From pattern to process: studies at the interface of gene regulatory networks, morphogenesis, and evolution

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Abstract

The development of anatomical structures is complex, beginning with patterning of gene expression by multiple gene regulatory networks (GRNs). These networks ultimately regulate the activity of effector molecules, which in turn alter cellular behavior during development. Together these processes biomechanically produce the three-dimensional shape that the anatomical structure adopts over time. However, the interfaces between these processes are often overlooked and also include counter-intuitive feedback mechanisms. In this review, we examine each step in this extraordinarily complex process and explore how evolutionary developmental biology model systems, such as butterfly scales, vertebrate teeth, and the *Drosophila* dorsal appendage offer a complementary approach to expose the multifactorial integration of genetics and morphogenesis from an alternative perspective.

Introduction

A major goal of developmental biology is to elucidate how the diverse anatomical structures throughout the organism take on their unique shapes from undefined embryonic tissues. The formation of even the simplest three-dimensional structure requires the deployment of tissue-specific gene regulatory networks (GRNs) that operate through transcriptional regulation to ultimately activate cellular effectors (Box 1). Once activated through transcriptional, post-transcriptional, or post-translational mechanisms, these effector molecules function to directly alter ubiquitously expressed proteins, such as the actin cytoskeleton (Box 1). Cumulatively, this results in a context-specific alteration to the mechanical properties of the cell, contributing to formation of the anatomical structure[1–4] (Fig. 1). However, it has been difficult to understand the interface of GRN-effector connections to understand how combinations of cellular effectors are precisely patterned to shape anatomy, and how these GRN-effector connections diversify to modify anatomical form during evolution. The goal of understanding how to connect GRNs to anatomy is

complicated by mechanical or signaling influences from neighboring tissues, that are regulated by different GRNs, painting a complex and interconnected picture of multiple GRNs activated in separate tissues affecting the morphogenesis of a single structure [3,4]. While development can be studied at the level of GRNs, cellular effectors, or biophysical mechanics, it is critical that we comprehend how these distinct systems are connected and influence each other in an integrated way. Here, we will review this developmental phenomenon in epithelial tissues, highlighting several recent studies that illuminate each facet of this problem while emphasizing the novel insights they provide. Further, we propose that the study of evolutionary modifications can provide insights into how these systems interface by examining how GRN-effector connections are modified during evolution, and review up-and-coming evolutionary developmental biology (evo-devo) model systems in which these questions can be explored.

The specification of developmental programs by gene regulatory networks

GRNs control the regulation of gene transcription in time, space, and intensity [1] to generate precise expression outputs that affect tissue morphology (Fig. 1A & Box 1) [5,6]. Recent work on the ventral furrow of *Drosophila melanogaster* illustrates how cell-to-cell variations in gene expression can be generated. A nuclear localized gradient of the transcription factor Dorsal establishes the dorsoventral axis of the embryo, and downstream genes Fog and T48 are expressed in a similar gradient along this axis (Fig. 1)[7]. One might hypothesize cells with the highest levels of nuclear Dorsal would transcribe *fog* and *t48* mRNA at a higher rate. However, the gradients of *fog* and *t48* are instead established in a progressive manner, with cells receiving the strongest Dorsal signal activating transcription earlier than cells receiving lower levels of Dorsal [8]. This causes higher levels of *fog* and *t48* transcripts to accumulate in cells with the highest amounts of Dorsal. These dynamic differences in *fog* and *t48* expression are vital for morphogenesis of the ventral furrow as both genes encode cellular effectors that help establish an activity gradient of non-muscle myosin II that drives apical constriction, which is essential for proper invagination of the ventral tissue (Fig. 1)[9]. Such observations emphasize the important role transcriptional dynamics can play by producing variation in gene expression, which can have fundamental mechanical consequences on morphogenesis.

At the interface between GRNs and effector molecules

As *Drosophila* ventral furrow formation illustrates, GRNs spatially and temporally pattern the level of expression of specific regulatory factors that impart on each cell a unique *trans*-regulatory environment, capable of activating a particular number of cellular effector genes, which control aspects of cellular behavior (Fig. 1B & Box 1) [3,4]. However, it is important to note that there are few universal correlations between the expression of a specific cellular effector and a certain cell behavior, because cellular context is important. Diverse cellular contexts can result from a change in the milieu of co-expressed cellular effectors, but can also result from changes in the cell's mechanical microenvironment, (e.g. strain applied to the cell), which will be discussed in the next section. As the effector repertoire of a cell is greatly influenced by its GRN, disentangling how GRNs interface with collections of

cellular effectors that encode the cell's physical responses represents a pressing need in this field.

The importance of understanding connections between GRNs and their target cellular effectors is particularly well demonstrated by the formation of morphologically diverse denticles on the larva of *Drosophila*. Denticles are actin rich epithelial projections that adorn the ventral surface of *Drosophila* larva[10]. Many signaling pathways interact to regulate the position and development of these structures, and converge to activate the transcription factor, *shavenbaby* (*svb*) [11], which is required for a cell to adopt the denticle fate. In the denticle, Svb regulates cellular effectors that promote various morphogenetic processes including actin reorganization, interaction with the extracellular matrix, and cuticle formation [10,12,13]. Interestingly, *svb* and several of its downstream cellular effector targets are also required for formation of other actin rich projections in *Drosophila*, such as the adult wing hairs, aristal laterals, and adult abdominal trichomes [11]. Although these epithelial projections all require *svb*, they are morphologically quite distinct, raising the possibility that *svb* regulates the formation of rudimentary actin rich projections, but that the final phenotype depends on the cellular context. Rizzo and Besjovec found that the transcription factor SoxNeuro (SoxN) is required to generate distinctive denticle morphologies observed on the larva of *Drosophila* [14]. Both *svb* and *SoxN* are required to activate shared, but also distinct sets of downstream cellular effectors with *svb* controlling denticle height and SoxN regulating width. The authors hypothesized that these two transcription factors respond differently to upstream signaling gradients to generate the diverse phenotypes observed across the *Drosophila* larva.

As this case highlights, similar GRNs can be responsible for generating generic structures whose diverse morphologies are specified by local context. Understanding how this localized context is generated at the GRN level is important, but also vital is understanding how downstream cellular effectors are precisely patterned. Essential to understanding the importance of the outcome of the GRN-effector connection is elucidating how cellular effectors exert influences on cell behaviors.

Intrinsic and extrinsic physical responses to cellular effectors

Once a cellular effector is activated, it can produce intrinsic effects by altering the function of other effectors, for instance, modulating adhesion, remodeling the cytoskeleton, changing the cell's polarity, or targeting of cellular effectors to specific subcellular locations (Box 1) [15]. Once positioned, multiple effectors operate together to dynamically regulate the cell's behavior, for instance, driving migration or initiating cell shape changes (Fig. 1C)[16]. Considering the interconnected nature of an epithelial tissue, these intrinsic changes can have extrinsic influences on neighboring cells and tissues. Extrinsic mechanics can influence or limit the range of shape changes the cells can adopt (Fig. 1D). Within an epithelium, these intrinsic and extrinsic mechanical processes add up to create an integrated physical response leading to distinct cell behaviors, such as cell rearrangement and shape changes. Although far more comprehensive reviews of both intrinsic and extrinsic mechanisms of epithelial cell shape change exist [16–21], we will examine recent examples that illustrate these interactions.

A case of oriented relaxation during dorsal closure in *Drosophila* highlights the importance of both intrinsic and extrinsic contributions to morphogenesis. Recent work has uncovered a mechanism whereby the cytohesin family member Steppke, an Arf-GEF, counteracts the assembly of actomyosin cables at the apical membrane of lateral epidermal cells [22]. Steppke allows cell-cell junctions to relax and the tissue to stretch in response to tension from neighboring tissues during the process of dorsal closure in *Drosophila*. Thus, Steppke operates as an intrinsic factor in lateral epidermal cells by relaxing junctions but also plays a role within the mechanically integrated dorsal closure movements as an extrinsic factor by reducing tension in the amnioserosa to aid in dorsal closure.

Intrinsic and extrinsic mechanisms also contribute to the formation of the ventral furrow during *Drosophila* gastrulation. As mentioned earlier, an intrinsically regulated gradient of non-muscle myosin II activity along the dorsal-ventral axis is important for invagination, however extrinsic mechanics are also vital for proper anterior-posterior orientation of actomyosin arrays within ventral furrow cells. Experimental treatments that changed the overall shape of the embryo or the dimension of the domain of gene expression in the surrounding tissue resulted in uniformly distributed actomyosin arrays in the ventral furrow [23]. This effect could be reversed through a variety of methods (e.g. laser ablations and knockdown of adhesion proteins), which restored directional tension to the ventral furrow cells and resulted in anterior-posterior actomyosin organization. This indicates that the overall shape of the embryo and the pattern of surrounding gene expression impose constraints that result in uneven tensions on the ventral furrow cells, which influences actomyosin organization in ventral furrow cells. This work highlights how intracellular force-generating and load-bearing structures might directly detect and respond to mechanical cues from the surrounding tissue to influence the final phenotype of the structure.

Finally, a striking example of the extrinsic mechanical influence of surrounding tissues on morphogenesis and gene regulation can be found in the patterning of periodic epithelial feather buds in chickens. A recent study reported that the dermal cells spontaneously aggregate below the epidermis due to their own contractility [24]. Dermal cell aggregates cause the overlying epithelial cells to bunch, resulting in the formation of a feather bud placode. Not only are epidermal cell mechanics affected by dermal cells movements, but gene regulation is also altered when β-catenin in the epidermal cells sense the dermal cell aggregation and responds by turning on a follicle GRN [24]. Overall, this suggests that the physical and regulatory state of a cell can be influenced by the mechanical movements of neighboring tissues, similar to how a signaling pathway can alter the regulatory state of neighboring cells through secretion of ligands. This and other similar cases of differentiation in response to extrinsic mechanical influences are being identified [25] and caution against focusing on intrinsic mechanical processes alone, highlighting the importance of examining the relative contributions of both intrinsic and extrinsic mechanical processes to morphogenesis.

Integrating an evolutionary perspective

So far, we have summarized recent work that is purely developmental, spanning a spectrum of morphogenetic processes that integrate GRNs, cellular effectors, and cellular mechanics. Such approaches can be complemented by evolutionary studies employing comparative methods. These studies can identify genetic variants that modify developmental processes, and have the potential to disentangle issues of cell autonomy raised by extrinsic mechanical influences and highlight which intrinsic processes were directly targeted during evolution. Below, we introduce comparative evo-devo model systems that have illuminated different aspects of morphogenesis and present new opportunities for deeper insights into the integration of GRNs, cellular effectors, mechanics, and morphogenesis.

Butterfly scales: connecting GRNs to the elaboration of single-cell appendages

Butterfly wings exhibit an enormous array of color patterns that has inspired numerous developmental, evolutionary, and ecological studies. The colors observed in butterfly wings can be formed by two mechanisms acting within the scale. The first way is through the use of different pigments, which selectively absorb certain wavelengths of light [26]. Many studies over the years have uncovered how some of these pigmentation patterns are genetically controlled. For example, Wnt signaling is responsible for regulating various pigmentation patterns across the wing [27–30].

More relevant to morphogenesis, the other way to form color is through structural changes to the butterfly scale that alters the way light is scattered [26]. The development of these complex scales begins with the projection of an epithelial cell that elongates and flattens to form the scale shape. Once scale morphogenesis is complete, the cell dies leaving both pigments and chitin, which forms the structural components of the scale [31]. The three dimensional shape of each scale is quite intricate, consisting of many chitinous substructures such as the upper lamina, which is composed of ridges and microribs, and the smooth lower lamina, both of which can contribute to structural color [32–34] (Fig. 2A). Because chitin is secreted during the development of this epithelial appendage, there are likely multiple morphogenetic processes during scale development that can be affected to alter structural color.

In the genus *Bicyclus*, structural violet/blue color has independently evolved twice [34]. To determine how this structural color may have evolved, researchers used artificial selection to evolve violet/blue color in a subset of scales of *B. anynana*, a predominately brown-pigmented species without structural color [34]. The authors detected an increase in thickness of the lower lamina of the scales that produce the structural color in both artificially selected and the naturally evolved species, suggesting that the lower lamina may be a common evolutionary target for violet/blue structural color evolution in *Bicyclus*.

How chitin is precisely secreted during butterfly scale development to form these architectural structures is unknown, however a previous study implicated F-actin [31]. In this study, the authors observed single bundles of actin in developing pigmented scales, with rows of chitin secreted between each actin bundle. However, in structurally colored scales of *Agrulis vanillae*, double bundles of actin were observed between the chitin ridges in addition

to an overall increase in the amount of F-actin present during scale formation. This suggests that F-actin organization may play a key role to direct chitin secretion to form the ridges that can impart structural color. In addition, recent research has implicated pigment biosynthesis genes as contributors to chitin structure in the butterfly scale[35]. Mutations in various pigment genes can alter both pigment production and the chitin structure of the scale, which may have possible implications in limiting the potential path of evolution.

The regulatory networks controlling structural color are understudied, but recent research has pinpointed the transcription factor Optix as a repressor of blue structural color in *Junonia coenia*. CRISPR/Cas9 induced knockout mutations of *optix* in *J. coenia* resulted in formation blue structural color in species that normally lack it (Fig. 2A). Interestingly, this study identified two cellular effectors with known roles in F-actin filament organization that were downregulated in *optix* knockouts[36], correlating with the previous findings that F-actin may play an important role in determining where chitin is secreted.

Going forward, butterfly scales represent an excellent comparative model system to identify genes that regulate the morphogenesis of epidermal organs through their role in generating structural color. Of particular interest, artificially selected *B. anynana* strains could be used to genetically map loci that contribute variation in scale morphogenesis. Complementing this approach with descriptions of cellular effectors that are progressively activated during scale formation would illuminate the broader coupling of GRNs with effectors that operate during the formation of these complex structures.

The vertebrate tooth: elucidating evolutionarily important intrinsic and extrinsic mechanisms

Developmental and evolutionary biologists alike have long used teeth as a model system for many reasons, such as the ability to develop them *ex vivo* in culture and their abundant fossil record. Through the years, great progress has been made in understanding the gene network that patterns tooth development, including several signaling pathways expressed in a signaling center required to pattern tooth development (reviewed here [37–39]). Within mammals, there is substantial diversity in tooth morphology, especially in number, shape, and orientation of cusps, which are elevations on the surface of the tooth that often form a point [40]. Much of our knowledge of tooth development comes from research in mammals, fueled by extensive knowledge and tools developed for mice [38], but one very useful approach to study the evolution of tooth morphology is to leverage the extensive fossil record of mammals to infer their ancestral and derived forms. For instance, reduction of Fibroblast Growth Factor 3 (Fgf3) levels in mice and mutations in Fgf3 in humans both lead to a more ancestral tooth morphology, suggesting its involvement in more elaborate morphologies [41]. Another study found that gradual decreases in ectodysplasin (Eda) and sonic hedgehog (SHH) signaling in the mouse were able to mimic a more ancestral phenotype including a reduction in cusp number and loss of cusps on lower molars, highlighting the importance of absolute levels of growth factor signaling for tooth morphology [42]. Together these studies underscore important pathways that may be altered during tooth evolution, establishing promising systems where the connections between signaling events and specific cellular effectors that alter cell shape can be elucidated.

How cellular effectors control tooth shape is largely unexplored, but recent work has identified Rac1 and RhoA, regulators of F-actin, as important players that contribute to differences in tooth shape between gerbils and mice. Between their cusps, gerbils have ridges known as lophs that are missing in mouse (Fig. 2B)[43]. Inhibition of Rac1 or increases in RhoA in gerbils induces cell shape changes that lead to tissue invagination, eliminating lophs between the cusps to mimic the mouse phenotype. Reciprocal experiments to reduce RhoA results in loph-like ridges in mice. This study illustrates how altering expression of cellular effector can elucidate their role in controlling cell behavior and their functional influence on gross morphological difference between species. Future work can begin to connect patterning events to these important cell shape changes.

In addition to changes in signaling pathway activity and intrinsic cellular effectors, surrounding tissues can also influence the shape of teeth. Cusps can form in either parallel or alternating arrays; variations in these patterns have repeatedly evolved (Fig. 2C). Recent work has elucidated that the surrounding jaw influences the pattern of cusp formation through physical constraints [44]. Overall, given the great evolutionary diversity in tooth development, it will be interesting to determine the differences in GRNs regulating the diverse shapes between different vertebrates, in addition to understanding how changes to GRNs in the jaw can extrinsically alter the overall shape of the tooth. More generally, comparative analyses of the co-evolution of signaling networks and morphogenesis operating during tooth formation may highlight ways that epidermal organs are malleable to processes primed to select new adaptive morphologies.

Drosophila dorsal appendage: how similar structures can form by different morphogenetic processes

The dorsal appendage is a tubular structure that forms on the eggshells of Drosophilid species and is utilized for respiration during embryonic development. Its formation is a well-studied developmental process that is accompanied by a striking diversity in number and morphology across species, making it an excellent model to examine the evolutionary origins of integrated GRNs, cell signaling, and cell mechanical systems (reviewed here [45,46]). Early development of these structures begins with the projection of cells from the flat surface of the developing eggshell. Despite gross morphological similarities among species, distinct mechanisms have been found to drive the protrusion of these structures. In *D. melanogaster*, cell rearrangements drive protrusion of the nascent appendages, while in *Scaptodrosophila lebanonensis*, cell shape changes appear to be a major mechanical process in projecting the cells out (Fig. 2D) [47]. Based on the observations of these two distinct cellular mechanisms, researchers recently examined patterning systems that might account for these differences in morphogenesis [48]. The intersection of the BMP and EGF pathways regulate the formation of several eggshell structures including the dorsal appendage. The BMP signaling pathway displays similar patterning in both *D. melanogaster* and *S. lebanonesis*, but, major difference in the patterning of EGF signaling pathway is observed between the species. These changes result in one domain of expression of the transcription factor *broad* from which several dorsal appendages originate, as opposed to the two domains observed in *D. melanogaster* which each produce one dorsal appendage. This detailed knowledge of changes in patterning combined with the drastic difference in morphogenetic

processes between *D. melanogaster* and *S. lebanonensis* positions the dorsal appendage for comparative analyses that can connect genetic changes in signaling pathways to downstream differences that regulate cellular effectors, providing a system to examine the importance of cell context on cellular effector function.

Conclusion

The process of developing an anatomical structure is not a simple one. It may involve many different levels that feedback upon one another and is best viewed from multiple complementary perspectives of GRNs, morphogenetic processes, and biomechanics. Studies often focus on individual steps of this process, but in order to move forward, integrative approaches must bridge these perspectives. We propose that the examination of evolutionary differences, combined with comparative morphogenetic studies can provide unique perspectives to help integrate these fields that will complement existing and upcoming developmental models. In particular, evo-devo model systems in which the contribution of individual genetic variants can be quantified (e.g. through genetic crosses) will be particularly powerful. Such systems will allow one to figure out which cells differ in cellular effector deployment and compare that information to quantitative measures of cell behavior and cell mechanics, facilitating the discrimination of intrinsic from extrinsic physical responses. Identifying these processes and how they have been altered during evolution can be a source of inspiration for engineers seeking novel methods to engineer tissues and treat disease. Above and beyond identifying genetic variants, both developmental and evolutionary model systems will require a deeper understanding of the key cellular effectors, their mechanical consequences, and how their cell-type specific influences on morphogenesis are realized.

Acknowledgements

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Glossary of Terms

Gene Regulatory Network (GRN)

GRNs are composed of signaling pathways, transcription factors, and cellular effectors. Signaling pathways pattern development through cell-cell communication, often resulting in the activation of a transcription factor. Transcription factors regulate gene expression by binding to individual enhancers of downstream genes (e.g. another transcription factor, cellular effector, etc). Each enhancer requires a different set of transcription factors to bind and gene activation will only occur in cells in which the correct sets of transcription factors are present. Overall this complex set of interactions patterns development and governs the final phenotype of the cell [5,6].

Cellular Effector

Any gene that functions to non-transcriptionally activate, localize, or alter other core cellular proteins (e.g. actin, myosin, cadherin, etc.). Cellular effectors can be turned on via transcriptional regulation or can be proteins already present in the cell that are activated by other cellular effectors. Together cellular effectors function to alter the behavior of a cell by changing the mechanics within the cell or through altering mechanical connections to neighboring cells. Cellular effectors can allow a cell to express multiple phenotypes in response to different context without new transcription [3,4].

Intrinsic Mechanics

The combined effect of the cell effectors present within a cell that affects the cell's mechanics.

Extrinsic Mechanics

The combined influence of effectors operating in neighboring cells, transferred via direct cell-cell contacts, to alter a cell's intrinsic mechanics.
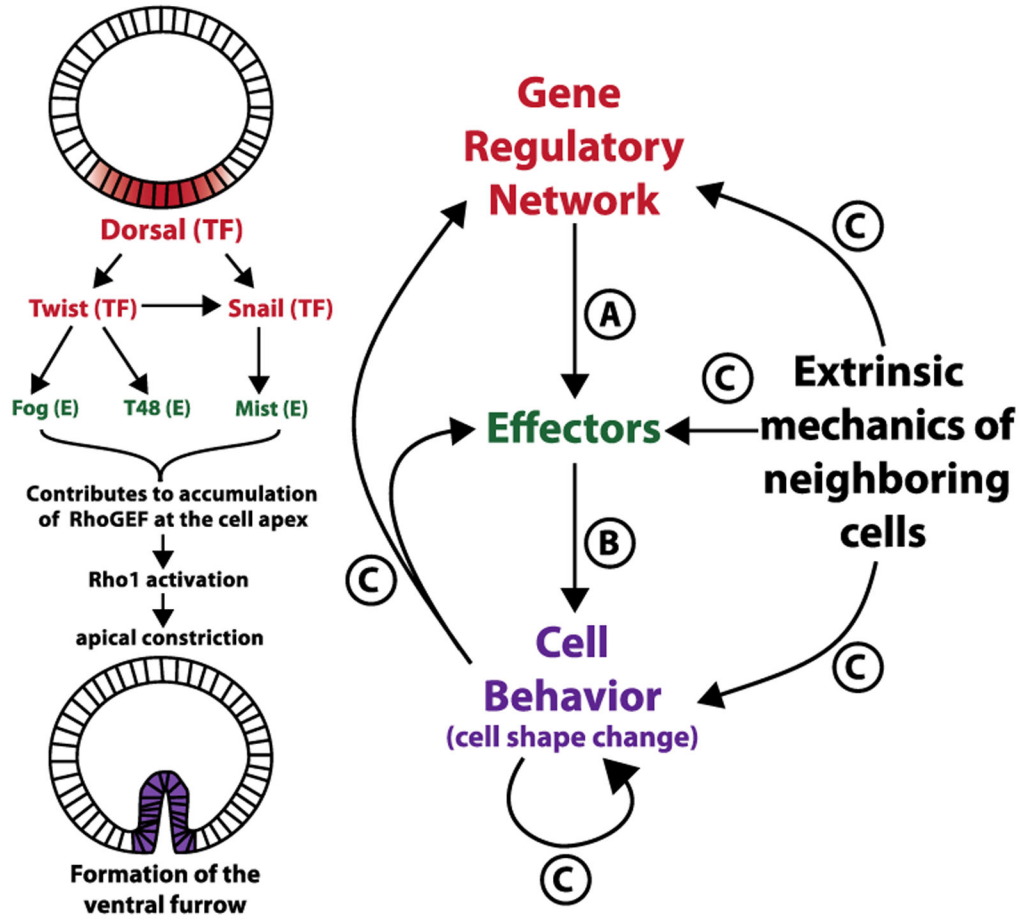
Figure 1. The influence of GRNs, cellular effectors, and neighboring mechanics on tissue morphogenesis.

Morphological structures are pre-patterned by GRNs (red) that turn on a precise set of effector molecules (green). These effectors control cell behaviors, here altering the shape of cells, together forming the final phenotype. (Left) An example of these processes occurring during ventral furrow formation where a nuclear gradient of the transcription factor Dorsal activates downstream transcription factors, which in turn activate effector molecules. These effector molecules accumulate at the apical membrane, causing the cells to apically constrict, and resulting in tissue invagination. (Right) Depiction of the interaction between gene regulatory networks, cellular effectors, and cell behaviors. While networks activate effectors (A), which drive changes in cell behaviors (B), development can also be influenced by signals and mechanical cues coming from neighboring cells in addition to feedback from different parts of the process (C).
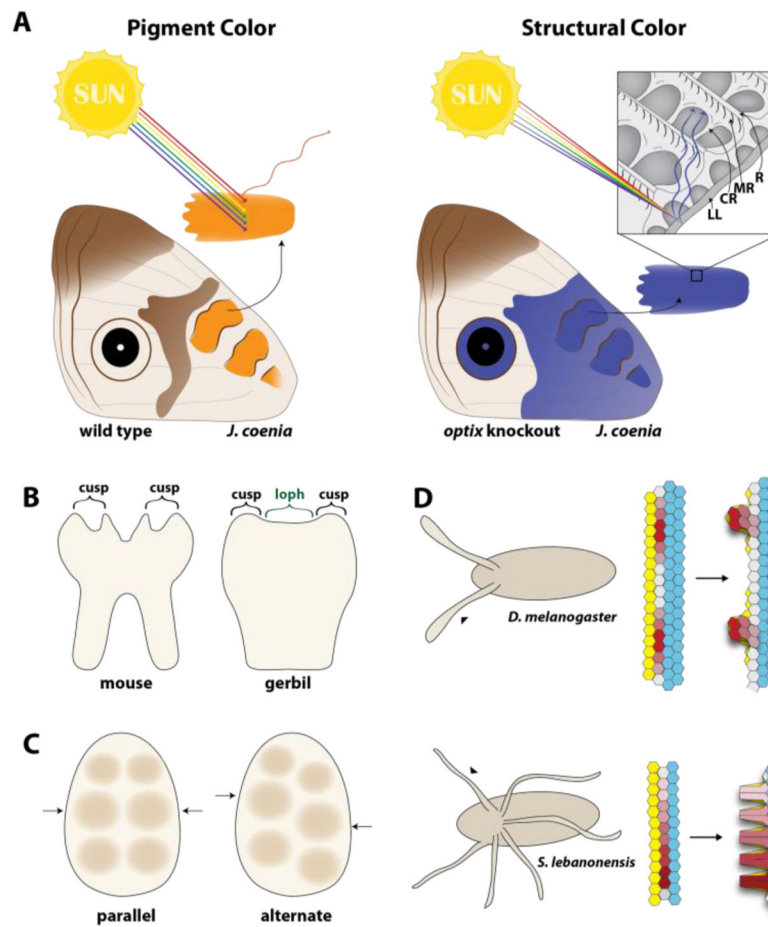
Figure 2. Evo-devo models offer many routes to explore patterning and morphogenesis.
(A) In butterflies, colors can be formed using pigments, such as the ommochrome pigment (orange), which works by absorbing all wavelengths of light except orange. In contrast, structural colors are formed by reflection of light interacting with components of the scale to selectively reflect blue wavelengths in the *optix* knockout. The structure of the scale is quite complex consisting of a lower lamina (LL) connected to an upper lamina composed of ridges (R), microribs (MR), and crossribs (CR). (B-C) Differences in rodent molar morphology. (B) In a cross-sectional view of the mouse molar, cusps are separated, but in gerbils cusps are connected by a ridge called a loph. (C) Occlusal view of an adult tooth with cusps represented by circles. Cusps can either be parallel or alternate in their placement on the tooth. (D) Morphology of the dorsal appendage (arrowhead) of the *Drosophila* eggshell differs in placement and number between species. Early morphogenesis of these homologous structures also differ, with the dorsal appendage of *D. melanogaster* forming through neighbor exchange events (red cells) and the dorsal appendage of *S. lebanonensis* (also known as *S. pattersoni*) forming through cell shape changes (red cells).