation analysis applied, and symbiont (eDNA) and metabolite analyses have started.

Session 2: Evolution and evo-devo (Chairs: Billie Swalla and Bradley Davidson)

Oikopleura dioica, the evolution of a free lifestyle plenty of losses, expansions and scrambling.

Alfonso Ferrández-Roldán1,2,3, Marc Fabregà-Torrus1,2, Gaspar Sánchez-Serna1,2, Nuria Torres-Águila1,2, Biel Cassà-Garcia1,2, Daniela Ascione1,2, Eva R. Quintana1,2, Ricard Albalat1,2, Aki Masunaga4, Nicolaus J. Wibisina4, Michael J. Mansfield4, Charles Plessy4, Nicholas M. Luscombe4, Cristian Cañestro1,2

1Departament de Genètica, Microbiologia i Estadística, Facultat de Biologia, Universitat de Barcelona (UB), Av. Diagonal 645, Barcelona 08028, Spain.

2Institut de Recerca de la Biodiversitat (IRBio), Universitat de Barcelona (UB), Barcelona, Spain.

3Institute of Evolutionary Biology (CSIC-Universitat Pompeu Fabra), Passeig Marítim de la Barceloneta 37, Barcelona 08003, Spain

4Genomics and Regulatory Systems Unit, Okinawa Institute of Science and Technology Graduate University (OIST), Onna-son, Okinawa, 904-0495, Japan

Understanding how was the last common ancestor of tunicates is important to illuminate the evolutionary origin of the different groups of tunicates, as well as to infer the adaptive innovations that facilitated the evolution of their diverse lifestyles. Recent work from our lab has shown that massive gene losses led to the deconstruction of the cardiopharyngeal gene regulatory in appendicularians, which facilitated the adaptive reacquisition of a free-living style of this group of tunicates from a biphasic sessile ascidian-like ancestor. Here, we will show further examples of how extensive losses and bursts of duplications affecting developmental signaling pathways (e.g. retinoic acid and Fgf), myogenic transcription factors, and muscle motor genes might be related to the evolution of the muscle of the tail and the innovation of the fully free-living style that characterizes appendicularians. Finally, we will also discuss how the sequencing of the genomes of Oikopleura dioica from around the world has revealed massive genome scrambling, and how this discovery might lead to discovering the presence of multiple cryptic species around the globe.

From solitary to colonial with zooid miniaturization: ancestral-state reconstruction based on NGS data of stolidobranch ascidians

Naohiro Hasegawa*

Faculty of Human Environmental Studies

Hiroshima Shudo University, Hiroshima, Japan 731-3195

The size of organisms has consistently intrigued researchers across various disciplines in biology. However, the evolutionary process of zooid miniaturization in colonial animals remained an enigmatic topic. The family Styelidae, within the ascidian order Stolidobranchia, showcases a diverse spectrum of coloniality, positioning it as an ideal candidate for delving into the intricacies of colonial evolution. In this research, we inferred a phylogenomic relationship mainly within Styelidae using transcriptomes of a total of 42 ascidians; from 17 species sampled in Israel and Japan and transcriptome data from 25 species sourced from a previous study and a database. Through ancestral-state reconstruction, our analysis indicated a clear directional change: following the acquisition of coloniality, zooids tended to become progressively smaller. This miniaturization is likely an adaptive response, enabling organisms to swiftly colonize limited marine substrate. We formulated a mathematical model suggesting that zooid miniaturization, due to living space constraints, would result in a faster asexual cycle and accelerated expansion in a colony. Our data also suggested that coloniality evolved independently three times within Styelidae. Moreover, once colonial traits are established, they appear to be consistently preserved, underscoring their biological importance in the colonial lineage.

The major effector of the canonical Wnt signalling pathway, Tcf/Lef, at the transition from invertebrates to vertebrates.

Nuria Torres-Aguila1,+, Marika Salonna2, Sebastian Shimeld3, Stefan Hoppler2, David E.K. Ferrier1

1. The Scottish Oceans Institute, School of Biology, University of St Andrews, UK.

2. Institute of Medical Sciences, University of Aberdeen, UK.

3. The Department of Biology, University of Oxford, UK.

+. Current affiliation, Departement de Genètica, Universitat de Barcelona, Spain.

The canonical Wnt (cWnt) pathway is a key intercellular signalling pathway that is integral to development, regeneration and disease. The major transcription factor that mediates the response of cWnt is Tcf/Lef. Typically, invertebrates have a single Tcf/Lef gene whilst vertebrates have multiple paralogues. Humans possess four TCF/LEF genes, for example. This change in gene number has functional consequences for how Tcf/Lef genes operate, particularly via subsequent evolution of the combinations and sequences of the various motifs and domains now found across these paralogues. In addition to this increase in gene number at the origin of vertebrates there has also been a step change in the level of alternative splicing of the Tcf/Lefs. This increase in complexity has made it challenging to infer the ancestral state of Tcf/Lef for vertebrates and thus how the subsequent diversification and specialisation of the paralogues in distinct vertebrate lineages evolved. We recently revised the model of the Tcf/Lef evolutionary pathway in chordates and hence the likely ancestral state of Tcf/Lef for vertebrates. Our analysis of chordate Tcf/Lef genes, in which the tunicate Ciona intestinalis has been pivotal, also revealed that this increase in complexity at the level of the effector transcription factor seems to be a major route for elaboration of the functional diversity in the cWnt pathway. Furthermore, this pattern may well be a generality that extends to other major signalling pathways. We are now starting to dissect the functionality of chordate Tcf/Lef by using the tunicate Ciona intestinalis, as a ‘living test tube’.

The evolution of reincarnation? – Acquisition of polymorphism in the doliolids.

Bradley Davidson*, CJ Pickett*, Joe RyanW

* Swarthmore College, W Whitney Marine Lab

Although sequence variants associated with novel traits have been identified, it is difficult trace their impact on intervening scales including gene networks and cell lineages. We aim to address this question by studying doliolids, a highly divergent tunicate taxa. Doliolids are the only polymorphic chordate, transitioning through four distinct morphs specialized for locomotion, feeding, asexual or sexual reproduction. We have initiated genomic and developmental comparisons between Dolioletta gegenbauri and the primary tunicate model Ciona robusta to explore how alterations in doliolid signaling genes drove reallocation of embryonic cell lineages to generate novel organs. We are currently exploring this process by examining the emergence of additional doliolid muscle bands. Tunicate muscle band induction relies on FGF signaling. We have detected substantial alterations in Dolioletta FGF signaling genes including loss of the primary FGF-dependent transcription factor Ets1/2 and loss of Ets1/2 binding sites in Dolioletta regulatory elements. Loss of FGF-dependent induction and subsequent lineage reallocation serves as a working, testable model for acquisition of additional doliolid muscle bands. We have also begun to investigate the origins of novel structures associated with polymorphism. In particular, these efforts will focus on a derived appendage extending from the dorsal side of the primary locomotive morph. This appendage plays a central role in polymorphism as it contains three spatially distinct stem-cell lineages that gradually differentiate into feeding, asexual and sexual morphs.

The Tale of Degenerate Ascidian Tails

Billie J. Swalla1,2,3, Sydney Popsuj3,5, Lenny Negrón-Piñeiro4, Anna Di Gregorio4 and Alberto Stolfi3,5

1Biology Department, University of Washington, Seattle, WA 98195, USA

2Friday Harbor Laboratories, University of Washington, Friday Harbor, WA 98250, USA

3Station Biologique de Roscoff, 29680 Roscoff, France

4Department of Molecular Pathobiology, NYU College of Dentistry, New York, NY 10010, USA

5School of Biological Sciences, Georgia Institute of Technology, Atlanta, GA 30332, USA

Ascidians are invertebrate chordates and develop into chordate tadpole larvae, with a head containing sensory organs and a tail with a notochord, dorsal neural tube, and muscle cells. However, in one clade of ascidians, the Molgulidae, both the larval tail and pigmented otolith have been lost multiple times independently during evolution. We have sequenced and analyzed the transcriptome of Molgula occulta embryos at different stages and compared it to those of a closely related tailed species, Molgula oculata and of hybrid embryos obtained from tailless eggs fertilized with tailless species sperm. These two sister species live sympatrically near Roscoff, France and can be hybridized to produce a half-tailed larva with an otolith. Larval tails are formed through convergent extension and then swelling of 40 notochord cells. This process fails to occur in tailless molgulid species, leaving a “notoball”, a 20-cell aggregate, expressing many of the notochord genes. In hybrid larvae, notochords undergo normal convergent extension, forming a smaller, 20-cell notochord that lacks muscle. We are using this system to study changes in the gene regulatory networks that are responsible for the breakdown of the larval regulatory gene networks and lead to the lack of functional tissues in tailless ascidians. We have discovered that a maternal SHARK tyrosine kinase, the larval muscle actins and the larval tyrosinase genes have become pseudogenes and produce nonfunctional RNAs and proteins in the tailless embryos. Therefore, the intact tailed paternal genes can rescue the function of the pseudogenes in the hybrid embryos. We have found both the neural and notochord larval gene regulatory networks are intact in the tailless species, M. occulta, but we have found some broken links and are examining how they are restored in the hybrids.

Exploring the diversity of nervous systems in Appendicularia

Mai-Lee Van Le and Thomas Stach (presenting author)

Humboldt University Berlin, Institute of Biology, Comparative Electron Microscopy, Philippstr. 13, Haus 14, 10115 Berlin, Germany

Appendicularia, a diverse group of marine, invertebrate chordates, encompassing approximately 70 species, plays a crucial role in marine ecosystems. Belonging to Tunicata, which is the probable sister taxon to Craniota, the study of appendicularians holds implications for our understanding of chordate evolution. Characterized by their small size, rapid development, and stereotyped cell lineage, researchers proposed that appendicularians evolved from an ascidian-like ancestor by progenesis. Despite considerable interest in appendicularians, their morphological diversity remains insufficiently explored. In this study, we present a 3D morphological analysis of the central nervous systems in oikopleurid and fritillariid appendicularians. By examining the structure of brain and brain nerves in four fritillariid species (Fritillaria pellucida, F. borealis, F. formica tuberculata, F. haplostoma) and three oikopleurid species (Oikopleura dioica, Bathochordaeus stygius, Megalocercus huxleyi), we aim to shed light on the evolutionary trajectory and adaptations within Appendicularia. Our results show differences in innervation pattern and number of cells in the central nervous system of the analyzed species and between families. Fritillariid brains possess on average half the number of cells found in oikopleurid species. Moreover, fritillariid species seem to directly innervate ciliated bands in the pharynx. We discuss the evolutionary implications of our findings, offering insights into the potential ancestral conditions. This study underscores the importance of further research into the morphological diversity in order to understand the evolutionary history of appendicularians and chordate evolution as a whole.