Critical Review

Membrane Patterns Carry Ontogenetic Information That Is Specified Independently of DNA

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Abstract

Embryo development (ontogeny) depends on developmental gene regulatory networks (dGRNs), but dGRNs depend on pre-existing spatial anisotropies that are defined by early embryonic axes, and those axes are established long before the embryo's dGRNs are put in place. For example, the anterior-posterior axis in *Drosophila* and the animal-vegetal axis in *Xenopus* and echinoderms are initially derived from the architecture of the ovary through processes mediated by cytoskeletal and membrane patterns rather than dGRNs. This review focuses on plasma membrane patterns, which serve essential ontogenetic functions by providing targets and sources for intracellular signaling and transport, by regulating cell-cell interactions, and by generating endogenous electric fields that provide three-dimensional coordinate systems for embryo development. Membrane patterns are not specified by DNA sequences. Because of processes such as RNA splicing, RNA editing, protein splicing, alternative protein folding, and glycosylation, DNA sequences do not specify the final functional forms of most membrane components. Still less does DNA specify the spatial arrangements of those components. Yet their spatial arrangements carry essential ontogenetic information. The fact that membrane patterns carry ontogenetic information that is not specified by DNA poses a problem for any theory of evolution (such as Neo-Darwinism) that attributes the origin of evolutionary novelties to changes in a genetic program—whether at the level of DNA sequences or dGRNs. This review concludes by suggesting that relational biology and category theory might be a promising new approach to understanding how the ontogenetic information in membrane patterns could be specified and undergo the orchestrated changes needed for embryo development.

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INTRODUCTION

In 1958—five years after he and James Watson discovered the molecular structure of DNA—Francis Crick proposed that the specificity of a DNA segment lies solely in its nucleotide sequence, which encodes the nucleotide sequence of a messenger RNA (mRNA) and thereby the amino acid sequence of a protein. Crick also proposed that the information encoded in DNA sequences can be transferred from DNA to protein, but not back again. He called the former the "sequence hypothesis" and the latter the "central dogma" of molecular biology [1], but many writers since that time have used "central dogma" to refer to the two proposals together. In this review I follow their practice.

Three years after Crick proposed the central dogma, François Jacob and Jacques Monod reported the discovery of a genetic switch in bacteria that regulates the synthesis of β-galactosidase, an enzyme (protein) needed to metabolize lactose. When the bacteria are in a medium with no lactose, a DNA-binding protein represses the transcription of β-galactosidase mRNA, so the cells do not waste energy producing an unneeded protein. When lactose is present it induces the release of the repressor from the DNA, thus permitting the production of β-galactosidase. Jacob and Monod called this genetic system the "lac operon" [2,3].

In 1968 Eric Davidson published a book analyzing gene activity in early animal development that started with two premises: the central dogma ("Since the cell owes its definitive characteristics to the characteristics and functional attributes of its proteins… the differentiated state must ultimately depend on the transcription of genomic information") and genomic equivalence ("Every living cell nucleus in a metazoan organism [with some exceptions, as Davidson noted] contains the same complete genome as was present in the zygote nucleus").

Arguing from these two premises, Davidson concluded that animal embryogenesis (ontogeny) depends on "selective variation in gene activity" that involves regulatory mechanisms such as the lac operon [4].

Up until that time, many biologists had maintained that cytoplasmic factors are more important than nuclear genes in controlling ontogeny [5,6]. Indeed, in his 1968 book Davidson reviewed evidence that in several animal phyla "the initial, visible events of embryogenesis are *not* under the direct control of the embryonic cell genomes." For example, the early development of cross-species hybrids in sea urchins as well as frogs follows the maternal pattern, with no detectable contribution from the paternal genome. Davidson concluded that early embryogenesis in these (and many other) animals is controlled by "morphogenetic determinants that are localized in the cytoplasm." He emphasized, however, that the localized morphogenetic determinants are ultimately encoded by DNA; so are the transcription factors that regulate gene activity [4].

In 1970, Jacob wrote that an organism is the realization of a "programme génétique" (genetic program) written in DNA sequences [7]. The same year, Monod wrote that "the sequence of nucleotides in a DNA segment entirely defines the sequence of amino acids in the corresponding polypeptide [protein]." And since "the polypeptide sequence specifies completely (under normal initial conditions) the folded structure that the polypeptide adopts once it is constituted, the structural and hence functional 'interpretation' of genetic information is unequivocal, rigorous. No supplementary input of information other than the genetic is necessary; none, it seems, is even possible" [8]. Thereafter the idea of cytoplasmic control was largely replaced by the idea that genetic programs direct embryo development. A popular formulation of the central dogma is now "DNA makes RNA makes protein makes us" [9,10].

The emphasis on genetic programs owes much to evolutionary theory—specifically, to the modern synthesis of Darwinian evolution and Mendelian genetics. According to the modern synthesis, new heritable variations originate in genetic mutations. In a 1970 interview, Monod said that with the establishment of the central dogma, "and the understanding of the random physical basis of mutation that molecular biology has also provided, the mechanism of Darwinism is at last securely founded" [11].

Yet even in 1970 it was clear that there is no simple correspondence between DNA sequences (the genotype) and an organism's observable characteristics (its phenotype), and therefore that the notion of "genetic program" needed—at the very least—further work. In what follows I summarize the subsequent elaboration of that notion in Eric Davidson's idea of "gene regulatory networks." I then survey evidence for the temporal and causal priority of spatial anisotropies in early development, and I argue that ontogeny in multicellular animals (metazoa) depends significantly on sources of spatial information in cells that are not reducible to DNA sequences. In particular, I focus on spatial information in plasma membranes, in the form of patterns of intracellular targets, complex carbohydrates, and ion channels. I conclude by discussing the implications of membrane information for evolutionary theory and our understanding of ontogeny.

GENE REGULATORY NETWORKS

In 1969, Stuart Kauffman wrote that the analogy between digital computing and the control system exemplified by the lac operon had already suggested to several authors "that the genome embodies complex switching circuits which constitute a program for metabolic stability and cell differentiation." Treating "genes" as binary switches, Kauffman used Boolean logic to model what he called "genetic nets" or "genetic regulatory circuits" [12].

In 1990, Davidson applied the term "gene regulatory networks" (GRNs) to sets of interacting DNA sequences, RNAs, and proteins that regulate transcription and other cellular processes. He argued that "the significant features" of early development in animal embryos "have to do directly with the distribution in embryonic space of gene regulatory molecules." Davidson acknowledged that the distribution depended on spatial anisotropies in early embryos, but he considered those anisotropies less significant than their implementation by genes and gene products: "Since the basic objective is to understand what makes the various spatial domains of the early embryo functionally diverse, in terms of cell structure, behavior and synthetic activity, the problem of regulatory architecture has to be considered in particular at the level of the histospecific [i.e., tissue-specific] structural genes that endow the embryo with its specific regional functions" [13].

Since 1990 many authors have dealt with gene regulatory networks, but Davidson has been the most prolific writer on the topic. In 2006, he wrote that animal embryos illustrate two features:

> The less important is the variable specifics of the initial cytoplasmic bases of spatial anisotropy. The other feature is of ultimate importance: This is the common functional endpoint of these very diverse initial stratagems for the spatial indication of future developmental domains. The principle is that whatever the bases of the anisotropies, however they come into being, whatever the cell fates that derive from what they set in train, they end up causing certain maternal transcription factors to be present and active in some spatially defined embryo nuclei, but not in others [14].

So Davidson maintained that GRNs are more important in ontogeny than the spatial anisotropies that precede them. In his view, the genetic programs embodied by GRNs control development, and mutations in the underlying genes lead to evolution. "Since the morphological features of an animal are the product of its developmental process," he wrote, "and since the developmental process in each animal is encoded in its species-specific regulatory genome, then change in animal form during evolution is the consequence of change in genomic regulatory programs for development" [14].

Despite this emphasis on the genome, Davidson made it clear in 2011 that he was not advocating "the classic neo-Darwinian concept that evolution of animal morphology occurs by means of small continuous changes in primary protein sequence," nor was he claiming that "evolution at all levels can be illuminated by detailed analysis of *cis*-regulatory changes in genes that are direct targets of sequence level selection." These perspectives, he pointed out, "often focus on changes at single gene loci, and both are framed within the concepts of population genetics." Instead, according to Davidson "the evolution of animal body plans is a system level property of developmental gene regulatory networks (dGRNs) which control ontogeny of the body plan" [15].

SPATIAL ANISOTROPIES IN ONTOGENY

Where do spatial regulatory domains come from? Far from being determined by the gene regulatory molecules that occupy them, as Davidson would argue, spatial domains determine the distribution of regulatory molecules. Spatial anisotropies precede—and are causally upstream of—the embryo's dGRNs.

Drosophila

Among the best-studied dGRNs are those involved in the development of the fruit fly *Drosophila melanogaster*. The earliest dGRNs in ontogeny are customarily taken to consist of the "maternal effect" genes, which are involved in elaborating the anterior-posterior and dorsal-ventral axes of the oocyte [16–18]. For the anterior-posterior axis, maternal effect genes are involved in setting up a gradient of Bicoid protein with its highest concentration at the anterior end, and a gradient of Nanos protein with its highest concentration at the posterior end [19–21]. Other dGRNs are subsequently involved in establishing the dorsal-ventral axis, setting up body segments ("segmentation genes"), and specifying segment identities ("Hox genes") [22–24].

The Bicoid gradient is commonly taken to be the starting-point of the first dGRN in *Drosophila* development [14,25]. The Bicoid gradient depends on a gradient of maternal *bicoid* mRNA that is highest at the anterior end of the oocyte [26,27]. When *bicoid* mRNA enters the oocyte from the nurse cells a microtubule-based mechanism concentrates it at the anterior end, where it becomes closely associated with the cell cortex [28–30]. As used here, "cortex" refers to the plasma membrane and an underlying layer of cytoplasm consisting primarily of cytoskeletal elements (e.g., actin microfilaments and spectrins) [31]. The cortical localization of *bicoid* mRNA also requires several proteins, especially the endosomal sorting protein ESCRT-II [32,33].

Yet the anterior pole has to be in place before *bicoid* mRNA can be localized there. In fact, the *Drosophila* oocyte acquires its anterior-posterior polarity much earlier. The oocyte starts out in a follicle consisting of sixteen interconnected cells, fifteen of which will become "nurse cells" that synthesize and transport maternal mRNAs into the developing oocyte. Anterior-posterior polarity first appears in the oocyte when it becomes distinct from the nurse cells (Fig. 1) [34]. The selection of one cell to become the oocyte involves polarization of the membrane and depends on the asymmetric segregation of a germline-specific organelle called the "fusome," an intracellular structure containing membrane-associated cytoskeletal components (such as spectrins) normally found in the cell cortex [35,36]. The fusome organizes the microtubule network early in oogenesis and directs the migration of centrosomes from the nurse cells into the new oocyte [37,38]. Intercellular signaling between the oocyte and the surrounding follicle cells then regulates the anterior-posterior patterning of the latter [39].

At first, the follicle containing the oocyte and its associated nurse cells is roughly spherical. Then molecular motors in a surrounding epithelial "corset" force the follicle to rotate several times, turning it into an oval with the oocyte at one end [40]. By this time, the anterior-posterior axis of the oocyte is well established—long before maternal effect gene products enter it from the nurse cells (Fig. 1, far right). So the anterior-posterior body axis in the *Drosophila* oocyte does not originate in maternal effect genes and their products, but in spatial asymmetries inherent in the architecture of the ovary and in polarized membranes and cytoskeletal components of the follicle [41].

Xenopus

In the frog *Xenopus laevis,* oocytes have neither anterior-posterior nor dorsal-ventral axes. Instead, a *Xenopus* oocyte has an animal-vegetal axis, with the animal hemisphere covered by dark pigment granules and the vegetal hemisphere filled with large yolk platelets. After fertilization the dorsal-ventral axis

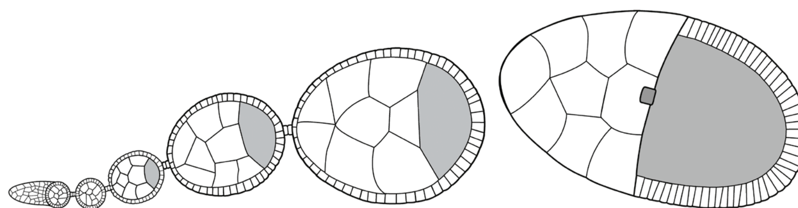

Figure 1: *Drosophila* oogenesis. The *Drosophila* oocyte originates in a follicle consisting of sixteen interconnected cells. Polarization of the cytoskeleton and membrane specify one cell to become the oocyte; its fifteen neighbors then become nurse cells that synthesize maternal mRNAs. The follicle is roughly spherical at first (left); molecular motors in the surrounding epithelium then rotate it, forming an oval with the oocyte at one end. By this time the anterior-posterior axis of the oocyte is well established, before maternal mRNAs are transported into it from the nurse cells (right). doi:10.5048/BIO-C.2014.2.f1

is established by a process of "cortical rotation," in which the cortex rotates approximately 30° relative to the interior of the zygote [42,43]. The movement is microtubule-dependent, and the cortex normally rotates away from the sperm entry point, which has suggested to some that the aster nucleated by the sperm centrosome initiates cortical rotation [44]. The rotation movement orients microtubules in the vegetal cortex, and these form a parallel array that provides tracks to transport various proteins from the vegetal pole to the future dorsal side of the embryo, thereby establishing a dorsal-ventral axis [45,46]. One of the most important of these proteins is β-catenin, a member of the Wnt signaling pathway that is involved in cell-cell communication and gene regulation in the early development of many animals, including *Drosophila* and *Xenopus* [47,48]. The cortical region in which these proteins accumulate subsequently becomes the "blastopore," an opening through which cells migrate inward during gastrulation to establish the anterior-posterior axis. The dorsal lip of the blastopore has been called the "organizer," because transplanting it to the presumptive future ventral side of another embryo induces the latter to organize a second tadpole conjoined to the first [49].

The dorsal-ventral and anterior-posterior axes of the *Xenopus* embryo depend on the prior establishment of the animal-vegetal axis in the oocyte. *Xenopus* oogenesis is customarily divided into six stages [50]. From stage II through stage V, maternally produced yolk is transported across the entire surface of the developing oocyte. Once inside, yolk platelets are concentrated in the vegetal hemisphere by intracellular transport (Fig. 2) [51]. More than twenty RNAs are also concentrated in the vegetal hemisphere, by two different mechanisms exemplified by the maternal RNAs *Vg1* and *Xcat-2* [52]. *Vg1* is subsequently involved in mesoderm induction during embryogenesis, and *Xcat-2* (which has some sequence similarity to *Drosophila nanos*) may be subsequently involved in germ cell development [53,54]. During stage IV, *Vg1* is transported to the vegetal pole by a microtubule-based mechanism and then anchored to the vegetal cortex by a mechanism requiring membrane-associated actin microfilaments [55]. But earlier in oogenesis a microtubule-independent mechanism localizes *Xcat-2* RNA to the vegetal pole along with a "mitochondrial cloud," also known as the "Balbiani body" [56,57].

The Balbiani body is an aggregate of mitochondria, cytoskeletal elements, endoplasmic reticulum, RNAs and proteins [58]. In early oocytes it is located next to the germinal vesicle (the maternal pronucleus) and is the first indicator of animal-vegetal polarity [59,60]. In this respect it is like the *Drosophila* fusome; indeed, a fusome entering a *Drosophila* oocyte resembles a Balbiani body [61]. During *Xenopus* oogenesis, RNAs diffuse into the Balbiani body and are trapped there by elements of the endoplasmic reticulum [62]. The Balbiani body then expands directionally toward the vegetal pole by an unknown mechanism, delivering RNAs such as *Xcat-2* to the vegetal cortex [63].

In the microtubule-dependent transport that occurs later (in stage IV), the localization of *Vg1* RNA to the vegetal pole depends on a "localization signal" in the RNA's 3′ untranslated region [64,65]. In this respect, the localization of *Vg1* is similar to localization of other mRNAs in a variety of organisms, including *bicoid* in *Drosophila* [66]. The *Vg1* localization signal works, however, only because its cortical target is already in place. Once RNAs are anchored at the vegetal pole of a *Xenopus* oocyte, isolated pieces of the vegetal cortex retain them [67]. The same is true of the dorsal cortex of *Xenopus* zygotes after cortical rotation [68]. Some RNAs might be anchored to cytoskeletal elements associated with the cortex (such as actin microfilaments), but evidence indicates that at least some RNAs are anchored to the vegetal cortex in a membrane-dependent manner [69].

Echinoderms

The oocytes of echinoderms (e.g., sea urchins and starfish), like those of frogs, have a primary animal-vegetal axis [70,71]. The second axis to form is the oral-aboral axis, which extends from the mouth to the opposite side of the organism [72]. Exactly when the oral-aboral axis forms and how it is determined have been controversial and vary from species to species [73–81].

Since the adults of most echinoderms exhibit five-fold radial symmetry, the identification of anterior-posterior and dorsal-ventral body axes in these animals has been problematic. The oral-aboral axis is commonly equated to the dorsal-ventral axis, because it is the second to form and most species orient with the mouth down, on the ventral side [74,78,79,82]. But the oral-aboral axis has also been equated to the anterior-posterior axis, because in bilaterally symmetrical animals the mouth is at the anterior pole and echinoderms are thought to have evolved from bilaterian ancestors [83,84].

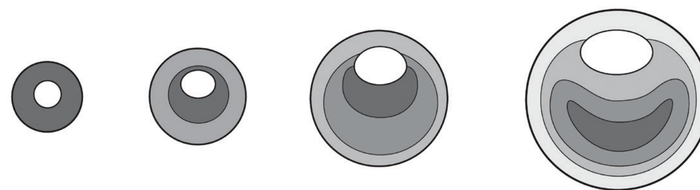

Figure 2: *Xenopus* oogenesis. An early *Xenopus* oocyte (left) is approximately spherically symmetrical. As yolk is transported into it from the surrounding ovary, it becomes more polarized. The different shades of gray in this simplified drawing represent layers of yolk; the lighter areas are younger. The clear area is the germinal vesicle (the maternal nucleus), which becomes more elliptical as the oocyte matures. Animal-vegetal polarity is clearly visible in the distribution of the yolk and the location of the germinal vesicle near the animal pole (second from left), before maternal mRNAs enter the oocyte (right) to be differentially distributed along the animal-vegetal axis. **doi:**10.5048/BIO-C.2014.2.f2

In any case, it is clear that an echinoderm oocyte has an animal-vegetal axis that precedes the formation of the oral-aboral axis and is necessary for proper development. When unfertilized *Paracentrotus lividus* or *Hemicentrotus pulcherrimus* eggs are surgically bisected in a plane that includes the animal-vegetal axis, both halves proceed through gastrulation, but when the eggs are bisected in a plane perpendicular to the axis only the vegetal half undergoes development past the blastula stage [73,85]. Since β-catenin is involved in vegetal development, it has been suggested that it accumulates at the vegetal pole of the oocyte while other molecules localize to the animal pole [86–89]. Several specific mRNAs are also differentially localized in the oocyte with respect to the animal-vegetal axis [90,91].

Just as in *Drosophila* and *Xenopus* oocytes, the localization of specific molecules to the animal and vegetal poles in echinoderm oocytes presupposes that those poles are already present. In several sea urchin species, the animal pole of a primary oocyte is marked by a "jelly canal" that admits foreign ink particles excluded elsewhere from the cell [70,85,92,93]. In immature starfish oocytes, the animal poles are distinguished by the adjacent eccentric germinal vesicle (the maternal nucleus) and centrosomes of the pre-meiotic aster [94,95]. In any case, it is clear that the animal-vegetal axis of an echinoderm oocyte is present at the earliest stage of oogenesis and presumably derived from the architecture of the ovary.

Furthermore, the animal-vegetal axis is clearly expressed in the cortex. In starfish, the patterning of the cortex depends on microtubules arrays [95,96]. In sea urchins, the oocyte cortex contains arrays of microfilaments, microtubules, and intermediate filaments [97,98]. So in sea urchins and starfish, as in *Xenopus laevis* and *Drosophila melanogaster*, some spatial information in oocytes is imparted by polarized cytoskeletal arrays, and some is imparted by patterns in the plasma membrane or cortex.

The cytoskeleton and plasma membrane interact in complex ways, so the patterns in one cannot be completely understood apart from patterns in the other. Nevertheless, the remainder of this review focuses primarily on patterns in the plasma membrane.

BIOLOGICAL MEMBRANES

In 1925, Evert Gorter and François Grendel proposed that biological membranes are composed of lipid bilayers, with the hydrophobic ends of the constituent lipid molecules embedded in the middle of the bilayer [99]. In 1970, L. D. Frye and Michael Edidin labeled cell surface antigens in mouse cells with an antibody that fluoresced at a particular wavelength, and they labeled cell surface antigens in human cells with an antibody that fluoresced at a different wavelength. Then they used a virus to induce fusion of the mouse and human cells into heterokaryons. After about 40 minutes at 37° C, the labeled antigens had intermixed, spreading over the entire membrane in most of the heterokaryons. When Frye and Edidin inhibited protein synthesis there was no effect on the rate of intermixing, but when they lowered the temperature to 15° C the rate decreased. The results were consistent with simple diffusion, and Frye and Edidin concluded that membrane-associated proteins are free to diffuse laterally in the plane of the membrane [100].

Citing the work of Frye and Edidin two years later, Seymour Singer and Garth Nicolson proposed a "fluid mosaic model" of cell membranes. In their model, the lipid bilayer is like a two-dimensional sea in which mostly monomeric proteins float, unrestricted in their lateral movements except for isolated local interactions [101].But a new technique called fluorescence recovery after photobleaching (FRAP) soon provided evidence that the fluid mosaic model was inadequate. In the FRAP technique, a membrane is first labeled with fluorescent probes; a highly focused bright light bleaches the probes in a small patch of the membrane; and fluorescent molecules then diffuse into the bleached patch [102,103]. Results showed that the rate of diffusion of many proteins in natural (as opposed to artificial) biological membranes was much slower than predicted by the fluid mosaic model [104,105].

In 1984 Wolfhard Almers and Charles Stirling reported, "It is becoming clear that in fully differentiated tissues, a large fraction of membrane protein is neither freely mobile nor randomly distributed… Evidently, Frye and Edidin's 1970 finding of unrestricted mobility was atypical" [106]. Evidence against unrestricted mobility continued to accumulate, and in 1991 Edidin (along with Scot Kuo and Michael Sheetz) concluded that "the lateral diffusion of most [membrane] proteins is hindered in several ways," producing spatially restricted domains or "patches" rather than a random distribution [107].

In 2005, Donald Engleman concluded that the fluid mosaic model needed to be re-examined, because each of its principal ideas was "misleading." Instead of being a lipid bilayer sea in which widely dispersed monomeric proteins float freely, biological membranes are "typically crowded" with oligomers and "preferential associations," such that "patchiness is the order of the day" [108]. The same year, Akihiro Kusumi and his colleagues modeled the plasma membrane "as a compartmentalized fluid, in which compartmentalization is caused by the fence (corralling) effects of the membrane skeleton [which is actin-based in most mammalian cells] as well as the hydrodynamic slowing effects of transmembrane-protein pickets anchored on the membrane-skeleton fence" [109].

Proteins are not the only molecules heterogeneously distributed in the membrane—lipids are, too. Various researchers in the 1970s and 1980s presented evidence for spatial domains in membrane lipids [110–112]. Domains rich in sphingolipids and cholesterol were called "lipid rafts" [113]. For several years their existence was controversial [114–117], but improved experimental techniques subsequently yielded abundant evidence for lipid raft nanodomains [118–122]. Like membrane protein compartments, lipid rafts may be formed and maintained, in part, through cytoskeletal interactions [123,124], and they play important roles in membrane organization and signal transduction [125–127].

So biological membranes are patterned in complex ways. Those patterns serve important functions in cells, tissues and embryos. The following sections summarize the roles of plasma membrane patterns in (a) providing targets and sources for intracellular transport and signaling, (b) regulating cell-cell

interactions by means of a "sugar code," and (c) generating endogenous electric fields that provide three-dimensional coordinate systems for ontogeny.

INTRACELLULAR TARGETS AND SIGNALING

The precise intracellular localization of mRNAs is functionally important in many animal cells—not just oocytes [128–131]. The localization of mRNAs commonly depends on specific sequences in their untranslated regions that have been called "zipcodes" [132]. Like postal zipcodes, such sequences identify the "addresses" in the cell to which the mRNAs are to be sent. Like a postal zipcode, however, an mRNA zipcode is meaningless unless it matches a pre-existing address—that is, a target.

Evidence from a variety of cells suggests that mRNA localization requires the binding of a protein to the zipcode to form a ribonucleoprotein particle (RNP); the combination is then transported to its destination [133]. A well-studied example is the RNA zipcode-binding protein ZBP-1, which is necessary for the localization of β-actin mRNA to the leading edges of migrating fibroblasts (where actin microfilaments are polymerizing) [134–136]. Another example is Vg1 RBP (for "RNA-binding protein"), which is necessary for localizing *Vg1* mRNA to the vegetal pole in *Xenopus* oocytes [65,137]. Still another example is heterogeneous nuclear ribonucleoprotein A2 (hn-RNP A2), which is involved in localizing mRNAs in neurons [138].

Like zipcodes themselves, however, zipcode-binding proteins do not specify the destination. Using the postal code metaphor, zipcode-binding proteins could be likened to cargo containers, cytoskeletal motor molecules to delivery trucks, and the cytoskeleton to the highway system on which the trucks travel. But destinations for intracellular transport—like the geographical addresses in a postal delivery system—must also be specified.

In some cases, destinations might be specified by the spatial arrangement of microtubules; in the postal metaphor, packages could be dispatched on a particular highway and then carried to the end of the road and simply dropped off. In some cases, however, destinations are known to be specified by targets in the form of membrane-bound proteins that respond to extracellular cues. For example, β-actin mRNA localization in fibroblasts is induced by receptor tyrosine kinases (RTKs) in the plasma membrane that respond to extracellular platelet-derived growth factor [139,140]. RTKs consist of a ligand-binding extracellular domain, a single α-helix that passes through the membrane, and an intracellular domain containing a protein tyrosine kinase [141,142]. Upon activation, the tyrosine kinase transfers a phosphate group from ATP to a protein, initiating a complex and dynamic intracellular signaling cascade that includes small guanosine-triphosphatases (GTPases) of the Rho family. These molecules—especially the cell division control protein Cdc42—polarize the microtubule network that delivers β-actin mRNA to the source of the signaling cascade [143–146]. The fibroblast then migrates in the direction of the external cue.

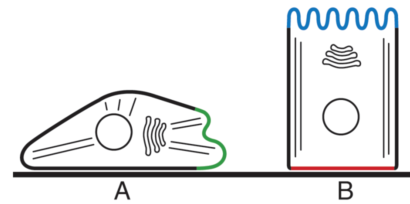

Figure 3: Comparison of mesenchymal and epithelial polarity. The illustration A) shows a highly simplified drawing of a mesenchymal cell moving along extracellular matrix (the heavy straight line at the bottom). Activated membrane-bound receptor tyrosine kinases (green) induce front-to-back polarity and actin polymerization at the leading edge. The illustration B) shows a highly simplified epithelial cell, resting on the extracellular matrix and tightly bordered on either side by other epithelial cells (not shown). Channels in the basal membrane (red) pump sodium ions out of the cell and channels in the apical membrane (blue) admit sodium ions into the cell. The result is a trans-epithelial potential, basal side positive. In both cells the large circle in the center is the nucleus, the parallel stack of inner membranes is the Golgi apparatus, and the straight lines are microtubules. **doi:**10.5048/BIO-C.2014.2.f3

In an animal embryo, cells that migrate easily (such as fibroblasts) are called mesenchyme, while cells that are immobilized in adherent sheets are called epithelium. Mesenchymal cells exhibit front-to-back polarity, with an axis oriented in the direction of movement. Immobilized epithelial cells exhibit top-to-bottom (apical-basal) polarity, with an apex oriented toward a lumen or the outside of a tissue and a base attached to an extracellular substratum (Fig. 3). Cells in epithelia are also attached to each other laterally by tight junctions to form an impervious protective layer [147,148].

Mesenchymal cells can transform into epithelial cells, and *vice versa*, in what are called the mesenchymal-epithelial transition and the epithelial-mesenchymal transition, respectively [149]. Both transitions are essential in metazoan ontogeny. Organizing cells into tissues depends on coordinating cell polarities, and in the process cells can switch between various polarized states [150]. After gastrulation, vertebrate embryos develop a neural tube composed of epithelial cells. At the end of neurulation, some of these (the neural crest cells) transform into mesenchymal cells that migrate elsewhere in the embryo to form craniofacial structures, the peripheral nervous system, pigment cells in the skin, and parts of the cardiovascular system [151,152]. Upon reaching their destinations, most of these mesenchymal cells then transform back into epithelial cells [153].

Polarity in an epithelial cell is normally initiated by contact with another cell [154]. The contact point produces an asymmetry in the plasma membrane that leads to the asymmetric localization of various proteins [155]. This asymmetric localization is mediated by differences in phosphorylated lipids in the apical and basal membranes [156,157], and by various sorting signals associated with the proteins. A particularly well-studied example of the latter is the glycophosphatidylinositol "anchor" [158,159]. Two key signaling molecules are the phosphoinositides PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2. In mesenchymal cells, these regulate protrusion of the front

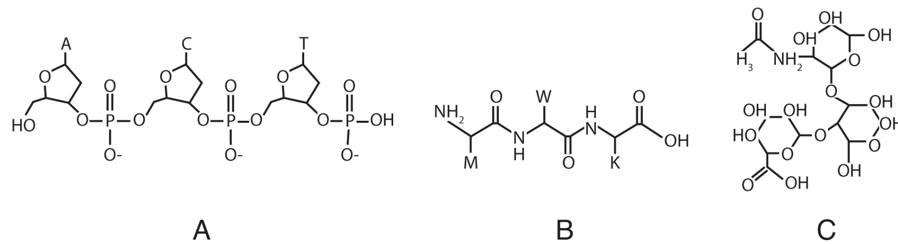

Figure 4: Linear vs. branched information. Illustrated are A) a trinucleotide, B) a tripeptide, and C) a trisaccharide. Nucleotides in DNA are covalently linked to each other only in linear chains; the same is true for amino acids in polypeptides (proteins), with a few exceptions. But monosaccharides can be covalently linked to each other through one or more of their hydroxyl groups, so they can be combined with other monosaccharides in several different ways to form branched glycans. **doi:**10.5048/BIO-C.2014.2.f4

and retraction of the back to produce forward movement. In epithelial cells, the same molecules maintain the functional differences between the apical and basal domains [160,161].

Although polarity in mesenchymal and epithelial cells is normally initiated in response to extracellular cues, the plasma membranes of many other animal cells have spatially heterogeneous patterns that are more intrinsic to isolated cells.

THE SUGAR CODE

The plasma membranes of all living cells studied to date are covered by arrays of carbohydrates called "glycans" [162]. Glycans can be attached to lipid molecules (glycolipids) or to proteins (glycoproteins), and many of them are quite complex [163]. In living cells, nucleotides in DNA are covalently linked to each other in linear chains; with some exceptions, the same is true for amino acids in proteins. But monosaccharides can be covalently linked to each other through one or more of their hydroxyl groups. Since D-glucose (for example) has five hydroxyl groups, one of which can assume two different positions, it can be covalently linked to other monosaccharides in six different ways (Fig. 4). As a result, carbohydrates can form branching chains that are far more elaborate than linear chains

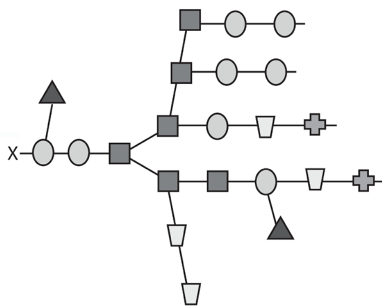

Figure 5: A branched glycan. The five different shapes here represent five different monosaccharides. This shows one way these monosaccharides could be covalently connected to form a branched glycan. The "X" on the left could be either a protein (in which case this would be a glycoprotein) or a lipid molecule (in which case this would be a glycolipid). More monosaccharides (including others not represented here) could be attached on the right (among other places), yielding trillions of different glycans carrying different glycocodes. **doi:**10.5048/BIO-C.2014.2.f5

of nucleotides and amino acids (Fig. 5) [164].

While the four nucleotides in the genome can form a maximum of $4^6 \approx 4 \times 10^3$ hexanucleotides, and the twenty amino acids in the proteome can form a maximum of $20^6 \approx 6 \times 10^7$ hexapeptides, the dozen or so monosaccharides in the "glycome" can theoretically form more than 10^{12} hexasaccharides. Clearly, the information-carrying capacity of the "glycome" far exceeds the combined capacities of the genome and the proteome. The information carried by the glycome has been called the "glycocode" or "sugar code" [165–169].

Glycosaminoglycans (GAGs) are unbranched polysaccharides composed of disaccharide subunits containing an amino group. Yet although they are unbranched, they can be assembled from dozens of different subunits, and sulfate groups can be attached to them in a wide variety of patterns. For example, a sulfate group can be attached to a trisaccharide in ten different positions, increasing its information-carrying capacity tenfold [170]. This makes GAGs some of the most information-dense molecules in biology [171–173]. Of the five types of glycosaminoglycans, four are covalently attached to proteins to form proteoglycans (PGs). Like glycolipids and glycoproteins, PGs are common in the plasma membranes of many cells.

The complexity of glycans has made it difficult to study them in living cells and tissues with standard biochemical methods, but antibodies have proven useful in identifying specific carbohydrate moieties [174]. Studies using monoclonal antibodies have shown that cell-surface glycans in early mouse embryos change in a highly ordered and stage-specific manner; the data suggest that they mediate cellular orientation, migration, and responses to regulatory factors during development [175–177]. Monoclonal antibody studies have also found cell- and stage-specific changes in cell-surface glycans during early embryogenesis in the chick [178,179], and during neural development in late-stage rat embryos [180].

Genetic analyses have produced comparable results. Mutations in genes encoding the protein portions of glycoproteins and sulfated PGs, and mutations in genes encoding various enzymes involved in glycosylation or sulfation, have revealed spatiotemporal expression patterns in the embryos of *Caenorhabditis elegans*, *Drosophila melanogaster*, mice and humans [181,182], as well as in zebrafish [183]. These patterns play important roles in development [184–186].

Another approach to studying cell-surface glycans is matrix-assisted laser desorption/ionization (MALDI) mass spectrometry imaging [187]. This technique has been used to study gangliosides, a class of glycolipids that are typically partitioned into lipid rafts and have hundreds of different carbohydrate side-chains. MALDI mass spectrometry has revealed striking differential distributions of gangliosides in mouse brain tissues [188].

The sugar code can be "interpreted" by proteins called lectins. Unlike antibodies, lectins are not produced by the immune system, and unlike enzymes they do not catalyze biochemical reactions, but like antibodies and enzymes they "recognize" specific three-dimensional structures of other molecules. They do this by means of "carbohydrate recognition domains" (CRDs) [189–191].

Lectins mediate a wide variety of cell-cell interactions [192–194]. They have also been useful in studying the distribution and functions of cell-surface glycans. For example, lectins with different specificities have revealed spatial and temporal changes in the distribution of cell surface oligosaccharides during gastrulation and neurulation in chick embryos [195]. Comparative analyses with lectins have also shown reproducible differences in the spatial distribution of cell-surface carbohydrates in the olfactory sensory neurons of mice [196].

Galectins are a taxonomically widespread family of animal lectins that bind glycans containing β-galactoside [197–199]. Certain galectins are differentially expressed in early human embryogenesis, suggesting a role in tissue differentiation [200]. Other galectins are temporally and spatially regulated in frog embryos [201]. By forming lattices with glycoproteins on the plasma membrane, galectins help to organize cell-surface domains and thereby affect cell signaling [202,203]. Recent work has shown that a network of two galectins interacting with cell-surface moieties regulates skeletal morphogenesis in chick limbs [204].

The examples cited above involve tissue-level differences, but spatial localization of cell-surface glycans has also been observed in single cells. Several examples involve fungal cells: Immunological analyses have demonstrated changes in cell wall glycoprotein distribution during germ-tube formation in *Candida albicans* [205]; *Cryptococcus neoformans* cells exposed to fluorescently labeled lectin showed marked localization of cell-surface glycans around sites of cell division [206]; and a similar approach revealed heterogeneous subpopulations of lectin ligands on hyphal bodies of *Beauveria bassiana* [207].

Studies using fluorescently labeled lectins have shown that several glycosidases—enzymes that catalyze the hydrolysis of the bonds joining carbohydrates to other molecules—occur in the plasma membrane of *Drosophila* spermatozoa. The glycosidases are localized to the sperm's acrosomal region (the tip of the head) and the tail [208,209]. Complementary carbohydrate residues have been identified in mature *Drosophila* oocytes, where they are localized to the micropyle—a hollow anterior projection through which the sperm enters. This pattern of cell surface glycans suggests a role for them in fertilization (Fig. 6) [210–212].

New techniques may soon make it possible to characterize more precisely the spatial localization of specific glycans on the surfaces of single cells. For example, labeled sugars can be incorporated into specific carbohydrates in a process called "metabolic oligosaccharide engineering" [213–215]. This technique has been used to study spatiotemporal differences in cell-surface glycans during zebrafish development [216]. Another potentially fruitful approach is to use functionalized quantum dots and glyconanoparticles for the fluorescent imaging of dynamic glycan expression on single cells [217,218].

ENDOGENOUS ELECTRIC FIELDS

It has long been known that probably all living cells (not just nerve and muscle cells) generate electric fields across their membranes [219–222]. In animal cells, a sodium-potassium pump in the membrane utilizes energy from ATP to move three sodium ions out of the cell while taking in two potassium ions [223,224]. This raises the intracellular concentration of potassium ions, which corrects the imbalance by flowing out of the cell through ion-selective channels in the membrane. The combined action of sodium-potassium pumps and potassium "leak" channels makes the interior of the cell electrically negative with respect to the exterior. The resulting voltage difference across the membrane is called the "membrane potential" [225–227].

The voltage difference (V) per unit distance is the electric

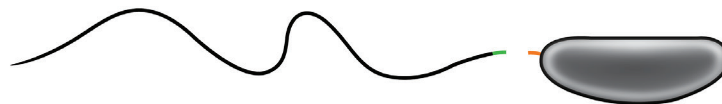

Figure 6. Glycans in *Drosophila* fertilization. This shows a *Drosophila melanogaster* spermatozoon and oocyte just before fertilization. The two are drawn to scale: The sperm is ~1.8 mm long, and the oocyte is ~0.5 mm long. The acrosome (the tip of the head of the sperm) contains the glycosylated enzyme α-L-fucosidase (green). The portion of the sperm head just behind the green label contains the nucleus; farther back, the sperm tail contains more α-L-fucosidase (not shown). The micropyle (a small hollow projection at the anterior end of the oocyte through which the sperm will enter the egg) contains α-L-fucose (orange). The two complementary glycans are thought to mediate fertilization. **doi:**10.5048/BIO-C.2014.2.f6

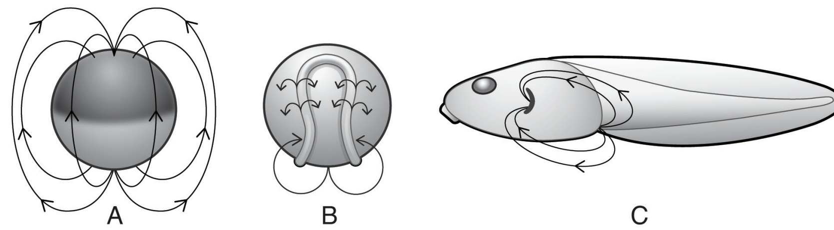

Figure 7. Endogenous electric fields in Xenopus embryos at progressive stages of development. A *Xenopus* oocyte (A) is shown with some of the field lines of the endogenous electric field (EEF) surrounding it. Positive ions exit through the vegetal pole and enter through the animal pole. A *Xenopus* neurula (B) is about the same size as the oocyte but consists of thousands of cells. The field lines show some of the paths of positive ions around the neural fold, which will close to form the embryonic spinal column. A *Xenopus* tadpole (C) has positive ions leaving the future site of hindlimb development and entering through the gills, creating an endogenous electric field. **doi:**10.5048/BIO-C.2014.2.f7

field (E), which in biological systems is usually expressed in units of mV/mm. The direction of an electric field is taken to be the direction in which positive charges flow; in living cells and embryos, electric currents consist of positive and negative ions rather than electrons. The amount of electric current (A) flowing across a unit area is the current density (J), which in biological systems is usually expressed in units of μA/cm^2. Electric field and current density are quantitatively related by the equation $E = J\rho$, in which ρ is the resistivity of the medium, measured in ohm-centimeters (Ω·cm). Endogenous electric current densities are usually of the order of 1–100 μA/cm^2 [228].

Cellular membrane potentials generally range from -20 to -200 mV DC. Multicellular organisms, and their organs, are covered by epithelia, which (as we saw above) are polarized. Among other things, the ion channels in the apical domain are different from those in the basolateral domain [161]. The result is a "transepithelial potential" (TEP) that depends on sodium ions. Unlike the transmembrane potential of individual cells, the TEP is usually negative on the outside of the organ or organism and positive on the inside. Most TEPs range from 15 to 60 mV (though they can be lower or much higher), and endogenous electric fields (EEFs) are typically in the 10–150 mV/mm range [229–231].

Membrane potentials were first measured with sharp microelectrodes developed in the 1940s [232]. In this technique, a very small microelectrode is inserted into the cytoplasm of a cell and the potential difference is measured between it and a reference electrode in the surrounding medium. In the 1970s, Erwin Neher and Bert Sakmann developed the technique of patch clamping, which uses a small glass pipette in contact with (but not penetrating) the plasma membrane to measure the membrane potential [233]. Since a sharp microelectrode unavoidably causes some leakage through the membrane, patch clamping is preferred [234].

Transmembrane voltage can also be measured using voltage-reporting dyes. In Förster resonance energy transfer (FRET), a dye molecule (the "donor") is stimulated to fluoresce at its characteristic wavelength; at the same time it transfers energy to a different dye molecule (the "acceptor") adjacent to it, which fluoresces at a different wavelength. If the two molecules are separated by even a small distance, the energy transfer does not occur and only the first molecule fluoresces. A useful donor is the phospholipid CC2-DMPE, which binds to the outside of the cell membrane, while a negatively charged hydrophobic acceptor such as $DiBAC_4(3)$ can move across the cell membrane in response to changes in membrane potential. When the donor is stimulated, the ratio between the amounts of fluorescence at the dyes' two wavelengths measures the degree of their separation and thus the membrane potential [235–237].

Endogenous direct current electric fields around cells and embryos can be measured with an ultrasensitive vibrating probe invented in 1974 by Lionel Jaffe and Richard Nuccitelli. The original probe measured the field difference between two points 30 μm apart while oscillating at 200 Hz to minimize noise [238]. Vibrating probes—enhanced by several technological improvements—are still being used to measure EEFs and ion currents in and around biological systems [239,240].

EEFs in oocytes and embryos

Mature *Xenopus* oocytes that have been dissected from their follicles have membrane potentials between 60 and 80 mV [241]. The resulting EEF is polarized along the animal-vegetal axis, and the oocytes drive electric currents with a density of about 1 μA/cm^2 through themselves, with positive charges entering around the animal pole and exiting through the vegetal pole (Fig. 7A) [242]. After fertilization the embryo enters the cleavage stage; without increasing its overall size, it subdivides into thousands of cells called blastomeres. In the first and second cleavage divisions, large electrical currents leave the new membrane forming in the furrows and return through the older membrane outside the furrow [243]. As a *Xenopus* embryo continues to cleave, it forms a fluid-filled central cavity called a blastocoele, and the blastomeres develop into an epithelium with an apical side facing the external medium. The resulting transepithelial potential is about 20 mV, blastocoele positive [244].

When cleavage ends gastrulation begins, and cells from the exterior migrate into the blastocoele through the blastopore (the location of which was previously determined by cortical rotation). A large current flows outward through the blastopore, carried mainly by Na^+ ions [245]. When the neural folds form, a steady ionic current is driven out of the lateral walls of the folds (Fig. 7B) [246]. After the folds close, the neural tube

maintains a potential difference of ~18 mV across its walls; since the walls are only ~40 μm thick, the resulting electric field across the neural tube cells is over 400 mV/mm [247]. The neurula then elongates into a tadpole, and electric currents flow outward from the two flank areas where the hind limbs will form and flow back inward through the gills (Fig. 7C) [248]. These currents reach their maxima (about 2 μA/cm^2) just after the limb buds become visible and decline after that [249].

Ionic currents are also present in developing chick embryos. The embryos of birds form a double-layered "blastodisc" of epithelial cells resting on the egg yolk; a groove called the "primitive streak" then forms in the outer layer. During gastrulation, cells migrate through the primitive streak into the space between the two layers. Measurements with a vibrating probe show that strong electrical currents leave the primitive streak, with densities of 10–20 μA/cm^2 [250]. The Na^+-dependent currents return through the periphery of the blastodisc [251]. Even larger currents have been detected at the posterior intestinal portal (PIP), located at the tail end of the primitive streak. In chick embryos between two and four days old, currents with densities of 46–60 μA/cm^2 begin to flow into the PIP, followed later by outward currents with densities as high as 192 μA/cm^2 [252].

Early mammalian embryos also develop as blastodiscs and form primitive streaks. Ionic currents with densities of 20–60 μA/cm^2 have been detected leaving the primitive streaks of mouse embryos [253]. Like frog embryos, mouse embryos produce outward currents associated with their limb buds. Chick embryos also produce very large outward currents (about 100 μA/cm^2) at the future sites of their limb buds, three developmental stages before the latter become visible [254].

Effects of electric fields in vitro

How might embryonic electric currents and fields influence development? One way might be to direct cell movements. For over a century electrically guided locomotion, called "galvanotaxis," has been observed in cells from a variety of organisms in the presence of artificially applied electric fields [255]. But can relatively small *endogenous* electric fields produce galvanotaxis? One way to test this is to apply artificial electric fields of physiological strength to embryonic cells *in vitro*.

In the 1980s, Robert Stump and Kenneth Robinson reported that *Xenopus* neural crest cells migrated toward the cathode (the negative pole) of an applied DC electric field of 10 mV/mm, well within the range of observed EEFs [256,257]. Richard Nuccitelli and Carol Erickson reported the same behavior for quail neural crest-derived fibroblasts [258,259]. By contrast, chick embryonic Schwann cells (which are derived from peripheral nerves) migrate preferentially toward the anode (the positive pole) in DC electric fields ranging from 10 to 100 mV/mm [260]. Not all embryonic cells, however, migrate directionally in an electric field of physiological strength [261,262].

Applied electric fields can also affect neural networks. Nerve cells establish contact with each other by extending projections called neurites. When embryonic chick ganglia were placed in DC electric fields ranging from 70–140 mV/mm, their neurites grew faster toward the cathode than the anode [263]. Embryonic *Xenopus* neurons reportedly do the same in fields as low as 7 mV/mm [264,265].

Disrupting bioelectric patterns in embryogenesis

The most compelling evidence that ion currents, transmembrane voltage potentials and EEFs play significant roles in ontogeny comes from artificially disrupting them *in vivo* and then observing the effects of their disruption on morphogenesis. As we saw above, a strong current flows outward from the posterior intestinal portal (PIP) in four day-old chick embryos. In 1992, Kenneth Robinson and Kevin Hotary implanted conductive shunts into such embryos and thereby reduced by 30% the endogenous electric currents leaving the PIP. Over 90% of the affected embryos exhibited developmental abnormalities, most (as expected) in tail development—ranging from abnormal morphology to complete absence. In a control group receiving non-conductive implants, only 11% displayed defects [266].

In gastrulating *Xenopus* embryos, as we saw above, a large current flows outward through the blastopore. This current is associated with an intraembryonic head-to-tail EEF of 27 ± 4 mV/mm. In 1994, Hotary and Robinson inserted microelectrodes into embryos at this stage and applied electric currents of various magnitudes. A group of twenty-three embryos received currents of 100–500 nA in the opposite direction of the normal outward current; of these, twenty developed abnormalities that included reduced heads and retarded eye formation. Microelectrodes were also inserted in a control group of fourteen embryos, but the applied current was either 10 nA or zero; of these, only one developed abnormally [267].

After gastrulation, as we saw above, the neural tube of a *Xenopus* embryo maintains a potential difference across itself. In the mid-1990s, Riyi Shi and Richard Borgens studied the transneural tube potential (TNTP) in axolotl embryos and reported it to be 40–90 mV. As in *Xenopus*, the lumen of the neural tube in axolotls is negative with respect to the outside. When Shi and Borgens injected Na^+ channel blockers into the lumen through microelectrodes, the TNTP dropped by 30% immediately and by 80% within fifteen minutes. Of fifty embryos treated in several different experiments all but one developed severe cranial defects, ranging from substantial deletions of central nervous system structures to the complete absence of a head. In one control group, eleven embryos were similarly injected, but without Na^+ channel blockers; all developed normally. As another control, the Na^+ channel blockers were injected just under the surface ectoderm (rather than into the neural tube) in twenty embryos, and all but one of these developed normally [268,269].

In 2011 Laura Vandenberg, Ryan Morris and Dany Adams disrupted cranial development in *Xenopus* tadpoles by inhibiting an ion channel protein. Different subpopulations of ectodermal cells in normal tadpoles are distinguished by different membrane voltages generated by ATP-dependent proton pumps in the

plasma membrane known as (H^+)-ATPases (or V-ATPases, because they were originally discovered in vacuolar membranes) [270–272]. Adams and her colleagues had previously shown that V-ATPases are necessary in left-right patterning and tail regeneration in *Xenopus* [273,274]. V-ATPases are also involved in the anterior-posterior patterning of the *Xenopus* nervous system [275]. Vandenberg and her colleagues made a mutant mRNA for ductin, the proton-pumping subunit of V-ATPases [276]. This mRNA was translated into non-proton-pumping ductin, and its injection into early *Xenopus* embryos caused craniofacial abnormalities such as small and deformed heads, malformed auditory organs, and abnormal or misplaced eyes. Ductin-independent reagents that affect membrane voltage produced similar results, so the abnormalities were not caused directly by the mutant protein. The voltage-reporting dyes CC2-DMPE and $DiBAC_4(3)$ confirmed that the treatments had altered the bioelectrical patterns of the embryos, and in situ hybridization showed that the altered bioelectrical patterns corresponded to disrupted expression patterns of several genes involved in craniofacial development [277].

Vaibhav Pai, Michael Levin and their colleagues also manipulated ion channels in *Xenopus* embryos to induce changes in transmembrane voltage potential that disrupted eye patterning. Using CC2-DMPE, they first showed that eyes normally develop from two small areas in the anterior neural field that are hyperpolarized (internally more negative, by about 10mV) relative to neighboring tissues. To determine whether hyperpolarization is required for normal eye development, the researchers injected the dorsal blastomeres of four-cell-stage *Xenopus* embryos with mRNAs for two ion channels that are not normally present: EXP1, which conducts positively charged ions into the cell [278], and GlyR, which can be chemically activated to enable the outflow of Cl^- ions [279,280]. Since hyperpolarization requires the exclusion of positively charged ions and the retention of negatively charged ions, these two channels strongly disrupted the hyperpolarization patterns in injected embryos. The resulting tadpoles had a high incidence of incomplete, small, or deformed eyes—or they were missing eyes entirely. Control embryos that were uninjected, injected with a non-channel protein, or injected with GlyR alone or its chemical activator alone, developed well-formed eyes. Various other ion channels were also used to perturb the normal pattern of transmembrane voltage potential. The results included not only the eye defects listed above, but also ectopic eyes well outside the anterior neural field—including eyes on the tadpole's side or tail [281].

EEFs as spatial coordinate systems

In the 1980s, Jaffe and Nuccitelli both proposed that ionic currents and/or voltage gradients could provide ontogenetic information for embryonic pattern formation, though they acknowledged that many experiments remained to be done [248,282]. Experiments such as those described above clearly demonstrate that the disruption of bioelectric patterns has significant local effects on ontogeny, but EEFs might play a larger role as well.

Harold Burr and Filmer Northrop suggested in the 1930s that endogenous "electro-dynamic fields" determine (or at least strongly influence) the global anatomy of an organism [220]. In 1995, Shi and Borgens reviewed the experimental evidence that embryonic cells are responsive to applied voltages in the physiological range and that disruption of EEFs produces developmental defects consistent with the disruption. They concluded that EEFs "may provide a three dimensional coordinate system" that helps to specify form in embryos [283].

The term "morphogenetic field" has had many meanings since the idea of embryonic fields was first proposed by Alexander Gurwitsch in the early twentieth century [284–286]. As used by Michael Levin, the field concept denotes both informational and regional relationships, and its essence is "non-locality"—the idea that "many diverse examples of pattern formation are best understood not as cell-level behaviors around any one locale but rather at higher levels of organization." Although gradients of morphogenetic molecules may specify some patterns, Levin (following Burr and others) argues that EEFs may also function as "templates of shape." Thus "it is likely that a full understanding of the morphogenetic field and its informational content will need to involve cracking the bioelectric code (the mapping between spatiotemporal ionic profile patterns and tissue morphology outcomes)" [287,288].

MEMBRANE PATTERNS AND DNA

So membrane patterns—the three-dimensional arrangements of membrane-associated proteins, lipids and carbohydrates, as they change over time—carry essential ontogenetic information. Yet (as I demonstrate below) the information carried by membrane patterns cannot be reduced to sequence information in DNA, for at least two reasons. First, the vast majority of proteins in eukaryotes are not completely specified by DNA sequences. Second, even if DNA sequences completely specified all proteins, DNA would not specify their spatiotemporal arrangements in membranes.

Most proteins are not completely specified by DNA sequences

The central dogma (which here includes Crick's sequence hypothesis) claims that (1) DNA specifies RNA and (2) RNA specifies protein. Yet this claim fails at both steps, because most RNAs are not uniquely specified by DNA sequences, and many proteins are not uniquely specified by RNAs—either in their amino acid sequences or in their final folded forms.

After transcription, RNAs from many eukaryotic genes undergo alternative splicing. Recent studies estimate that transcripts from approximately 95% of multi-exon human genes are spliced in more than one way [289–291]. By intervening between transcription and translation, alternative splicing generates RNAs with sequences that differ from DNA sequences [292]. The differences are functionally significant. In humans and other animals, alternatively spliced transcripts are expressed in tissue- and developmental stage-specific ways [293,294]. Among other things, they regulate physiological

changes [295], neuronal development [296], and stem cell pluripotency [297]. Alternative splicing is essential for the transition between epithelial cells and mesenchymal cells [298,299], and it produces enormous diversity in membrane proteins, including ion channels [300,301]. With alternative RNA splicing, cells make thousands more RNAs and proteins than are encoded in DNA sequences. One immunoglobulin gene in *Drosophila melanogaster* generates more than 18,000 protein isoforms through alternative splicing [302].

In addition to alternative splicing, many metazoan transcripts undergo RNA editing, which can (a) modify cytidine to uridine; (b) modify adenosine to inosine; or (c) insert additional nucleotides. Several recent analyses have demonstrated extensive RNA editing in the human transcriptome [303–305]. The editing of an mRNA often alters the amino acid sequence of the encoded protein so that it differs from the sequence predicted by the DNA [306,307]. RNA editing can also regulate the amount of functional RNA in the cell and expand the functionality of a limited set of transcripts [308]. One example of functional editing is found in two species of octopus, one living in the Antarctic and the other in the Caribbean. Both possess very similar genes for a particular potassium channel, but in the Antarctic species RNA editing recodes an isoleucine to a valine in the channel's pore, greatly accelerating its gating kinetics so that it can function properly in the extreme cold [309]. Other examples of functional RNA editing involve adenosine deaminases that act on RNAs (ADARs), which selectively convert adenosine (A) to inosine (I) [310]. ADARs are widespread in metazoan nervous systems, where they edit pre-mRNAs of proteins involved in electrical and chemical neurotransmission [311–314]. In mice, ADARs are necessary for the survival of the stem cells that generate mature blood cells [315], and they play an important role in the gastrointestinal tract [316].

Not only are the nucleotide sequences of many RNAs not fully specified by DNA sequences, but also the amino acid sequences and final folded forms of many proteins—on which their functions depend—are not fully specified by RNA sequences. Some proteins undergo splicing, a phenomenon first reported in yeast in 1990 [317,318]. In protein splicing, an "intein" (analogous to an intron in RNA splicing) is removed from the protein and the flanking pieces ("exteins") are spliced together without the need for cofactors or enzymes [319–323]. Although protein splicing has been found only in unicellular organisms, a somewhat analogous process occurs in the biogenesis of various metazoan proteins. The process in metazoans is called "autoproteolysis," in which a protein cleaves itself into two pieces. Autoproteolysis is widespread in the hedgehog family of proteins (needed for signal transduction in animal development) and in nuclear pore complex proteins [324–328].

Even in many proteins that do not undergo splicing, the amino acid sequence does not completely specify the final folded form. Protein folding can be affected by the intracellular milieu, including factors such as pH, the variety of molecular chaperones, and the degree of macromolecular crowding [329–334]. Some proteins adopt similar forms and/or functions despite having very dissimilar amino acid sequences [335–339]. Others assume different three-dimensional forms despite having the same or very similar amino acid sequences. Some well-known examples of the latter are prions—misfolded proteins that are pathogenic and heritable [340–343]. But there are also non-pathogenic proteins in which the same amino acid sequence can fold into more than one shape; these are known as "metamorphic" proteins [344,345]. One example of a metamorphic protein is Mad2 (for "mitotic arrest deficient"), a protein that monitors the attachment of microtubules to kinetochores [346,347]. Another example is lymphotactin, a small secreted signaling molecule [348,349]. A third example is the CLIC family of proteins, which function in chloride channels [350,351]. Still another example is IscU, which is involved in iron-sulfur cluster assembly in bacteria and eukaryotic mitochondria [352,353].

Some proteins are "intrinsically disordered." Approximately 20–30% of proteins in mammalian cells are inherently devoid of any ordered three-dimensional structure and adopt folded conformations only after interacting with other molecules [354]. More than 50% of eukaryotic proteins have at least one domain longer than 30 amino acids that is intrinsically disordered [355–358]. Intrinsically disordered proteins play crucial roles in regulating ion channels and serving as molecular hubs in intracellular signaling networks [359–361].

Finally, most eukaryotic proteins are post-translationally modified by glycosylation [362]. First a glycan is attached to the protein, usually through an asparagine, serine or threonine residue [363–366]. In some cases the residue is part of a consensus sequence, but in other cases it is not [367,368]. For branched glycoproteins, enzymatic networks then add or remove carbohydrate residues in tissue- and developmental stage-specific patterns [369,370]. For unbranched proteoglycans, other enzymatic networks vary the order and sulfation patterns of the constituent disaccharides to yield different spatial and temporal distributions [371,372]. These enzymatic networks are quite complex, involving dozens of interacting glycotransferases, and they can vary glycosylation patterns epigenetically depending on the needs of the cell in a given environment or at a specific developmental stage [373–375]. The final glycosylated form of a protein is thus very far removed from direct specification by a DNA sequence. As various biochemists have put it, the modification of proteins through enzymatic glycosylation imparts "an additional level of 'information content' to underlying polypeptide structures" [376]; it "is an event that reaches beyond the genome" [377]; and if DNA-centered biology is compared to Newtonian physics, protein glycosylation may pave the way for a "quantum mechanics of biology" and a "scientific revolution analogous to the one which transformed the field of physics in the early 20th century" [378].

Membrane patterns are not specified by DNA

On the second point, even if DNA sequences uniquely specified the molecular structures of proteins, DNA would

not specify the spatial distribution of proteins in the plasma membrane. Some membrane patterns are templated by the membranes from which they are derived, with proteins from the cell interior being incorporated during membrane growth only if they match the existing matrix [379]. Such templating has been well studied in protozoa. In 1965, Janine Beisson and Tracy Sonneborn induced one member of a conjugating pair of *Paramecium aurelia* to transfer to its partner a section of cortex that had been surgically inverted 180° relative to the surrounding cortex. Although the DNA was unchanged, the altered pattern of cilia in the cortex was heritable. Indeed, ciliates with artificially inverted rows have been stably maintained for thousands of generations [380–382]. In 1977, Stephen Ng and Joseph Frankel observed the same phenomenon in *Tetrahymena pyriformis* and concluded, "The cell as an architect thus not only makes use of the genomic information to produce the appropriate building blocks, but, in addition, also arranges the building blocks according to the blueprint as defined in the preexisting architecture" [383]. Frankel called this extra-genic blueprint the "corticotype" [384]. Similar results were reported in *Tetrahymena pyriformis* by David Nanney and in *Stylonychia mytilus* by Gary Grimes [385,386].

Cortical templating is not limited to protozoa. In 1930, Sonneborn reported it in a metazoan, the flatworm *Stenostomum incaudatum* [387,388]. In 1990, Michael Locke observed paired patterns in caterpillar epidermis cells that "imply that a part of the epigenetic sequence leading to the formation of the pattern has replicated [and been] inherited by daughter cells. It is not just genetic material that is inherited but part of a cell in a particular state." According to Locke, such inheritance "requires more than number and kind of molecule. The duplication of pattern involves relative position and orientation," factors that "cannot be specified only by a base sequence" [389].

In 1983, Robert Poyton proposed that biological membranes carry "spatial memory," the units of which are spatially localized hetero-oligomeric proteins. According to Poyton, when phospholipids are incorporated into a growing membrane in preparation for replication, the hetero-oligomers dissociate into their subunits. Newly synthesized subunits in the cytoplasm then associate with the corresponding older subunits to form hybrid hetero-oligomers that are chemically identical to the originals. Thus each unit of spatial memory would replicate semi-conservatively, like DNA (Fig. 8) [390]. Consistent with Poyton's hypothesis is evidence that local lipid bilayer properties influence the stability of transmembrane oligomers [391–393]; that protein dimers in membranes dissociate when diluted with phospholipids [394,395]; that membrane proteins selectively recruit other proteins in ESCRT complexes and Ras nanoclusters [396–401]; and that intracellular membranes grow by incorporating new proteins into pre-existing templates [402]. Poyton concluded, "Realizing that genetic memory is one-dimensional, along a DNA molecule, whereas spatial memory is likely to be two-dimensional, along membrane surfaces, and three-dimensional within the cellular interior, it is probable that spatial memory is more complicated and diverse than genetic memory" [390].

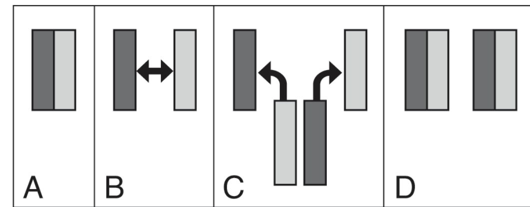

Figure 8. Membrane replication. In Robert Poyton's theory of membrane replication, the unit of membrane memory is a hetero-oligomeric protein, represented here in simplified form by the two rectangles (A). As the membrane grows from the addition of new phospholipids, the parts of the oligomer separate (B). Newly synthesized proteins from the cytoplasm associate with the complementary subunits from the original oligomer (C). The result is two new oligomers that are chemically identical to the originals (D). **doi:**10.5048/BIO-C.2014.2.f8

A eukaryotic cell contains many different types of membranes, which Thomas Cavalier-Smith has collectively designated the cell's "membranome" [403]. Each membrane is characterized not only by its chemistry but also by its topology. Some recent writers have used "membranome" in a reductionist sense to mean the list of individual proteins found in membranes, though this is clearly at odds with Cavalier-Smith's original topological formulation [404–406].

Cavalier-Smith distinguished between "genetic membranes" and "derived membranes." Genetic membranes, which include the endoplasmic reticulum and plasma membranes of many cells, "always arise by growth and division of the same type." Derived membranes, which include lysosomal membranes, "form instead by differentiation from dissimilar membranes." Some genetic membranes are "primary," that is, they grow by direct insertion of individual protein and lipid molecules. Other genetic membranes are "secondary," in the sense that they grow (in eukaryotes) by incorporation of vesicles from a primary genetic membrane [407]. If any type of genetic membrane were lost, it could probably not be regenerated from its constituent molecules—even if all the genes encoding its proteins and lipid-synthesizing machinery remained—because the requisite spatial pattern would be gone.

The closest thing to an exception to this last statement is found in Bryopsis, a genus of green algae consisting of large multinucleate cells. When one of these cells is damaged or fragmented it forms protoplasts in seawater that are not enclosed in plasma membranes, but in gelatinous envelopes made of polysaccharides. After about 12 hours a lipid bilayer might form underneath the polysaccharide coat—but only if a protoplast retains at least 15% of the original cell membrane. If such a protoplast also contains a nucleus, it might regenerate into a complete plant [408–413]. So even in the case of Bryopsis protoplasts, new plasma membrane is regenerated "by growth and division of the same type" of membrane, as Cavalier-Smith pointed out. It does not form de novo. Furthermore, the

membrane regeneration seen in Bryopsis appears to be unique; it is not found in other plants, in bacteria, in protozoa, or in metazoa—in which membrane patterns carry more information than can be preserved in a small fragment.

The importance of global spatial information can be illustrated by the first cell division of a *Xenopus* zygote. The animal-vegetal axis and cortical rotation divide the cortex into four broad zones. In Figure 9, the intersection of planes A and B corresponds to the animal-vegetal axis, while the intersection of planes A and C corresponds to the direction of cortical rotation. If the zygote divides along plane A, each of the two daughter cells will inherit not only a nucleus but also portions of all four zones of cortical information, so if the daughter cells are separated they can both develop into complete tadpoles. If the zygote divides along plane B or C, however, each daughter cell will inherit a nucleus, but not a full set of cortical information, so they cannot develop into two tadpoles.

Thus, according to Cavalier-Smith, the idea that the genome contains all the information needed to make an organism "is simply false. Membrane heredity, by providing chemically specific two-dimensional surfaces with mutually conserved topological relationships in the three spatial dimensions, plays a key role in the mechanisms that convert the linear information of DNA into the three-dimensional shapes of single cells and multicellular organisms. Animal development creates a complex three-dimensional multicellular organism not by starting from the linear information in DNA... but always starting from an already highly complex three-dimensional unicellular organism, the fertilized egg, which membrane and DNA heredity together have perpetuated" [403].

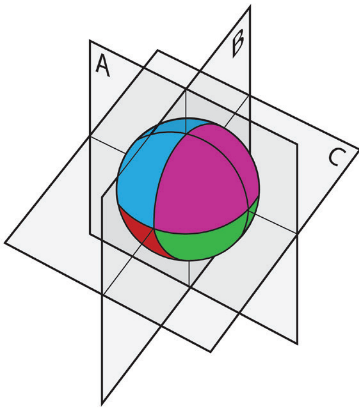

Figure 9: Zones of cortical information in a *Xenopus* zygote. The animal-vegetal axis and cortical rotation divide the cortex of a *Xenopus* zygote into four broad zones. Here the intersection of planes A and B corresponds to the animal-vegetal axis, while the intersection of planes A and C corresponds to the direction of cortical rotation. If the zygote (after duplication of the nucleus) divides along plane A, each of the two daughter cells will inherit not only a nucleus but also portions of all four zones of cortical information; if these daughter cells are separated, they can both develop into complete frogs. If the zygote divides along plane B or C, however, each daughter cell will inherit a nucleus, but not a full set of cortical information; their development will be blocked. **doi:**10.5048/BIO-C.2014.2.f9

Membrane heredity, however, cannot explain how membranes change in specified ways during ontogeny. In most cases, embryonic cells do not simply transmit duplicate membrane patterns to their daughter cells when they divide. The membrane patterns of nerve cells are very different from those of muscle cells, blood cells, bone cells, and liver cells—to name only a few—so membrane patterns have to change in the course of differentiation and morphogenesis. The changes are not haphazard, however; they are highly regulated to generate the integrated organ systems of an adult of the proper species. If membrane patterns are not specified by DNA sequences or by patterns in the cells from which they are derived, how are they specified? I will return to this question below.

SUMMARY AND IMPLICATIONS

In 1958, Francis Crick proposed that the specificity of a DNA segment lies solely in its nucleotide sequence, which encodes the nucleotide sequence of a messenger RNA (mRNA) and thereby the amino acid sequence of a protein. Crick also proposed that sequence information can be transferred from DNA to RNA to protein, but not in the other direction [1]. Crick called these the "sequence hypothesis" and the "central dogma," respectively. For brevity (and in accordance with common usage), I subsume both under the title "central dogma," which has been popularly stated as "DNA makes RNA makes protein makes us."

The central dogma logically implies that embryo development (ontogeny) is directed by a genetic program encoded in DNA sequences. Elaborating on this, Eric Davidson maintains that developmental gene regulatory networks (dGRNs) "control ontogeny of the body plan" [15]. But dGRNs must be distributed in embryonic space, and (as Davidson acknowledges) their distribution depends on spatial anisotropies in the egg that are defined in relation to the embryonic axes that establish the basic body plan.

Yet the evidence from oogenesis in *Drosophila*, *Xenopus*, and echinoderms shows that the major embryonic axes are established long before the embryo's dGRNs are put in place—much less activated. The anterior-posterior axis in *Drosophila*, the animal-vegetal axis in *Xenopus*, and the animal-vegetal axis in echinoderms are initially derived from the architecture of the ovary, through processes mediated by cytoskeletal and membrane patterns rather than dGRNs. Some might argue that the architecture of the ovary was set up by GRNs in the mother, but in the absence of sufficient evidence such an argument is simply a restatement of the central dogma. In embryo development, there is no empirical justification for giving DNA sequences ontogenetic priority over architectural constraints and cytoskeletal and membrane patterns.

Although cytoskeletal and membrane patterns interact in complex ways and cannot be completely understood apart from each other, this review has focused primarily on the latter. Biological membranes were once thought to be like two-dimensional seas in which proteins float and diffuse freely, unconstrained except for local interactions. It is now clear, however, that many membrane proteins and lipids are compartmentalized into spatially restricted domains and

arranged in global patterns that affect ontogeny.

Some plasma membrane patterns are formed in response to extracellular signals, as is the case with mesenchymal and epithelial cells; others are less dependent on the extracellular environment and more intrinsic to the cell, as is the case with spatially localized glycans and ion channels. Plasma membrane patterns serve essential ontogenetic functions by providing targets and sources for intracellular signaling and transport, by regulating cell-cell interactions, and by generating endogenous electric fields that provide three-dimensional coordinate systems for embryo development.

Membrane patterns are not specified by DNA sequences. First, DNA sequences only partially specify RNAs and proteins. After transcription, many RNAs undergo alternative splicing and/or editing, so thousands of different mRNAs can be generated from a single DNA sequence. After translation, some proteins are edited to produce different amino acid sequences, and many proteins with similar amino acid sequences can adopt more than one folded structure—or they are intrinsically disordered. Furthermore, most eukaryotic proteins are post-translationally modified by glycosylation. Given the enormous number of possible glycan structures, a protein can be modified in trillions of possible ways. If "makes" is taken to mean "specifies," then "DNA makes RNA makes protein" fails at each step.

Second, even if DNA completely specified proteins, it would not follow that one-dimensional DNA sequences specify the two- and three-dimensional arrangements of proteins in membranes. Many membrane patterns are templated by the membranes from which they are derived, and proteins from the cell interior are incorporated during membrane growth only if they match the existing matrix. Such templating has been especially well studied in protozoa, but it has also been observed in planaria and insect epidermis. According to evolutionary biologist Thomas Cavalier-Smith, the idea that the genome contains all the information needed to make an organism "is simply false. Membrane heredity, by providing chemically specific two-dimensional surfaces with mutually conserved topological relationships in the three spatial dimensions, plays a key role in the mechanisms that convert the linear information of DNA into the three-dimensional shapes of single cells and multicellular organisms" [403].

In embryo development, however, membrane heredity cannot be the whole story. During ontogeny many new membrane patterns arise that cannot be traced back to patterns in pre-existing membranes. The new patterns do not arise haphazardly; they are highly specified. Yet there is no evidence that they—any more than the patterns that precede them—are determined by a program in the organism's DNA. Whether membrane patterns are templated or form *de novo*, they carry ontogenetic information that is specified independently of DNA sequences [414]. This fact has serious implications both for evolutionary theory and for our understanding of ontogeny.

Implications for evolutionary theory

Of course, there is no single theory of evolution. First, the word "evolution" has many meanings, including simple change over time, the history of the cosmos, or (in biology) the transformation of one species into another. Second, even in biology there are several theories of evolution. I will focus on one of these, which I will call Neo-Darwinism.

Like evolution itself, Neo-Darwinism has had many meanings. The term was first used by Samuel Butler in 1880 to distinguish Charles Darwin's theory from that of his grandfather, Erasmus Darwin. Butler used "Darwinism" to refer approvingly to Erasmus's theory that new variations arise "due to the wants and endeavours of the living forms in which they appear," while Butler used "Neo-Darwinism" to refer disparagingly to Charles's ascription of new variations "to chance, or, in other words, to unknown causes" [415,416].

In 1895, Georges Romanes used "Neo-Darwinian" to describe the view (which he attributed to August Weismann and Alfred Russel Wallace) that "natural selection is the only possible cause of adaptive modification;" Romanes used "Neo-Lamarckian" to describe the view (which he attributed mainly to Americans) that "much greater importance ought to be assigned to the inherited effects of use and disuse than was assigned to these agencies by Darwin." According to Romanes, Charles Darwin's view (which he called "Darwinism") stood "between these two extremes" [417].

When Charles Darwin's theory of evolution was combined in the 1930s with Gregor Mendel's theory of genetics, the resulting "modern synthesis" attributed new variations to genetic mutations and excluded the inherited effects of use and disuse [418,419]. Since then, many people have used the term Neo-Darwinism to refer to the modern synthesis, as supplemented by molecular genetics.

I use Neo-Darwinism here to mean the view that all living things are descendants of one or a few ancestral organisms that have been modified principally (though not exclusively) by the natural selection of advantageous variations, and that new variations originate from changes in DNA sequences. This was Jacques Monod's view, though he called it Darwinism [11]. Eric Davidson distances himself from what he calls the "neo-Darwinian concept" that evolution occurs by "small continuous changes in primary protein sequence," and he attributes evolution instead to system level changes in developmental gene regulatory networks (dGRNs) [15]. But I would argue that in so far as he attributes control of ontogeny to a program in DNA, Davidson's view is still a variant of Neo-Darwinism.

As we have seen, however, the idea that embryo development is controlled by a genetic program is inconsistent with the biological evidence. Embryo development requires far more ontogenetic information than is carried by DNA sequences. Thus Neo-Darwinism is false.

Yet population genetics, which assumes that evolution is due to changes in gene frequencies, is based on the assumption that Neo-Darwinism is true. As originally formulated in the 1920s and 1930s by J. B. S. Haldane, Ronald Fisher, and Sewall Wright, population genetics relied on Mendelian genes [421–423], while modern population genetics assumes that those genes are equivalent to DNA sequences. A recent advocate of the centrality of population genetics is Michael Lynch, who

wrote in 2006 that "evolution is a population genetic process governed by four fundamental forces." One force is selection, "whose central role in the evolution of complex phenotypic traits is universally accepted, and for which an elaborate formal theory in terms of genotypic frequencies now exists." The other three forces are mutation (including DNA insertions, deletions and duplications), recombination, and random genetic drift. "Given the century of empirical and theoretical work devoted to the study of evolution," Lynch wrote, "the only logical conclusion is that these four broad classes of mechanisms are, in fact, the only fundamental forces of evolution." Thus "nothing in evolution makes sense except in the light of population genetics" [424].

Of course, no one denies that DNA is essential for ontogeny, and that DNA mutations can affect phenotypic traits. Furthermore, population genetics may be useful for tracing lineages within existing species. But since the ontogenetic information in an embryo far exceeds that in its DNA, evolution must necessarily involve far more than changes in gene frequencies.

In 1992, Brian Hall noted that population genetics is "neither sufficient nor inclusive" as an explanation for evolution. He proposed the term "evolutionary developmental biology" (which others had used before him) to describe the study of "how developmental processes effect evolutionary change and how development itself has evolved." Hall concluded: "Evolution acts at the three levels of changes in gene frequencies, the appearance of new characters, and the adaptation and radiation of new species. The common denominator of all three is genetic change through time, the common agent of that change is alteration in ontogeny, the common integrator of the three is epigenetic organization. The science of the study of these interactions is evolutionary developmental biology" [425].

Despite Hall's inclusion of ontogeny and epigenetic organization, many advocates of "evo–devo" (the nickname for evolutionary developmental biology) have perpetuated the Neo-Darwinian emphasis on genetic programs. For example, Sean B. Carroll wrote in 2005 that evo-devo focuses on "tool-kit genes"—developmental genes that are common to a wide variety of multicellular animals but controlled by different genetic switches "that integrate inputs from other tool-kit proteins acting a bit earlier in the embryo." The embryo has a coordinate system that "imposes some spatial order on how the program of tool-kit genes unfolds," but Carroll emphasized the primacy of interacting genes and gene products: "The important point to know is that the throwing of every switch is set up by preceding events, and that a switch, by turning on its gene in a new pattern, in turn sets up the next set of patterns and events in development." Thus the rules for generating animal form are found in DNA: "In the entire complement of DNA of a species (the genome), there exists the information for building that animal." The "evolution of form is ultimately then a question of genetics" [426]. So I would argue that Carroll's view (like Davidson's) is a variant of Neo-Darwinism, and thus false.

An adequate theory of evolution would not try to force organisms into the Procrustean bed of the central dogma—though basing a theory of evolution solely on changes in membrane patterns would be equally mistaken. The latter carry ontogenetic information that is specified independently of DNA sequences, but a case could be made (though I have not made it here) that the same is true of cytoskeletal patterns. So an adequate theory of evolution would have to explain how various information sources in the organism (including its DNA, membrane patterns, and cytoskeletal patterns) change in a coordinated fashion to produce new species, organs, and body plans. Before attempting to explain how organisms change in the course of phylogeny, however, we need to address the question of how they change in the course of ontogeny.

Implications for our understanding of ontogeny

A developing animal embryo undergoes thousands of changes in its molecules, cells, organs, and overall anatomy. Such changes are integrated to produce, with great fidelity, a specific outcome. Although disrupting development can produce death or deformity, embryos have a remarkable ability to recover from disruptions and produce normal adults of their species. Ontogeny is thus orchestrated, reliable, robust, and stereotypical. What is the best way to understand it?

One way is molecular biology, which takes an essentially Newtonian approach. Living systems are analyzed in terms of molecules (analogous to atoms in Newtonian physics), their locations and movements in the cell (position and velocity in Newtonian mechanics), and their chemical and physical interactions (the forces among them). Given an embryo in its initial state, all subsequent states (including new membrane patterns) would follow from the ordinary laws of physics acting on the constituent molecules. This does not preclude the emergence of unexpectedly complex and dynamic patterns, analogous to those we see in the Belousov-Zhabotinsky reaction [427,428], but such patterns would be only "weakly" emergent: Though unexpected, they arise from the lower-level constituents of a system by the ordinary operation of the laws of classical (and perhaps quantum) mechanics [429,430].

But what if embryo development involves "strongly" emergent properties? What if ontogeny requires higher-level information that cannot be reduced to the characteristics and interactions of lower-level constituents [431]? In a 2012 discussion of the bioelectric code, Michael Levin proposed that "biological structures encode maps for their shape"—what he called "target morphologies." According to Levin,

> What makes target morphology models distinct from [weakly] emergent models is the hypothesis that there are some measurable quantities contained in the living system that are directly isomorphic to the anatomy that is being constructed or maintained. In [weakly] emergent models, there is no such process, the shape being assembled as the result of low-level rules and not by comparison to (or directives from) any informational structure that encodes a final shape" [287].

A Newtonian approach permits weakly emergent properties

but excludes strongly emergent ones; yet it is not the only way to approach living systems scientifically. In the 1950s, mathematical biologists Nicholas Rashevsky and Robert Rosen introduced a new approach they called "relational biology" [432–434]. Unlike Newtonian biology, which gives ontological priority to matter (i.e., molecules), relational biology (as the name implies) gives ontological priority to the relations that constitute an organized system. Although molecular biology has been successful at a certain level, its methods involve discarding the organization of a cell while keeping the matter. Yet the former, once discarded, cannot be recovered from the latter—and living things are fundamentally characterized by their organization.

Relational biology has radical implications not only for biology, but also for physics. A Newtonian approach assumes that living things embody a subset of the natural laws governing atoms and the universe; thus the former are in principle reducible to the latter. But if living things embody natural laws in addition to those that govern non-living matter, the Newtonian approach is mistaken. Relational biology begins with the opposite assumption—namely, that atoms and the universe embody a subset of the natural laws governing living things. The latter can then be studied in their own right, without trying to reduce them to the former [435]. Of course, to be scientifically fruitful relational biology must move beyond mere assumption.

In the 1940s, seventy years after the formulation of classical set theory, Samuel Eilenberg and Saunders Mac Lane proposed a more general theory of structures and systems of structures called "category theory," which was further developed in the 1950s and 1960s by Alexander Grothendieck, Peter Freyd and William Lawvere [436–440]. A category consists of "objects" and "mappings." A category-theoretic mapping (also called a transformation or morphism) is a process for getting from one object to another object; it is customarily represented by an arrow. A category-theoretic object is a vertex from which one or more arrows originate or at which one or more arrows terminate. It is a logico-mathematical entity (such as a set), not necessarily a physical thing (though a set can consist of one or more physical things). Category theory is primarily concerned with the analysis of mappings and their interrelationships. In set theory, a "function" is a mapping from an element of one set to an element of another set; in category theory, a "functor" is a mapping from one category to another that preserves the relational structures of the mappings and objects. Since 1945, category theory has been logically and mathematically worked out in great detail [441–443].

Category theory provides a rigorous conceptual foundation for relational biology. Starting with Rosen in 1958, biologists have now applied it to various aspects of living systems [444–448]. Much more theoretical and experimental work remains to be done, of course. One promising research program will involve applying relational biology and category theory to the DNA-independent specification of ontogenetic information in membrane patterns.

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