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Dissecting Organismal Morphogenesis by Bridging Genetics and Biophysics

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Abstract

Multicellular organisms develop complex shapes from much simpler, single-celled zygotes through a process commonly called morphogenesis. Morphogenesis involves an interplay between several factors, ranging from the gene regulatory networks determining cell fate and differentiation to the mechanical processes underlying cell and tissue shape changes. Thus, the study of morphogenesis has historically been based on multidisciplinary approaches at the interface of biology with physics and mathematics. Recent technological advances have further improved our ability to study morphogenesis by bridging the gap between the genetic and biophysical factors through the development of new tools for visualizing, analyzing, and perturbing these factors and their biochemical intermediaries. Here, we review how a combination of genetic, microscopic, biophysical, and biochemical approaches has aided our attempts to understand morphogenesis and discuss potential approaches that may be beneficial to such an inquiry in the future.

1. INTRODUCTION

Development entails a complex interplay between patterning and morphogenesis. Developmental patterning refers to the emergence of order in a seemingly unordered biological system through the specification of different cell fates in a position- and time-dependent manner. Morphogenesis, instead, is the process by which a system attains its form (or shape) by integrating chemical and mechanical signals with the spatiotemporal information provided to it via patterning.

The word morphogenesis has a Greek etymology (from morphê, meaning shape, and genesis, meaning creation). This process occurs at different scales, from cellular to tissue, organ, and organismal. It is through morphogenesis that the correct (and often characteristic) form is attained at each of these scales. An incorrect form can cause serious defects, such as anencephaly in humans, a severe malformation of the brain caused by the failure of neural tube closure. Thus, unraveling the guiding principles of morphogenesis is crucial for understanding not only development but also the basis of various congenital diseases. It is, therefore, not surprising that morphogenesis has been a subject of intense scientific investigation over the past several decades.

Early research in morphogenesis includes pioneering work on Entwicklungsmechanik (developmental mechanics) by Wilhelm Roux and Wilhelm His in the nineteenth century, who changed morphogenesis from being a descriptive science to one exploring the underlying mechanisms (36, 97). Roux was one of the first researchers to propose that epigenesis can be explained as a mechanical outcome of cell dynamics. However, it was His who, through a combination of improved experimental designs (e.g., the microtome for thin sectioning) and integration with cell theory, demonstrated a role for cellular mechanics (behaviors such as cell migration) in the genesis of the nervous system and the growth of embryonic nerve cells (36). His also showed that germ layers developed through cell delamination, thus refuting Ernst Haeckel's theory, which proposed their formation during phylogeny from an ancestral form, the gastrea (50, 167). In addition to the work of Roux and His, D'Arcy Thompson authored two editions of *On Growth and Form* (1917 and 1942), a book in which he pioneered the use of mathematics to explain the shape of animals and plants, thereby laying the foundation for much of the recent research on morphogenesis at the interface between developmental biology, physics, and mathematics.

Initial discoveries in morphogenesis were particularly impressive, given that they were made despite severe technological limitations. Since then, the advent of novel technological tools, such as high-resolution microscopy and genetically encoded fluorescent proteins, combined with the establishment of new model organisms amenable to both genetic and cell biological manipulations, such as the fruit fly *Drosophila melanogaster*, the nematode *Caenorhabditis elegans*, and the zebrafish *Danio rerio*, has led to many new discoveries on the molecular, cellular, and biophysical mechanisms by which an organism takes shape. In this review, we summarize and discuss how research on organismal morphogenesis has benefited from recent technological and methodological advances in the fields of genetics, microscopy, biophysics, and biochemistry.

2. UNDERSTANDING MORPHOGENESIS THROUGH GENETIC APPROACHES

2.1. Forward Genetic Screens

Classical genetics relies on the manifestation of phenotypes that provide geneticists a gateway into biological processes. Using this idea, several researchers have performed forward genetic screens, which mostly involve inducing random mutations (e.g., through treatment with mutagenic agents such as ethyl methanesulfonate or N-ethyl-N-nitrosourea) in the animals and screening for those that exhibit phenotypes of interest. For example, a screen aimed at identifying genes required for locomotion may select mutants with impaired motility. Such mutants are then characterized

and analyzed further to identify the gene(s) that were mutated to generate the phenotype, thus enabling geneticists to assign functions to genes. This makes forward genetics especially effective in contexts in which limited information regarding genes involved in a process is available. In this section, we present examples in which forward genetic approaches were successfully employed in uncovering novel genes or pathways responsible for some of the most fundamental morphogenetic processes in the early development of three of the most widely studied model organisms in developmental biology.

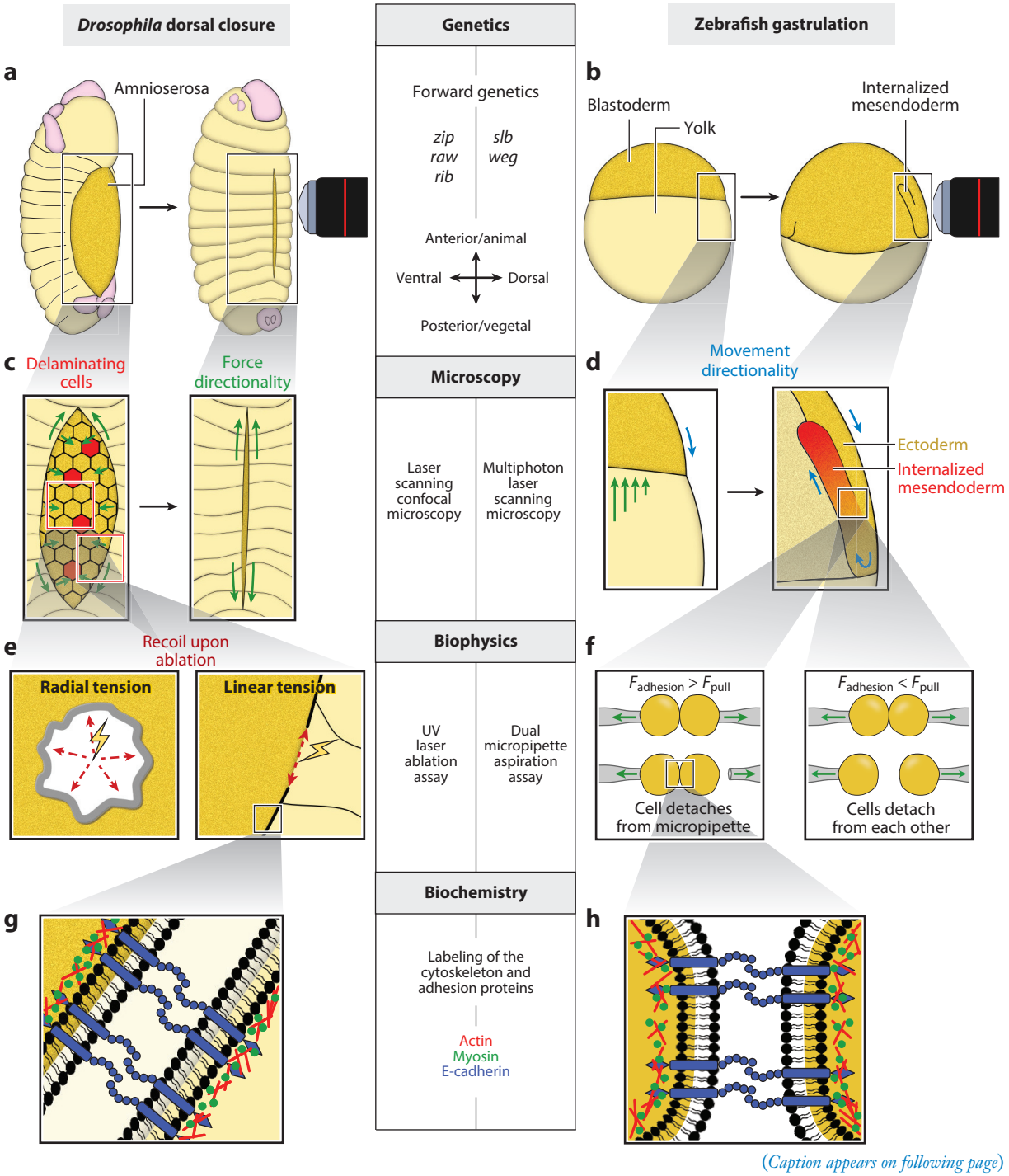
2.2. Studies in *Drosophila melanogaster*

Genetics is an extremely powerful tool, especially in pursuit of novel regulators of biological processes. The potential of this tool was most clearly demonstrated by the work of Christiane Nüsslein-Volhard and Eric Wieschaus in 1978 dissecting the genetic basis of early embryonic patterning in *Drosophila* through a forward genetic screen (177). The screen identified and characterized 600 mutants, the mutations in which were mapped to 120 genes (177). Some of the most important polarity and patterning genes were identified in this screen. Subsequent forward genetic screens also identified other important regulators of embryo polarity establishment and body axis formation, such as *nos* (*nanos*), *tor* (*torso*), *dl* (*dorsal*), and the first ever identified morphogen *bcd* (*bicoid*) (42, 115, 139, 146, 159).

Furthermore, forward genetic screens have also proven fruitful in dissecting how embryo patterning affects various morphogenetic processes during *Drosophila* early embryogenesis, such as ventral furrow formation, mesoderm and endoderm invagination, germ band extension and retraction, and dorsal closure. Two genes, *twi* (*twist*) and *sna* (*snail*), for example, were identified through unbiased screening approaches (117). Both genes encode transcription regulators important for ventral furrow formation and mesoderm fate specification and act downstream of *dl*, another transcription factor–encoding gene (16, 151, 166) known to be highly enriched in the ventral nuclei (139). The expression of *dl* leads in turn to the expression of *sna* and *twi*, which together define the mesoderm primordium (48, 65, 86). *sna* represses the expression of ectodermal genes, whereas *twi* activates genes required for a mesodermal fate (93). In embryos lacking *twi* or *sna*, ventral furrow formation is defective, and in double mutants, it completely fails (48, 151). Similar observations have also been made in embryos lacking *dl* (151). Thus, at the most fundamental level, embryo patterning and polarization along the dorsoventral axis are essential for ventral furrow formation and the specification of mesodermal fate.

In contrast, embryo patterning, mainly along the anteroposterior axis, is important for germ band extension (66, 83). Germ band extension is driven by cell intercalation along the anteroposterior axis, which relies on the presence of stripes of cells with different adhesive properties (66). The striped nature of the embryo depends on the genes that establish the various body segments and their polarities. Therefore, in embryos with altered segmentation gene expression, such as mutants of *pair-rule* genes, both cell intercalation and germ band extension are significantly reduced (66). Similarly, several genes required for germ band retraction were also identified through forward genetic screens, e.g., *tup* (*tail up*), encoding a transcription factor; *ush* (*u-shaped*), encoding a member of the FOG (friend of GATA) protein family; and *hnt/peb* (*hindsight/pebbled*), encoding a transcriptional attenuator (13, 28, 117, 178).

The opening of the epidermis appearing on the dorsal side of the embryo as a result of germ band retraction is sealed through a process called dorsal closure. Forward genetic screens led to the discovery of several genes regulating different aspects of dorsal closure. For instance, through a screen for mutations on the second chromosome, *zip* (*zipper*), *raw*, and *rib* (*ribbon*) mutants were identified, all of which exhibit defects in dorsal closure (117) (**Figure 1*a***). *zip* encodes myosin heavy chain, which, along with actin, forms a large supracellular actomyosin cable or ring at



(*Caption appears on following page*)

Figure 1 (*Figure appears on preceding page*)

Multiple approaches used to dissect key morphogenetic processes in *Drosophila* and zebrafish embryogenesis. This schematic representation shows the use of different experimental approaches to study *Drosophila* dorsal closure (*left panels*) and zebrafish gastrulation (*right panels*). (*a*,*b*) Schematic illustrations showing the sealing of the amnioserosa in *Drosophila* (panel *a*) and the internalization of the mesendoderm in zebrafish (panel *b*). The genes listed in the center exemplify regulators of both processes isolated through forward genetic screens. (*c*,*d*) Illustrations of the use of high-resolution confocal or multiphoton microscopy techniques to visualize cellular-, tissue-, and embryo-scale events occurring during both processes. During *Drosophila* dorsal closure, several cells in the amnioserosa exit the plane of the tissue through delamination, and the lateral epithelial cells elongate dorsoventrally concomitant with a purse-string-like behavior of the leading edge cells to seal the amnioserosa (panel *c*). Similarly, in zebrafish gastrulation, cells at the margin of the blastoderm internalize and form the mesendoderm, while the non-internalizing cells further away from the margin give rise to the ectoderm (panel *d*). (*e*,*f*) Illustrations showing the use of biophysical techniques to analyze the mechanical properties of cells and tissues during both processes. During *Drosophila* dorsal closure, ultraviolet (UV) laser cutting was used to determine mechanical tension in the amnioserosa and the neighboring epidermis (panel *e*). The amnioserosa experiences isotropic, radial tension, whereas the leading edge of the epidermis experiences anisotropic, linear tension. During zebrafish mesendoderm internalization, the adhesion strength between germ layer progenitor cells was determined by measuring the cell-cell detachment force using dual micropipette aspiration (panel *f*). Ectoderm progenitors display higher homotypic adhesion strength (*F*) than do mesoderm or endoderm progenitors. (*g*,*h*) Visualization of the subcellular localization of the cortical actomyosin network and E-cadherin in adhering cells through labeling (e.g., fluorescence labeling) of cytoskeletal and adhesion proteins during *Drosophila* dorsal closure (adhesion between epidermis cells and amnioserosa cells) (panel *g*) and zebrafish mesendoderm internalization (adhesion between ectoderm progenitor cells) (panel *h*).

the leading edge of the epidermis surrounding the amnioserosa required for zipping it shut, while *raw* encodes a JNK (c-Jun N-terminal kinase) signaling protein, and *rib* encodes a nuclear BTB-domain protein predicted to have DNA-binding transcription factor activity (17, 21, 184). All three genes are required for the dorsoventral elongation of epidermal cells bordering the dorsal opening, and the localization and activation of myosin at the leading edge of those cells (14). Several other genes controlling cell adhesion, polarity, signaling, and transcription during dorsal closure were also identified through forward genetic screens (75, 110, 130, 178).

Finally, forward genetic screens in *Drosophila* have also been instrumental in elucidating the genetic basis of organ morphogenesis. For example, fly wing morphogenesis, an excellent model for organogenesis, is driven by planar cell polarity (PCP) signaling, which is required to polarize cells within the plane of the tissue (185). Depending on the genes involved, PCP pathways can be classified as core or global PCP pathways: The core PCP pathway involves some of the same genes as the classical Wnt pathway but acts in a β-catenin-independent manner and is, thus, also referred to as a noncanonical Wnt/PCP signaling pathway, while the global PCP pathway centers around the activity of the atypical cadherins Fat and Dachsous (1, 30, 181). Both core and global PCP genes were first isolated through forward genetic screens in flies (24, 116, 130, 131). Several PCP components exhibit an asymmetric cortical distribution, which is essential for the establishment of polarity and drives directed cell migration, cell division, and tissue growth (185). In flies with compromised PCP signaling, such as loss-of-function mutants for *fz* (*frizzled*) genes, wing morphogenesis is defective (24). More specifically, such flies exhibit a loss of wing margin bristles and the absence of nearby regions, producing a notched wing. In addition, the wing bristles are also disoriented in these flies, indicating the loss of planar cell polarity. Similarly, flies lacking global, Fat- or Dachsous-mediated PCP signaling also exhibit defects in wing size and hair polarity (182). More recently, several new genes participating in the PCP pathways have been isolated that were likely missed previously due to their roles in additional processes (182).

2.3. Studies in *Caenorhabditis elegans*

Drosophila is not the sole model organism in which forward genetic approaches were instrumental in dissecting the genetic basis of embryogenesis and organogenesis. In fact, the *Drosophila* screen by Nüsslein-Volhard and Wieschaus was modeled on screens previously performed by Sydney

Brenner between 1965 and 1974 using the nematode *C. elegans* (18). Brenner was interested in studying the development of the nervous system. He was convinced that he required a simple genetic model organism in which it would be possible to "determine the structure of the complete nervous system" (18, p. 72). However, *Drosophila*, the more established genetic model at the time, with approximately 10^5 neurons (in adults), did not fit this criterion (18). Therefore, he established the nematode *C. elegans* as a model for his study. *C. elegans* has a short life cycle (~3 days at 20°C) and is sexually dimorphic; i.e., both male and female reproductive machineries are present within one hermaphroditic individual (18). Lastly, and most importantly, it has a small number (959) of somatic nuclei and only 302 neurons in total (161, 163). Given its simple construction, the ease of its genetic manipulation, and its short developmental time, *C. elegans* was an obviously powerful model organism for genetic analyses.

Brenner performed a landmark forward genetic screen by introducing random mutations in the *C. elegans* germline and selecting mutants defective for movement (as a proxy for defects in the development of the nervous system), morphological abnormalities, and differences in size and shape (18). He isolated 619 mutants and classified them based on their phenotypes, thus laying the foundation for several broad categories of *C. elegans* genes and phenotypes. Some of the most important of these genes are those for movement defects [*unc* (*uncoordinated*) and *rol* (*roller*)], abnormal size [*lon* (*long*) and *sma* (*small*)], and morphological defects [*dpy* (*dumpy*)].

Subsequent forward genetic screens from Robert Horvitz's group (27, 37, 59) identified genes constituting the highly conserved apoptotic pathway in *C. elegans*: *egl-1* (*egg-laying defective*), *ced-9* (*cell death abnormal*), *ced-4*, and *ced-3*. The apoptotic pathway genes have since also been shown to play important morphogenetic roles, such as in the regulation of cell and body size, asymmetric cell division, and cell fate specification (26, 108, 176). Another forward genetic screen by Thorpe and colleagues (168) identified genes required for endodermal fate specification by selecting for mutants with excess mesoderm. Five *mom* (*more mesoderm*) genes (*mom-1* to *mom-5*) were identified and were subsequently shown to encode members of the Wnt signaling pathway.

Forward genetic screens have also identified genes underlying organogenesis. For instance, by selecting for mutants with defective feeding behaviors, genes required for pharyngeal morphogenesis and functioning were identified, thereby establishing three classes of genes: *pha* (*defective pharynx development*), *phm* (*pharyngeal muscle*), and *eat* (*eating*: abnormal pharyngeal pumping) (7). Similarly, other genetic screens have uncovered entire pathways driving organogenesis, for example, for vulval development through selection for morphologically recognizable phenotypes [e.g., *vul* (*vulvaless*) and *muv* (*multivulva*)] (162). Collectively, these examples clearly demonstrate the success of forward genetic approaches in *C. elegans* in dissecting morphogenetic processes in varied biological contexts.

2.4. Studies in Zebrafish

Till the early 1990s, the most effective approach for identifying genes performing necessary functions in vertebrates was based on their high degree of homology with similar genes previously identified in invertebrates, such as *Drosophila* and *C. elegans*. In 1996, however, two groups—Christiane Nüsslein-Volhard's group in Tübingen, Germany, and Wolfgang Driever's group in Boston, USA—published results obtained from forward genetic screens for embryonic patterning and morphogenesis mutants performed through random mutagenesis of zebrafish (35, 51, 114).

The Tübingen and Boston screens identified and analyzed 1,858 mutants that were assigned to 592 genes. These genes were identified as being necessary for normal embryonic development. Besides various mutants with defects in embryo polarization and patterning, several other mutants were identified that appeared to be predominantly affected in morphogenesis. For instance, four

mutants, *half baked*, *lawine*, *avalanche*, and *weg* (which were later identified to harbor mutations in the same gene, *cdh1*, encoding a type-1 E-cadherin), display defective epiboly movements and germ layer boundary formation during gastrulation, eventually causing embryonic lethality (8, 9, 76, 77, 109) (**Figure 1*b***). In addition, *cdh1* is provided maternally and is also important for blastomere cohesion at early cleavage stages (9). Another example is the *pac* (*parachute*) mutant, which was discovered to carry a mutation in a gene encoding N-cadherin and shows defects in neurulation as a result of reduced neuroectodermal cell cohesion (71, 92).

In addition, numerous mutants (e.g., *pipetail*, *silberblick*, *knypek*, and *vang-like 2*), which were later identified to encode different components of the Wnt/PCP pathway, show disturbed gastrulation and altered patterns of convergence and extension through defects in cell adhesion and cytoskeletal organization, processes that are key to cell polarization and migration (52, 56, 57, 70, 126, 133, 137, 155, 170) (**Figure 1*b***). Likewise, genes mutated in *bashful*, *grumpy*, and *sleepy* mutants were later shown to encode different laminin-α1 subunits. These mutants exhibit defective axonal guidance as well as abnormal brain and notochord development (78, 118, 127, 128). Collectively, these findings show that, similar to the situation in invertebrate model organisms, forward genetic screens led to the identification of key pathways and gene families involved in regulating vertebrate early embryo morphogenesis.

2.5. Is Genetics Sufficient to Understand Morphogenesis?

These examples are a testimony to the success of genetics as a tool in our pursuit of mechanisms underlying key developmental processes, which range from cell birth (cell division) to the specification of its fate (death, differentiation, or pluripotency) and from the establishment of polarity in a tissue to its patterning, thereby leading to organogenesis. The scientific community has exploited this strength of genetics and made significant progress in understanding the genetic underpinnings of development. However, purely genetic approaches have certain limitations, some of which are well exemplified by the type of genes identified in the screens described in this section. For instance, most genes identified in the screen for patterning in *Drosophila* embryos by Nüsslein-Volhard and Wieschaus were upstream regulators, such as transcriptional regulators, which, when mutated, typically induce defects in both embryo patterning and morphogenesis. In contrast, mutants from forward genetic screens, which only display defects in morphogenesis, are less frequent, likely due to downstream effector proteins being involved in various morphogenetic processes and, thus, when mutated, leading to pleiotropic phenotypes. That said, classic forward genetic approaches, in which gene function is derived from embryo-wide loss-of-function phenotypes, might be insufficient to uncover the mechanistic basis of embryo morphogenesis. Rather, describing certain morphogenetic processes of interest using various analytic tools and techniques is required to predict certain molecular and cellular mechanisms at work, the genetic basis of which can then be addressed through reverse genetic approaches such as candidate gene knockouts. Controlling those reverse candidate gene inactivation approaches in both space and time is of particular importance, given that uniform gene inactivation might lead to early or pleiotropic phenotypes that mask the gene's function in a specific morphogenetic process. In addition, rapid progress in genomic techniques, such as single cell sequencing, has considerably facilitated the identification of candidate molecules and genes involved in cell fate specification and differentiation, the morphogenetic function of which can then be analyzed through reverse and conditional genetic approaches. In the following sections, we summarize and discuss how the development of various analytic tools has helped in dissecting the molecular, cellular, and biophysical bases of organismal morphogenesis in development.

3. HIGH-RESOLUTION MICROSCOPY TECHNIQUES USED TO STUDY MORPHOGENESIS

3.1. Microscopy Techniques Complement Genetic Approaches

Morphogenesis is the result of a complex interplay of events occurring at different scales, ranging from cell- and tissue-scale rearrangements and deformations to molecular-scale relocalization, (in)activation, and (de)stabilization of the underlying components. While genetics has been successful in identifying new genes responsible for various morphogenetic processes, much of our understanding of how these genes function to regulate such processes is the result of a combination of genetics with other tools and techniques. One necessary aspect to building a mechanistic model of morphogenesis is the ability to visualize such processes, a need that is addressed by microscopy. Recent decades have seen unprecedented improvements in microscopy techniques, which have enabled us to gain insights into morphogenesis through investigation at both small and large scales. In this section, we discuss examples demonstrating how our improved abilities to visualize morphogenetic processes in great detail through high-resolution microscopy have aided our attempts to understand morphogenesis. Depending on the strengths of each microscopy technique, we have classified them into three mutually nonexclusive categories, each of which indicates the scale at which a technique is best used.

3.2. At the Cellular and Tissue Scale

Cells and tissues are the fundamental units of organs and organisms. They act as mediators to link events occurring at the subcellular, molecular scale to those at the larger, organ/organismal scale. We therefore begin by describing the microscopy techniques available to investigate events occurring at the cellular and tissue scale.

Depending on the process and the biological system, specimens examined for a cellular- and tissue-level understanding of morphogenesis can be labeled or unlabeled. Differential interference contrast (DIC) microscopes are excellent for the latter case, as they exaggerate the contrast within a sample without the need for dyes to color the specimen and are especially effective with transparent samples. For instance, the first apoptotic genes were identified by examining *C. elegans* embryos and larvae for the presence of dead cells, which, unlike live cells, appear highly refractile when visualized using DIC microscopy (37, 55). In addition, hundreds of genes necessary for distinct processes during *C. elegans* early embryonic development, including the regulation of embryonic shape and cell divisions, were also identified through DIC microscopy (46). However, except in certain instances, DIC microscopy fails to provide the ability to unequivocally distinguish the identity of visible structures. This limitation is addressed by fluorescence microscopy, with which specific molecules, structures, and organelles can be distinguished based on their fluorescent labels.

Perhaps the most widely used fluorescence microscopy technique is confocal microscopy, two forms of which—laser scanning confocal microscopy (LSCM) and spinning disk confocal microscopy (SDCM)—are regularly employed. Confocal microscopes provide extremely thin optical sectioning of biological samples, thus improving our ability to distinguish between signals from objects in close proximity to each other, and have been employed in diverse scenarios in which spatial resolution is key. For example, it was through confocal microscopy that the cortical actomyosin flows in the *C. elegans* zygote were first visualized (111). In addition, these anteriorly directed flows were also shown to transport anterior polarity regulator proteins, such as PAR-3 and PAR-6, to the anterior, which in turn promote the flows, whereas PAR-2, a posterior determinant, inhibits the accumulation of actomyosin in the posterior, thus leading to the establishment of anteroposterior polarity (111). Furthermore, confocal microscopy enables the simultaneous

tracking of morphogenetic processes at different scales, such as cellular, tissue, and cytoskeletal dynamics during *Drosophila* dorsal closure (68) (**Figure 1*c***). The simultaneous illumination of large portions of live samples with powerful lasers, however, can also cause undesirable outcomes, such as photobleaching and phototoxicity. Therefore, although faster acquisition speeds with SDCM ameliorate such effects, they still remain a major drawback of confocal microscopy.

3.3. At the Organismal Scale

Cells exhibit diverse behaviors that collectively shape an organism. To understand how these behaviors are integrated at the organismal scale, it is usually necessary to visualize events occurring at scales much larger than that of individual cells or tissues. This necessity is usually associated with certain technical challenges. For instance, large samples need to be exposed to laser beams for extended periods of time, a problem that is further compounded by the use of stronger lasers in cases in which the sample is also thick. This leads to high levels of photobleaching and phototoxicity, both of which are detrimental to the object of the examination. This challenge can be overcome through the use of the gentler, yet still effective, microscopy techniques described next.

Light sheet microscopy (LSM) is a highly effective tool for fast, long-term live imaging. LSM selectively illuminates only those planes within the sample that are being scanned by the detector at any given time. This reduces phototoxicity while simultaneously providing thin optical sectioning (64, 173). Perhaps the most impressive examples of the applications of LSM are the in toto imaging of fish and fly embryos during early embryogenesis in which individual nuclei or cells were successfully tracked (79, 169). Other examples include the visualization of embryonic symmetry breaking, germ layer formation, and neuronal circuit formation and activity during fish, fly, and worm embryonic and postembryonic development (3, 90, 95, 120).

Another limitation of the microscopy techniques discussed so far is poor image quality deep inside thick samples without strong laser illumination. This limitation can be addressed through the use of multiphoton microscopy. Multiphoton microscopes employ high wavelength laser beams that penetrate deeper into the sample while causing limited phototoxicity. Through the excitation of fluorescent molecules in a subfemtoliter volume by simultaneous illumination by multiple (mostly two) photons, multiphoton microscopes render pinholes unnecessary (29). This technique has been successfully used, for instance, to image nuclear and cell divisions in fly and zebrafish embryos, to track invaginating mesoderm cells during fly and zebrafish gastrulation, to visualize zebrafish neural crest cell migration, and to determine neuronal structure and activity in the brains of living mice (22, 106, 121, 125, 171, 179) (**Figure 1*d***). A key limitation of multiphoton microscopy, however, is that the speed of image acquisition is slow, usually similar to that of LSCM, thus providing recordings with lower temporal resolution as compared to, for example, SDCM.

3.4. At the Molecular Scale

All morphogenetic processes have an underlying cause at the molecular scale. For instance, the molecular structure, stability, and organization of cytoskeletal components and adhesion molecules dictate the choice of cell behaviors and assist in the chosen behavior's execution. Therefore, to understand the molecular mechanism of morphogenesis, we must also be able to visualize events occurring at this scale. However, the light microscopy techniques described so far lack the ability to resolve signals closer than the diffraction limit, which presents a formidable challenge to molecular imaging. This limitation can be addressed through the use of

super-resolution microscopy techniques, such as structured illumination microscopy or stimulated emission depletion microscopy, which provide spatial resolutions greater than that provided by conventional light microscopy. The power of these techniques for studying morphogenesis becomes evident, for instance, with experiments that have addressed the subcellular organization of integrins at cell–extracellular matrix (ECM) contacts, key regulators of cell and tissue morphogenesis, showing that integrins segregate into distinct nanoclusters, each enriched in inactive or active integrins (157). Thus, super-resolution microscopy is an effective tool to gain a molecular understanding of morphogenesis. One major drawback of super-resolution microscopy techniques, however, is that they often adversely impact the health of live samples, such as embryos, due to high excitation intensity and long exposure times.

Another microscopy technique that can be used to obtain a high spatial resolution is electron microscopy, which relies on the scattering of electrons by heavy atoms in a sample. Electron microscopy can provide a spatial resolution of up to 47 pm (versus 200 nm for light microscopes), thus enabling the detailed visualization of biological samples, and have, therefore, been previously used to determine the ultrastructure of fibrillar polymers such as microtubules (5, 38). Scanning electron microscopy (SEM), for instance, has recently been employed to study the formation of the micropyle, a structure formed by granulosa cells surrounding the maturing zebrafish oocyte (102, 148, 180). More specifically, an enrichment of intermediate filaments in granulosa cells giving rise to the micropyle, observed by SEM, was key to uncovering the mechanism driving micropyle formation. However, while electron microscopy provides high spatial resolution, one of its major limitations is that it requires fixed samples, thus rendering live imaging impossible.

While microscopy is an excellent tool to study morphogenesis, there are some important considerations that investigators should note, especially when employing fluorescence microscopy. For instance, with fluorescently labeled reporters, it is important to be mindful of the undesirable effects that the tagging of proteins can have on their localization and function. Such artificial setups can impede, enhance, or altogether alter a protein's function, thus providing an inaccurate representation of its dynamics and activity. These effects can be ameliorated to some extent through the use of comparatively shorter fluorescent proteins or dyes that may be relatively unlikely to interfere with a protein's folding and/or its interactions with other partners. Furthermore, most traditional tools for fluorescent labeling require an overexpression (or introduction of high levels) of the protein of interest. Such high concentrations, even in the absence of additional fluorescent tags, can lead to artifacts. These artifacts can be minimized through single-copy transgene insertion techniques, such as MOS1-mediated single copy insertion (MosSCI), which introduces only one additional copy of a given gene into a haploid genome, or preferably CRISPR-Cas9, which can fluorescently label the endogenous protein without overexpressing it (43, 44, 72).

In summary, microscopy enables us to visualize morphogenesis on both the macroscopic and microscopic scales. This is an important step toward building mechanistic models of morphogenesis; to address this functionally, however, requires further approaches providing insights into the biophysical and biochemical basis of morphogenesis. In Sections 4 and 5, we describe these approaches.

4. BIOPHYSICAL TOOLS USED IN MORPHOGENESIS

4.1. Physical Properties of Cells and Tissues in Morphogenesis

So far, we have mainly discussed examples of forward genetic approaches that have been instrumental in identifying key genetic regulators of various morphogenetic processes and tools that have allowed visualizing morphogenesis in action. However, genetic instructions, such as gene

expression, need to be translated into biologically interpretable effectors or stimuli, such as mechanical forces, which are the executors of the small- and large-scale growth and rearrangements driving morphogenesis.

Mechanical forces in biology can be generated in various ways, e.g., by the contraction of the actomyosin cytoskeleton or the polymerization/depolymerization of microtubules (32, 144). These forces, when strong, are sufficient to effect shape, size, and position changes at the level of single cells (32, 144). In addition, if transmitted to neighboring cells (e.g., through cell-cell adhesion), these forces can also drive large-scale, tissue-level changes (91). Force generation and transmission rely on gene expression and may, therefore, also depend on genes dictating cell fate. In turn, the specification and execution of cell fate can be influenced by mechanical forces, thereby forming a mechanochemical feedback loop between the gene regulatory networks underlying cell fate specification and the mechanical processes driving cell and tissue morphogenesis (25, 100, 101, 105, 113, 129, 174). Finally, how cells and tissues respond to forces also depends on their material properties (e.g., viscoelasticity). Therefore, understanding the forces at play, their transmission and sensing, and the material properties of cells and tissues are necessary first steps toward constructing a mechanical model of morphogenesis. In this section, we describe tools and techniques that enable such measurements.

4.2. Tension Measurements

Cytoskeleton-derived forces drive several morphogenetic processes, including cytokinesis, spindle positioning, chromosome separation, and cell shape regulation (32, 143, 144, 152). The expansion or contraction of cytoskeletal networks, when resisted by coupling to other cellular structures, generates mechanical tension on a cellular scale but also on a tissue scale when several cells are mechanically coupled. Therefore, mechanical tension is often used as a proxy for the forces that generate it. Some of the most commonly used biophysical tools and techniques to measure mechanical tension are described in this section.

One frequently used method to determine mechanical tension is ablation with an ultraviolet (UV) microbeam. For example, the *C. elegans* zygote divides asymmetrically through the asymmetric positioning of its spindle in the posterior half of the zygote. UV laser ablation of the central spindle was used to show that a greater external pulling force acts on the posterior spindle pole than on the anterior spindle pole (49). More specifically, upon laser ablation, the posterior pole recoils with a greater velocity and exhibits greater displacement. However, for the zygote to position the spindle asymmetrically along the anteroposterior axis, an initially symmetric zygote must first polarize, a process that largely depends on actomyosin cortical flows (45, 60, 111). UV ablation experiments have also shown that anisotropies in cortical tension drive these flows in the zygote, thereby polarizing it (104). Similarly, laser ablation experiments during *Drosophila* dorsal closure have also shown that the leading edge of the lateral epidermis is under tension and that it acts like a supracellular purse string to seal the amnioserosa, which experiences isotropic tension (81) (**Figure 1*e***).

Another means of measuring mechanical tension is through the use of single cell force spectroscopy (SCFS). For instance, SCFS has been employed to measure the cortical tension in different germ layer progenitors in zebrafish (87). To that end, a colloidal force probe was used to cause indentations in individual progenitors, and the force-indentation curves were recorded using atomic force microscopy. These curves were then used to calculate the differential amounts of surface tension in the germ layer progenitor cell types that were influencing their segregation behavior.

In addition to SCFS, micropipette aspiration has been used to measure surface tension in cells. It involves applying known magnitudes of negative pressure on a cell through micropipettes to

determine the minimum pressure required to aspirate it into the micropipette, which again is determined by its surface tension. Micropipette aspiration has been employed to determine surface tension in several contexts. For instance, using this technique, it has been shown that compaction in mammalian embryos, which is essential for cell fate specification in the blastocyst, is driven by an actomyosin-dependent increase in surface tension at the cell-medium interface (99).

4.3. Viscoelasticity Measurements

The degree to which cells and tissues deform in response to mechanical forces is determined by their material properties. It is therefore equally important to assess cell and tissue viscoelastic properties to understand the mechanical basis of morphogenesis. This can be accomplished by using the techniques described in this section.

Like surface tension, the application of suction pressure through a micropipette can also be used as an assay to measure tissue viscoelasticity. More specifically, the flow profile of the cells inside the micropipette in response to the suction is used to calculate their viscosity. As for any fluid, the greater the tissue viscosity (or the lesser the fluidity), the lesser or slower their displacement is into the micropipette. Recently, this technique was used to establish that the deep cell tissue in the zebrafish embryo fluidizes at the onset of epiboly and that this fluidization is essential for proper epiboly movements (132).

Another method to measure tissue viscoelasticity involves injecting ferrofluid droplets into the sample and profiling their response to external actuation. Each droplet is displaced by the application of an external magnetic field, and upon removal of this field, the recoil of the droplet is tracked. This technique has, for instance, been used to measure the viscous properties of the cytoplasm of *Drosophila* early embryos, revealing that it is 1000× as viscous as water (33).

Viscoelasticity can also be measured using optical stretchers. Such stretchers employ two counterpropagating, identical diverging beams of infrared light to trap individual cells and stretch them along the axis of the beams. This stretch and the restoration of the cells upon the release of the stretching force are measured to determine the viscoelastic properties of the cell. For example, studies using this technique have shown that fibroblasts stiffen with age in an F-actin-dependent manner (145).

Another noninvasive technique to determine the viscoelastic properties of biological samples is Brillouin microscopy (31, 85, 142). It relies on the principle that light is scattered upon interaction with acoustic waves generated by thermally induced density fluctuations within a sample (Brillouin scattering). The shift in the frequency of the scattered light depends on the material properties of the sample, especially its complex longitudinal modulus. Therefore, stiffer samples cause greater Brillouin frequency shifts and vice versa. Brillouin microscopy has been successfully employed to generate an elasticity map of the mouse embryo and ovaries as well as to study the mechanical properties of the eye, and has also provided insights into the properties of the neuroepithelium during and after neural tube closure (11, 23, 136, 141, 186). A stiffness gradient has been demonstrated to exist along the dorsoventral axis of the neural tube such that the fusion region is softer and likely more deformable.

4.4. Adhesion Force Measurements

Cell-cell and cell-ECM adhesion are key determinants of cell behaviors during morphogenesis. For instance, weaker adhesion between migrating cells could lead to a mechanical uncoupling of neighboring cells, thus causing them to exhibit less coordinated migratory patterns. Similarly, loss of cell-cell adhesion can also hamper the transmission of mechanical forces over longer

distances, such as in tissues. Therefore, measuring adhesion is key to building a mechanical model of morphogenesis. Some assays enabling such measurements are described in this section.

One technique to assay the adhesion strength between cells involves micropipette aspiration. It involves pulling neighboring cells apart with the help of micropipettes with known magnitudes of force. The force that is required to separate the cells (the detachment force), as determined by the pressure in the pipette needed to hold them attached to the pipette during the separation process, is an indicator of the strength of adhesion between them. The easier it is to separate neighboring cells from each other, the lower is the adhesion strength. Recently, this technique was used to demonstrate the differential strengths of homotypic and heterotypic adhesion among germ layer progenitors and neural progenitors in zebrafish, which is likely key to the sorting of the different cell types during gastrulation and spinal cord development, respectively, amid a noisy morphogen gradient (98, 172) (**Figure 1*f***).

Finally, in addition to measuring cortical tension, SCFS has also been used to measure cell-cell and cell-ECM adhesion forces in gastrulating cells (135). More specifically, cells glued onto a cantilever were pressed either against a surface decorated with ECM for measuring cell-ECM adhesion or onto another cell to measure cell-cell adhesion. After a predetermined dwell time, the cantilever was withdrawn, and the maximal force required to detach the cell from the surface or cell was determined and used as an indicator of the adhesion strength. Using this method, the germ layer progenitor cell types from zebrafish embryos were shown to display different cell-cell adhesion strengths, which again depend on differences in actomyosin cortex tension between these cells.

4.5. The Need for Additional Tools and Approaches

In this section, we have discussed the role of biophysics in driving morphogenesis and described tools to determine the physical properties of biological samples. One limitation of several of these techniques, however, is that they provide a stationary view of the biophysical properties. For example, unlike live microscopy, when measuring viscoelastic properties through Brillouin microscopy, the assessment is mostly limited to a single time point and/or a section of the sample. In addition, most of these techniques are invasive in nature and need direct contact with the sample, which may not always be possible. Some of these limitations can be addressed by using force inference methods such as CellFIT (19). CellFIT uses model equations to calculate the forces at play from cell shapes and motions. This and other such tools have been previously used to estimate the forces driving apical constriction during *Drosophila* ventral furrow formation, and to explore the contributions of various cell behaviors in the successful execution of dorsal closure, germ band extension, and zebrafish neuroectoderm formation (15, 19). Nevertheless, improvements in the tool kit available to us to make such measurements will enhance our ability to further dissect the role of biophysics in morphogenesis.

5. BRIDGING FROM GENE REGULATION TO CELL AND TISSUE MECHANICS: THE ROLE OF BIOCHEMISTRY IN MORPHOGENESIS

Morphogenesis is fundamentally driven by an interplay between genetic and physical factors. However, to understand how those factors relate to each other, the molecular mechanisms by which genes influence cell and tissue mechanics and vice versa need to be explored. For example, mechanical forces are generated, transmitted, and sensed by cells and tissues through several kinds of biochemical entities, ranging from cytoskeletal polymers and the motors that trek on them to adhesion proteins and protein macrostructures such as mechanosensitive ion channels. The interactions of such biochemical entities with their mechanical environment can, in turn, induce a

variety of changes in their localization, conformation, stability, and activity, thus influencing their ability to generate, transmit, and/or sense forces. In this section, we review our understanding of the biochemical and molecular mechanisms underlying morphogenesis and discuss approaches for exploring these mechanisms.

5.1. The Identification and Isolation of Molecular Players

A key prerequisite for understanding the molecular mechanisms underlying morphogenesis is the identification of the molecular players relevant to the process. In this regard, holistic approaches, similar to forward genetic screens for the identification of genetic players, are highly effective. One such approach is known as omics.

Omics involves the analysis of the entire pool of RNAs (transcriptomics), proteins (proteomics), lipids (lipidomics), etc., present in a given system at a specific time. Such analyses can be performed at the level of entire tissues or embryos or at that of single cells. Comparative analyses between different cell types or different genotypes are informative about the differences in genes expressed or proteins present between the groups, which, in many cases, are the cause for their distinct phenotypes. For instance, a comparative RNA-sequencing analysis between untreated and rescued zebrafish *ichabod* mutants, which exhibit severe ventralization due to defects in the Wnt/β-catenin pathway, led to the identification of genes differentially upregulated in the rescued embryos, suggesting that their expression is positively regulated by Wnt/β-catenin signaling (41, 80). Further analyses revealed that the transcripts of some of these genes localize specifically to the dorsal part of the embryo, further strengthening the idea that they may be required for dorsoventral patterning.

Similarly, a comparative proteomics analysis revealed >50 proteins with potential roles in *Drosophila* ventral furrow formation (47). Here, the proteomes of ventralized and lateralized embryos were compared at the precellularization stage as well as at the early and late gastrulation stages to identify those proteins that were differentially enriched among the two groups. The authors found that most differential abundance already existed long before gastrulation, suggesting that the ventral cells are set up to undergo shape changes leading to ventral furrow formation very early in development. They later verified that RNA interference knockdown of the genes encoding some of the differentially enriched proteins indeed produced ventral furrow defects, confirming their role in the process.

In *C. elegans*, egg shells begin to form around oocytes shortly after they enter the spermatheca. One layer of the egg shell, the permeability barrier, whose formation is dependent on *seip-1* (*seipin*), protects the embryos from mechanical and osmotic stress and is therefore critical for development (10, 122). Lipidomic analysis of wild-type and *seip-1* mutant embryos through liquid chromatography–mass spectrometry revealed large-scale alterations in the lipid profile of *seip-1* mutant embryos (10). These alterations were found to be mainly caused by a significant reduction in the proportion of polyunsaturated fatty acids (PUFAs). Indeed, upon dietary supplementation with PUFAs, the mutant oocytes successfully formed the permeability barrier, leading to a significant increase in embryo survival. These examples demonstrate that omics approaches enable the identification of potential regulators of a variety of morphogenetic processes across model organisms.

Another approach for identifying the molecular players involved in morphogenesis is to focus on molecular interactions. Such assays usually begin with a molecular candidate already known to play a role in a process and screen for its interactors. These screens can be performed inside living organisms (e.g., a yeast two-hybrid screen) or in test tubes (e.g., coimmunoprecipitation). One such screen in mice, for instance, identified Rnf2 (RING1B) as an interactor of Bmi-1, a

protein most known for its role in neoplastic transformation (58). Further investigation revealed that Rnf2 is essential for gastrulation in mice and functions by modulating cell proliferation and differentiation in early embryos (175).

5.2. The Visualization of Molecules

Once identified, molecules need to be visualized to understand their role in morphogenesis. For example, one mechanism of force generation by microtubules and actin filaments involves the dynamic polymerization and depolymerization of their subunits. Such behavior is essential for the dynamic reorganization of the cytoskeleton that drives morphogenesis and is therefore an important topic of investigation. Cytoskeletal dynamics can be tracked through several means, ranging from artificially produced dyes to transgenic fluorescent proteins (34, 140, 150, 160) (**Figure 1*g*,*h***). Specifically tracking one end of the polymer is also possible. For instance, the expression of fluorescently labeled end-binding proteins enables the visualization of the plus or the minus end of microtubules (12, 63, 94, 183). Similarly, the spatiotemporal localization and dynamics of other players involved in morphogenesis, such as adhesion molecules, can also be visualized using these approaches (**Figure 1*g*,*h***). Finally, transgenic fluorescent labeling has been extremely successful in enabling the live visualization of several cell behaviors, from apical constriction and cell division to cell migration (6, 108, 124).

Other techniques, such as fluorescence in situ hybridization, that rely on the detection of DNA or RNA molecules through fluorescent probes can also be used to determine the localization and levels of individual transcripts. Using this technique, the localization of ~6,000 mRNAs involved in *Drosophila* oogenesis were determined, thus also demonstrating the application of the technique for large-scale analyses (69). Likewise, proteins of interest can be detected through the use of antibodies against specific epitopes via Western blotting and immunostaining. While Western blotting is informative about characteristics such as the molecular size of the proteins, immunostaining is useful to determine their localization. Immunostaining, for instance, was used to visualize the localization of several proteins ranging from force generators, such as myosin, to force transmitters, such as E-cadherin during epithelial morphogenesis in *Drosophila* embryos (107).

5.3. Interfering with Molecular Processes

As for genetics and biophysics, techniques must also be in place to perturb molecular players in order to determine their role in morphogenesis. This can be accomplished through multiple approaches. One such approach involves the treatment of cells, tissues, or embryos with chemical agents stabilizing or depolymerizing cytoskeletal components such as F-actin, microtubules, and intermediate filaments. For instance, by exposing zebrafish embryos to Nocodazole or Taxol, which depolymerizes or stabilizes microtubules, respectively, microtubules were demonstrated to play a necessary and active role in the movement of the yolk syncytial nuclei but not in that of the enveloping layer (EVL) during epiboly (154). In contrast, upon treatment with blebbistatin, a myosin II inhibitor, embryos exhibit impaired epibolic movements of the EVL margin cells (84). Thus, chemical treatments proved effective in understanding the cytoskeletal requirements of different tissue movements during epiboly, a key process in zebrafish morphogenesis. One limitation of this approach, however, is the lack of spatial specificity of the treatments.

This limitation can be addressed by optogenetics, an approach that provides significantly greater spatiotemporal specificity in molecular perturbations. Optogenetics involves the selective illumination of genetically encoded proteins with light. In most cases, when exposed to light of a specific wavelength, the localization, folding, stability, and activity of these proteins can be altered,

allowing researchers to understand the protein's role in its native state or even to induce specific cell behaviors ectopically. For example, chromophore-assisted laser inactivation, which relies on the spatially confined oxidation of nearby molecules by reactive oxygen species produced upon the illumination of fluorophores such as enhanced green fluorescent protein (eGFP), was used to investigate the role of myosin II in asymmetric cell division in worms (123). The authors showed that anisotropic myosin contractility in *C. elegans* neuroblasts is necessary for their asymmetric division. Another method, CRY2 (cryptochrome 2)-CIBN (cryptochrome-interacting basic helix-loop-helix), which enables the relocalization of proteins to specific subcellular compartments, was used to reversibly localize Raf kinase to the plasma membrane, thereby ectopically activating the Raf/MEK/ERK signaling pathway in *Xenopus* embryos (88). Similarly, it was used to induce apical constriction in the dorsal cells of early fly embryos through the ectopic activation of Rho signaling, which showed that apical constriction is sufficient to drive tissue folding (67). A modified method of CRY2-CIBN, called CRY2olig, can also be used to oligomerize proteins and is useful to study protein-protein interactions (164). Other prominent optogenetic methods include the use of phytochromes (PHYA-PHYB) and light-oxygen-voltage (LOV) domains to control protein localization, activity, and stability (89).

Protein function and localization can also be perturbed using functionalized protein binders such as those based on nanobodies. Nanobody binding to proteins can lead to their scaffolding, and, in some instances, to their inhibition and/or degradation. This approach can also be used to relocalize proteins of interest to regions where they do not normally localize. For example, nanobody-based GFP traps were used to mislocalize several cytoplasmic and transmembrane proteins to different domains along the apicobasal axis in *Drosophila* wing imaginal disc cells and to investigate the effect of this mislocalization on wing development (54). Using this tool, the extracellular gradient of decapentaplegic in the imaginal discs was shown to be necessary for proper wing patterning.

5.4. Bottom-Up Approaches: Reductionist and Synthetic

Like all complex processes, morphogenesis is dictated by multiple factors, which renders its study challenging. Therefore, it is often beneficial to adopt reductionist or synthetic approaches that limit the number of factors at play in order to assess the functions specific players of interest perform. Results from such bottom-up approaches can then be combined to construct a more comprehensive model of morphogenesis.

For instance, reductionist approaches involving cytoplasmic extracts were used to investigate molecular details associated with cell division in eggs and early embryos. *Xenopus* egg extracts can be artificially induced to enter interphase or mitosis through the addition of calcium or cyclin B, which leads to nuclear reassembly and spindle reorganization (53, 112). The mechanical and biochemical details of such events can then be studied in a test tube. This technique has recently been used to explore the biochemical factors governing spindle scaling in various *Xenopus* species (20, 82, 96). Cytoplasmic extracts can also be prepared from individual *Drosophila* syncytial embryos, and, given that flies are amenable to genetic and transgenic manipulations, the potential of this technique in flies is huge (165). Furthermore, unlike *Xenopus* egg extracts, *Drosophila* embryo extracts also exhibit nuclear cycles and are the only extracts so far that show this behavior ex vivo (165). This technique has successfully been applied to study repeated nuclear divisions, the cytoskeleton dependence of nuclear positioning, and the role of confinement in nuclear encapsulation (165). Furthermore, extracts make it easier to perform drug or small molecule treatments on cellular components, thus enabling pharmacological perturbations. Finally, the improved accessibility to these components due to their observation in smaller volumes, as opposed to

when inside the embryo, makes it possible to visualize cellular processes and also to mechanically perturb, for instance, the cytoskeletal elements involved in order to assess their functions.

Another reductionist approach has recently been used to demonstrate the mechanism driving one of the first steps during embryogenesis: the reorganization of the ooplasm. More specifically, zebrafish oocytes were spatially confined in a cuboidal box, thereby breaking their plasma membranes and actomyosin cortices (149). In these egg extracts, ooplasm segregated normally despite a disintegrated cortex, demonstrating that the cortex is not necessary for this segregation and redrawing the then-existing model explaining this process.

Like reductionist approaches, one very effective approach for understanding morphogenesis is the synthetic approach, which entails mimicking natural processes in an environment in which these processes do not normally occur. This not only provides an independent verification of our understanding of biological processes but also serves as a simplified means of addressing additional questions to advance the same. A synthetic approach has, for instance, been used to study the processes whereby alternate cell fates are determined in neighboring cells within a homogeneous tissue through lateral inhibition via the Delta-Notch pathway. Using culture cells that do not endogenously express Delta and Notch, a genetic circuit was synthetically constructed in which Delta was ectopically expressed with or without additional Notch expression (103). This revealed that when the cells were comparable with respect to their Delta and Notch expression profiles, they differentiated into alternate cell types, which is consistent with observations made in model organisms in which this interaction governs cell fate. In contrast, when Delta levels were significantly higher than those of Notch, such cell-type bifurcation was not observed. This paradigm has subsequently been used to explore the impact other factors, such as cell division, density, and contacts, have on the process of fate bifurcation. Collectively, reductionist and synthetic approaches enable investigators to systematically explore the molecular mechanisms that control cell fate specification and morphogenesis in an experimentally amenable and manageable context.

6. CONCLUDING DISCUSSION

In this review, we have discussed genetic, biophysical, biochemical, and microscopy-based approaches and how these can be combined to understand morphogenesis. However, owing to space constraints, we have provided only an incomplete list of such approaches. For instance, in Section 2, we discussed primarily forward genetics, omitting reverse genetics, an approach that complements the former. Furthermore, we have also not ventured into embryological procedures, such as cell or tissue transplantations, Keller explants, and organoid cultures, all of which have proven extremely successful in unraveling mechanisms underlying morphogenesis. For more information on those approaches, we refer the reader to recent reviews in particular on the use of organoid cultures in dissecting the mechanisms underlying embryo morphogenesis (61, 138, 147, 153).

One other very active area of research we have not covered in this review is the development of mathematical models to understand morphogenetic processes. These models are typically used to test the plausibility of certain assumptions that were made on the basis of experimental observations and to generate predictions on potential mechanisms that might be difficult to obtain otherwise and can subsequently be tested experimentally. Such models have turned out to be particularly useful for generating biophysical and/or mechanical explanations of morphogenetic processes. For instance, the development of an active gel theory has been crucial in describing the behavior of cytoskeletal networks consisting of cross-linked filaments that are rearranged by energy-consuming molecular motors (73, 74, 134). Likewise, the development and use of vertex models, in which cells are geometrically approximated as polygons with vertices changing their positions as a result

of growth, interfacial tension, and pressure, have been indispensable for understanding cellular rearrangements and deformations of epithelial monolayers (2, 39, 62, 156, 158). For more details on the use of these and other theoretical models in describing cell and tissue morphogenesis in development, we refer the reader to recent reviews on this topic (4, 40, 119).

Morphogenesis so far has predominantly been studied in model organisms, such as *Drosophila*, *C. elegans*, zebrafish, *Xenopus*, chicken, and mice. However, while the focus on those model organisms is justified for practical and historical reasons, it does not cover the breadth of different shapes and morphologies of animals within the tree of life and, thus, might provide a rather simplistic and biased view of the underlying mechanisms. Consequently, for dissecting the core modules of morphogenesis, this process also needs to be studied in non-model organisms, allowing us to identify evolutionarily conserved and divergent features. With the rapid advancement of imaging, transgenesis, and sequencing techniques, a systematic study of non-model organisms is now feasible and will likely considerably advance our understanding of the fundamental principles underlying the emergence of various shapes and morphologies in animal development.

Finally, improved image processing and analysis tools also have an important role to play in the quest for morphogenetic mechanisms. Despite major advancements in deep-tissue imaging, a key limitation of microscopy-based methods is the low signal-to-noise ratio deep inside samples. Post-imaging enhancement of such features often helps researchers to extract useful information from images that would have been previously considered unfit for analysis. Further improvements along similar lines would greatly enhance our ability to investigate the processes occurring deep inside embryos without the need for performing invasive embryological procedures.

In summary, while we are presently at a juncture at which we are better placed to understand morphogenetic processes than we historically have been, future technological improvements are desired to push the horizons of this understanding further.

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