Session 1 – Genomic Evolution of Developmental Mechanisms I

Possible intrinsic factors limiting animal body plan diversification

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How difficult is it to make pegasus from existing horse species? Fossil records and current animal diversity imply that anatomical patterns, especially, the body plan (basic anatomical pattern) of animals appear have less changeability and is often referred as a typical case of phylogenetic inertia. Then, what makes body plan so much conservative? Many study attributes this to the conservation of body plan establishing embryonic period, or the phylotypic period. However, the mechanism behind the conservation of this phylotypic period remains under debate. Various mechanisms based on the modern evolutionary theories, including natural selections, have been proposed, however, it still remains to be tested if classically argued intrinsic factor, such as developmental constraints could also be one of the factors. By performing comparative transcriptomic analyses of chordates, we found possible contribution of pleiotropic constraints toward the conservation of the phylotypic period. Re-utilization of existing genes has long been known to facilitate evolutionary diversification (e.g., creating novel traits etc.), however, our findings imply that gene re-utilization has a double-edged sword effect toward evolution, limiting effect on the diversification. Further, we also found that the phylotypic period is robust against mutations and environmental perturbations and shows developmental stability. These results suggest that the body plan-establishing period has less potential to make phenotypic variations, having less chance to diversify even under strong positive selections. In other words, this kind of intrinsic factors could have been one of the factors that contributed to conservation of body plan.

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Investigating the epitranscriptomic mark m5C on embryonic mRNA

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Development relies on post-transcriptional regulation of a large maternal RNA contribution to the egg, which is initially mobilized to activate the embryonic genome, then later targeted for clearance as the embryonic transcriptome is reprogrammed. A new paradigm is emerging in which covalent RNA base modifications such as N6-methyladenosine (m6A) and pseudouridine distinguish specific mRNA, affecting their expression and fate. With over 100 characterized RNA base modifications, it is likely that many contribute to an "epitranscriptomic code" that helps guide pluripotency induction and early development. Here, we apply comparative transcriptomics to understand the regulatory logic of 5-methylcytosine (m5C) in embryos. We mapped m5C sites over a time course of *Xenopus laevis* development using bisulfite RNA-seq and found that m5C correlates with heightened mRNA stability, thus protecting a subset of maternal mRNA from clearance. However, we additionally uncovered two intriguing phenomena: first, de novo methylation was rare to non-existent, suggesting that m5C methyltransferase activity on mRNA is restricted to the germ line and inhibited in the embryo. Second, nearly identical mRNA sequences deriving from gene homeologs were differentially methylated - *X. laevis* is a hybrid tetraploid with two copies of most genes, often subject to differential regulation. Since differentially methylated homeolog pairs can have the same local sequence around the m5C site, the mechanisms for recruiting m5C methyltransferases to mRNA may be more nuanced than previously thought. We are currently expanding our analysis to include three additional *Xenopus* species, coupled with *in vitro* and *in vivo* reporter methyltransferase assays, to deduce specificity determinants for m5C on embryonic mRNA. These findings underscore how epitranscriptomic information can influence transcriptome regulation and cellular identity during development.

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Differential binding of p63 targets among jaw primordia in a new gene regulatory network driving tooth development

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The ancient, highly conserved transcription factor p63 is expressed in epithelium and vital in mediating the development of epithelium-derived structures including teeth. Recently, p63 and a putative downstream gene regulatory network (GRN) were implicated as a driver of early odontogenesis. This GRN includes a suite of genes not previously linked to odontogenesis and/or p63, validated by recent analyses probing transcript and protein expression in tooth organs. The functions of these GRN candidates in odontogenesis remain to be clarified. Here we explored these functions using bioinformatics tools including functional enrichment analysis (g:Profiler) and enrichment mapping (Cytoscape-EM). Also, using ChIP Sequencing, we probed the binding dynamics of p63 in mandibular (MdP) and maxillary (MxP) prominences. The second branchial arch (BA2) served as a control prominence. Prominences were collected from E10.5 mouse embryos from *p63+/+* wildtypes, *p63+/-* heterozygotes, and *p63-/-* mutants, and sequenced on Illumina Next-Seq platforms. Enrichment results provided new evidence for this GRN in regulating epithelial tissue folding, lipid-mediated cellular signaling, and epithelial/mesenchymal signaling. ChIP-Seq results indicated a gene dose-specific p63 binding behaviour in *p63+/+* versus *p63+/-* mandibular and maxillary prominences. We saw a trend where p63 was bound to the greatest number of genes in the MxP (3993 genes), then in the MdP (696 genes), and least in the BA2 (52 genes). We found an overlap of 408 bound genes between the MxP and MdP, and less than 30 bound genes shared between MxP, MdP and BA2. Altogether, this study offers a new avenue via which to probe the evolution of diverse tooth phenotypes including tooth reduction and loss in jawed vertebrates.

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Shared epigenetic controls link post-natal development and later aging in marsupial and placental mammals

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The universality of aging across mammals has engendered much speculation on its causes. We generated genome-wide DNA methylation profiles for 58 tissue types and 185 mammalian species and used the data to develop pan-tissue aging clocks. These clocks' ability to accurately estimate age across mammals suggests that aging might result from defined mechanisms that are largely shared across tissues and species, rather than the accrual of random damage to cells. The clocks themselves also provide clues to these possible mechanisms; the cytosines that comprise them, i.e., those whose methylation levels change with age, are located near genes enriched in developmental processes, PRC2 binding sites, and H3K27me3 marks. The tandem enrichment of these factors is likely not coincidental; PRC2 maintains genes in a repressed state during development and beyond by marking chromatin with H3K27me3. To further explore these possible links, we generated genome-wide methylation data for tissues from the first six weeks of post-natal development in two distantly related mammals, opossum (*Monodelphis domestica*) and mouse (*Mus musculus*). As in adults, we found that age-related CpGs in post-natal mice and opossums are located near genes associated with developmental processes and PRC2 binding sites, among other processes. These findings suggest that postnatal development and aging are generally coupled across most mammalian tissues and species because of their shared reliance on PRC2 activity. They also suggest that the epigenetic processes shaping postnatal development are at least partially conserved in opossums and mice and perhaps most mammals, given these species' deep evolutionary divergence. However, we also uncovered some potentially biologically relevant differences including a strong association of age and CpGs located near immune system-related genes in opossums but not mice.

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The regulatory mechanism behind limb heterochrony during tetrapod evolution

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Developmental events must occur at the right time and place to produce well-patterned tissues and organs. Many studies have focused on the spatial control of developmental events, while the regulation of developmental tempo is less well understood. During the evolution of tetrapod species, the timing to produce the two sets of appendages - the forelimb and hindlimb become significantly varied from one to another clade. This is illustrated by the almost concomitant development of limbs in bird embryos and the delayed hindlimb development in mammals. This trait allows embryo development to coordinate with the differential organization of the extraembryonic tissue. We explored the mechanisms behind tetrapod limb heterochrony using chicken and mouse embryos as primary model systems. We first determined the timing of cellular events featuring the limb outgrowth and the expression kinetics of components in the gene-regulatory network that controls the onset of limb development. This revealed a regional rather than global delay to activate the hindlimb outgrowth gene, *TBX4*. By using ATAC-seq, enhancer bashing assay, as well as dCas9-Krab approach, we identified an evolutionarily conserved enhancer that is responsible for *TBX4* expression in chicken and mice. Although the genomic region to activate *TBX4* is conserved, comparative transcriptomics suggested genes that were higher expressed in the mouse hindlimb field but not that of the chicken embryo. Ectopic expression of top candidate in the chicken hindlimb field caused delayed expression of *TBX4*. Together, our work suggests a mechanism whereby altered gene expression causes heterochrony of tissue development during the evolution of tetrapod species.

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Evo-Devo of integumentary regional specificity: Diverse stem cell niche topologies and novel epigenetic control by scale–feather convertors

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The evolution of feathers is an important event in the Evo-Devo of amniote integuments. It is critical to learn how an integument of one individual can generate diverse regional specificity. Recently we used the chicken feather/scutate scale and alligator scale as models to study the controlling mechanism regulating fate choice. Using transcriptome analysis and gene misexpression, we identified a group of scale-feather convertors which can induce a spectrum of scale-to-feather intermediate morphotypes in chick shanks (Wu et al., Mol Biol Evol. 2018). Here we further study the downstream and upstream events of these feather converters. 1) Downstream. Using time-lapse tissue explant imaging, we demonstrated different collective cell behaviors during the morphogenesis of the sole skin, scutate scale and feather follicles (with increasing novel complexity). We compare the topology of quiescent and actively proliferating cells in physiological and injury induced regeneration. From epidermis to follicular configurations, we observe the emergence of new cell types (dermal papilla, epidermal stem cell clusters) and new homeostasis strategies. 2) Upstream. Through profiling genome-wide chromatin accessibility (ATAC-seq), we found feather converters program chromatin landscapes of shank mesenchyme from the pre-scale state to a pre-feather state, suggesting that the scale-feather converters function on global chromatin configurations instead of simple activation of gene promoters. This additional layer of epigenetic control in the dermis dictates the morphogenetic movement flow in development and determines the formation of specific integuments in different regions. Sox 18, CTCF, gremlin and others will be highlighted. This work presents cell type evolution in skin regional specification and advances our understanding on how epigenetic regulation is involved in the plasticity and commitment of integument formation.

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Uncovering the genetic and developmental underpinnings of trait evolution in the blind Mexican cavefish

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Similar environments often result in the repeated evolution of the same traits in multiple, independently evolved populations. To understand how and why these traits evolve, it is necessary to identify and functionally validate the causal mutations underlying the evolution of these traits. Astyanax mexicanus is a small freshwater fish that exists in two forms: a blind, cave-dwelling form and a sighted surface-dwelling form. Cavefish have evolved a number of traits relative to their surface fish counterparts including eye regression, loss or reduction in pigmentation, and reductions in sleep and social behaviors. In recent years, many loci contributing to trait evolution in cavefish have been identified by genetic mapping approaches. However, assessing the functional consequences of genes within these loci has remained challenging. We have pioneered genome editing approaches in A. mexicanus to elucidate the genetic basis of complex trait evolution, and to evaluate how traits evolve repeatedly. Using the CRISPR-Cas9 gene editing system, we have functionally assessed and verified a role for multiple candidate genes in the evolution of traits within this species, and we have assessed how these genes impact development. Moving forward, these approaches can be used to understand the genetic basis of complex traits, how specific genetic changes lead to alterations in developmental programs, and ultimately, how genetic variants in cavefish contribute to fitness.

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How to build a gliding mammal

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Understanding the molecular basis of phenotypic diversity is a core goal in biology that requires elucidating links between genotypes, developmental mechanisms, and phenotypes. To accomplish this, our work focuses on dissecting the mechanisms by which morphological adaptations, and the gene regulatory networks (GRNs) that control their development, originate and evolve. We have developed a variety of genomic and experimental approaches in a novel marsupial model species, the sugar glider (*Petaurus breviceps*), to uncover the molecular basis of the mammalian gliding membrane, or patagium – a specialized skin structure that allows animals to thrust forward as they fall through the air. Sugar gliders complete much of its physical development ex utero, inside of their mother's pouch, which provides unparalleled access for tissue sampling, experimental manipulation, and development of molecular tools. In addition, sugar gliders belong to a clade of closely related species whose members have independently evolved patagia at least three times, enabling powerful comparative studies. Using a wide variety of experimental approaches, including transcriptomics, epigenomics, chromosome conformation assays, and functional experiments in sugar gliders, we identify a genomic locus implicated in marsupial patagium formation and patterning. Moreover, using bat transcriptomics and functional experiments in laboratory mice, we find that many components of the marsupial GRN act to pattern patagia in placental mammals. Taken together, our results provide mechanistic and conceptual insights into the molecular processes that generate phenotypic novelty.

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Reptile to chicken enhancer swaps to understand the molecular basis underlying morphological diversity in the vertebrate lung

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There is a vast diversity in tetrapod lung branching morphology. Phylogenetically, much of the pulmonary diversity among vertebrates appears to arise from the way epithelial tubes branch or form saccular (cyst) structures. *Fgf10* activity has been shown to play a critical role in regulating branch versus cyst morphology. We hypothesize that the species-specific differences in lung morphology may be primarily due to species-specific differences in *Fgf10* expression. To test this hypothesis, we have performed bioinformatic analyses on the *Fgf10* locus and have identified a conserved 11 kb noncoding region that potentially contains the *Fgf10* lung enhancer. We are taking large DNA sequences upstream of the *Fgf10* gene of three reptile species (a crocodilian, a turtle, and lizard) and swapping them into the orthologous locus in the genome of chicken primordial germ cells (cPGCs). We are accomplishing these swaps by using a combination of homology directed repair (HDR) and recombinase mediated cassette exchange (RMCE) in cPGCs. These edited cell lines can be used to generate germline chimeric chickens capable of producing offspring that putatively drive *Fgf10* expression in the lung under control of regulatory sequences from various other reptiles. We have also generated a cPGC cell line where, through RMCE, we can easily target any enhancer from any organism to drive a GFP reporter as a means to test the temporal and spatial regulatory characteristics of these enhancers.

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Deconstructing the matters of the heart and the origin of ancestral tunicate sessility

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A central question in chordate evolution is the origin of sessility in adult ascidians, and whether the appendicularian complete free-living style represents a primitive or derived condition among tunicates. According to the “a new heart for a new head” hypothesis, the evolution of the cardiopharyngeal gene regulatory network (GRN) appears as a pivotal aspect to understand the evolution of the lifestyles of chordates. During our studies to investigate gene loss as an evolutionary force, we have discovered that appendicularians suffered the “deconstruction” of cardiopharyngeal GRN due to massive ancestral losses of cardiopharyngeal genes and subfunctions. Using Oikopleura dioica as a model to understand heart development in appendicularians, our results show that these losses can be related to the loss of cardiopharyngeal multipotency. Our findings suggest an evolutionary scenario in which the deconstruction of the cardiopharyngeal GRN can be related to processes of regressive and adaptive evolution during the transition from an ancestral tunicate with an ascidian-like sessile lifestyle to a free-living style in appendicularians. Our results, moreover, highlights the relevance of also studying gene loss and the deconstruction of GRNs to better understand the evolution and diversification of life.

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Shaping vertebrate appendages: deep conservation of fin and limb developmental programs

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How vertebrate appendages first emerged and changed during evolution, are important questions in biology as those structures justify (in part) the evolutionary success of the vertebrate group. By using CRISPR/Cas9 in medaka fish (*Oryzias latipes*), we recently demonstrated that tetrapod paired limbs evolved from fish paired fins, as ZRS defective fish lack *shh* expression in fin buds and show complete truncation of paired appendages, mimicking the phenotype observed in mouse limbs lacking *Shh* activity.
In contrast to *Shh*, *Gli3* transcription factor expression is restricted to the anterior region of the developing bud. Mouse limbs lacking *Gli3* activity show polydactyly in a *Shh*-independent process, as limbs defective in both *Gli3* and *Shh*, mimic the polydactyly phenotype found in *Gli3* single mutants. Removal of *gli3* in the ZRS medaka mutant background completely rescued paired fins in a phenotype comparable to what observed in mouse limbs deficient for both *Shh* and *Gli3*. Moreover, single *gli3* medaka mutants show additional bones in pectoral and dorsal fins, a phenotype strikingly similar to the polydactyly defect found in mouse limbs lacking *Gli3*. Interestingly, *in situ* hybridization analysis in medaka *gli3* mutant pectoral fins revealed no obvious change in the expression domain of genes known to be mis-expressed in *Gli3* deficient limb buds (e.g., *pax9*).
These experiments suggest that the formation of fish fins and tetrapod limbs mainly rely on deep evolutionary conserved developmental programs that, with some modifications but maintaining the same toolkit of genes, can generate a diverse array of appendages forms.

Exploring the origin of vertebrates brain with comprehensive single-cell transcriptome lineages of a proto-vertebrate

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Ascidian embryos highlight the importance of cell lineages in animal development. As simple proto-vertebrates, they also provide insights into the evolutionary origins of cell types such as cranial placodes and neural crest cells. Recently, single-cell RNA-sequencing methods are revolutionizing our understanding of how cells are specified to become definitive tissues during development. Here we have determined single-cell transcriptomes for more than 90,000 cells that span the entirety of development—from the onset of gastrulation to swimming tadpoles—in *Ciona intestinalis*. We used single-cell transcriptome trajectories to construct virtual cell-lineage maps and provisional gene networks for 41 neural subtypes that comprise the larval nervous system. The resulting high-resolution transcriptome trajectories, regulatory cascades and provisional gene networks provide insights into the evolution of novel cell types in vertebrates, including the dual properties of the *Ciona* notochord and the expansion of the vertebrate forebrain. Moreover, we found coronet cells express melanopsin and share additional properties with the saccus vasculosus, a specialized region of the hypothalamus that mediates photoperiodism in nontropical fishes. Comparative transcriptome analyses also identified orthologous cell types for mechanosensory switch neurons, and VP+ and VPR+ relay neurons in different regions of the mouse hypothalamus. These observations provide evidence that the hypothalamus predates the evolution of the vertebrate brain.

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The little skate genome illuminates the evolutionary emergence of exceptionally wide paired fins

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Batoids, including skates and rays, evolved exceptionally wide pectoral fins as well as the flat body to adapt to benthic habitats. The molecular underpinnings of this unique trait, however, remain mostly elusive. Here we investigate the origin of this phenotypic innovation by a combinatorial approach of genome sequencing and embryonic experiments using the little skate *Leucoraja erinacea* as an experimental model. Analysis of a high-quality chromosome-scale genome sequence for the little skate shows that it preserves many ancestral jawed vertebrate features compared with other sequenced genomes. Combining genome comparisons with extensive regulatory landscapes in developing fins – gene expression, chromatin occupancy and three-dimensional (3D) conformation – we find skate-specific genomic rearrangements that alter the 3D regulatory landscape of genes involved in the planar cell polarity pathway. Functional inhibition of PCP signaling resulted in marked reduction of skate fin size, confirming this pathway as a major contributor of batoid fin morphology. Consistently with the previous data demonstrating redeployment of Hox gene expression in anterior pectoral fins, we also identified a fin-specific enhancer that interacts with 3' Hox genes and confirmed the potential of this element to activate transcription in the anterior fin using zebrafish reporter assays. Our findings highlight the central role of genome rearrangement and regulatory changes in the evolution of batoids, shedding light on the molecular origin of exceptionally wide paired fins.

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