

Evo-Devo: Variations on Ancestral Themes

E.M. De Robertis^{1,*}

¹Howard Hughes Medical Institute and Department of Biological Chemistry, University of California, Los Angeles, CA 90095-1662, USA

*Correspondence: ederobertis@mednet.ucla.edu

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Most animals evolved from a common ancestor, Urbilateria, which already had in place the developmental genetic networks for shaping body plans. Comparative genomics has revealed rather unexpectedly that many of the genes present in bilaterian animal ancestors were lost by individual phyla during evolution. Reconstruction of the archetypal developmental genomic tool-kit present in Urbilateria will help to elucidate the contribution of gene loss and developmental constraints to the evolution of animal body plans.

Introduction

During the last quarter century, molecular biologists have begun to reconstruct the history of life by comparing the sequences of genes between different organisms. Previously, animal relationships had to be deduced by observation of external morphological characteristics (Darwin, 1859). The discovery of conserved gene networks that control embryonic development and the ability to examine genomic records has revolutionized Darwinian evolutionary theory. This synthesis between developmental biology and evolution has been named Evo-Devo (described in books by Carroll et al., 2001; Gould, 2002; Kirschner and Gerhart, 2005; Carroll, 2005; Davidson, 2006). An anecdote illustrates the profound significance of conserved developmental gene networks. About twenty years ago at a meeting, I was having breakfast next to geneticist Edward B. Lewis from Caltech, who studied the Bithorax gene complex from 1946 until his passing in 2002. To strike up a conversation, I mentioned how amazing it was that Hox genes were conserved between *Drosophila* and vertebrates and was struck by the simplicity of the response of the great man: "Well, what this means is that we all come from a planarian." In this one brief statement, Lewis encapsulated the profound meaning of Evo-Devo. The last common ancestor shared by all bilaterally symmetrical (bilaterian) animals—called Urbilateria—must have been a complex creature (Figure 1A) possessing most of the developmental gene pathways from which animals are built (De Robertis and Sasai, 1996). Understanding how Urbilateria was constructed is one of the key questions of the Evo-Devo field. Another central challenge is to explain how conserved gene networks already present in this archetypal ancestor were modified to generate the wonderful diversity of animal life on Earth today. This Review presents recent advances in the study of the signaling pathways controlling animal development and examines the implications of these discoveries for the evolution of the body plans of animal phyla. We argue that reconstructing the genome of our urbilaterian ancestors will shed light on the origin of animal body plans, particularly the role played by gene loss, and we will illustrate how developmental constraints may have had more of an impact on evolutionary history than previously thought.

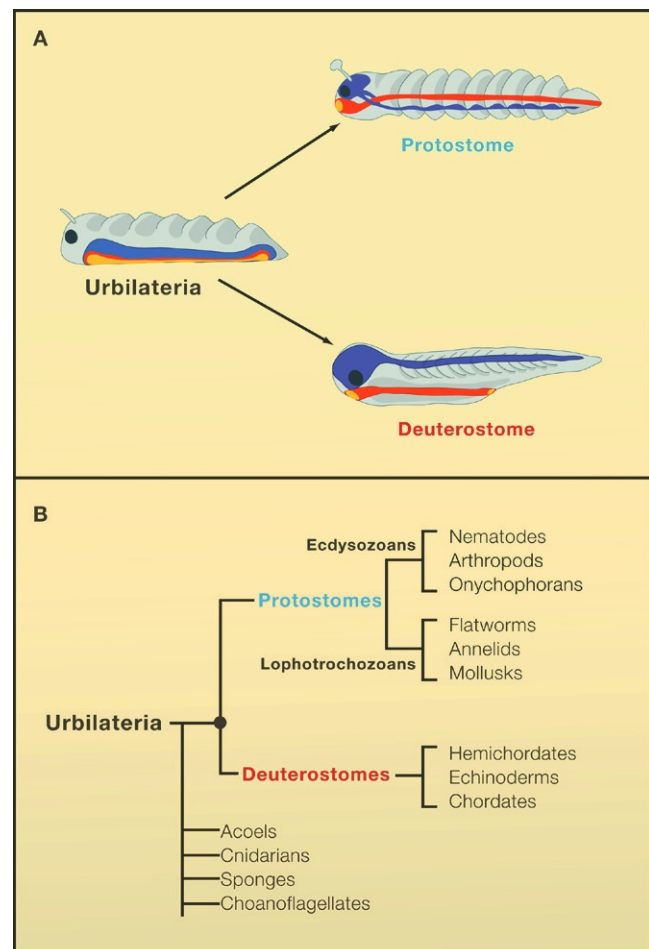


Figure 1. Evolutionary Relationships among Animals

(A) Urbilateria is the archetypal animal that was the last common ancestor shared by protostomes and deuterostomes. The Urbilateria in this image is depicted as a segmented bottom-dwelling (benthic) animal with eyes, central nervous system, a small appendage, and an open slit-like blastopore. Endoderm is shown in red, central nervous system in dark blue, and surface ectoderm in light blue.

(B) The new animal phylogeny, showing that cnidarians are basal to bilateria and that protostomes are divided into two branches, the molting Ecdysozoans and the nonmolting Lophotrochozoans.

Animal Phyla and the Cambrian Explosion

The field of Evo-Devo began in the pre-genomic era when genetic studies in *Drosophila* and gene cloning in *Xenopus* revealed that the Hox genes that control the antero-posterior (A-P) axis were unexpectedly conserved. Once the Chordin-BMP gene network, which mediates dorsal-ventral (D-V) development, was also found to be conserved between vertebrates and invertebrates, it became evident that their last common ancestor was a very complex organism. In the genomics era, it is now apparent that most, perhaps all, of the genetic tool-kit that controls animal development was already present in Urbilateria and its ancestors (Carroll et al., 2001; Carroll, 2005).

About 35 different animal phyla with distinct body plans currently exist (Valentine, 2004). Almost 30 of them are bilaterians, which are traditionally subdivided into protostomes and deuterostomes (Figure 1A). The protostomes (mouth-first) develop the mouth close to the blastopore and have a ventral nerve chord traversed by the foregut, as well as a dorsal brain ganglion. The deuterostomes (mouth-second) develop the blastopore close to the anus and have a dorsal central nervous system (CNS). More recently, it was discovered that protostomes could be further subdivided into molting (Ecdysozoans) and nonmolting (Lophotrochozoans) animals (Aguinaldo et al., 1997). The Ecdysozoa phyla include arthropods, nematodes, and onychophorans (velvet worms) among others, and Lophotrochozoa phyla include annelids, flatworms (such as planarians), and mollusks (Figure 1B). Previous studies compared the genes of chordates (such as humans) to those of the Ecdysozoans *Drosophila* and *Caenorhabditis elegans*. The introduction of a Lophotrochozoan, the marine annelid *Platynereis dumerilii* (Arendt et al., 2001), and of a bilaterian sister group, the cnidarian sea anemone *Nematostella vectensis* (Technau et al., 2005), as model systems are now uncovering a much broader panorama of the genetic machinery of animal development (Figure 1B).

Adult bilaterian fossils made their appearance suddenly, during the period between 535 to 525 million years ago (mya) called the Cambrian explosion (Gould, 2002; Valentine, 2004). However, earlier fossils of phosphatized ancient sea-bottom bilaterian tracks and trails and possible bilaterian embryos dating to 630 mya have been found (Knoll and Carroll, 1999; Yin et al., 2007). As recorded in the fossils of the Burgess Shale in British Columbia, Canada, and of the Chengjiang formation in Yunnan, China, all extant bilaterian body plans, as well as some extinct ones, can be traced back to the Cambrian explosion. Before these animals, a long line of Precambrian ancestors must have existed, but they left very few adult bilaterian fossils. Amazingly, all the body plans characteristic of the 35 phyla that exist today were already present 525 mya. Individual species thrived and developed many new variations, but the overall body plans were maintained. The enduring mystery of the Cambrian explosion is why no new phyla appeared during this long period of evolutionary time (Gould, 2002; Valentine, 2004), and many possible explanations have been proposed (Valentine, 2004; Davidson and Erwin, 2006).

One intriguing environmental possible explanation is provided by the Snowball Earth scenario (Hoffman et al., 1998). The earth underwent several massive glaciations between

750 and 550 mya, which may have frozen the oceans completely (Hoffman et al., 1998) or partially (Hyde et al., 2000). These periods of extreme glaciations decreased the biological productivity of the oceans for millions of years and may have presented animals with repeated bottlenecks of intense natural selection coincident with the radiation of metazoans. Animal populations might have become isolated geographically, surviving in regions of shallow waters, perhaps kept warmer by volcanic hydrothermal activity, so that "evolution might well be stimulated by this prolonged genetic isolation" (Schrag and Hoffman, 2001). Once the environment started to warm up again—through the volcanic release of CO₂ into the atmosphere—animals surviving this evolutionary bottleneck dispersed throughout the oceans once again, but now bearing the adaptations in body plan resulting from this intense natural selection. As will be seen below, an efficient way of adapting rapidly to new environments is through gene losses. I would like to propose here that gene losses during periods of intense natural selection could have played a role in the origin of phyla. What makes this proposal attractive is that it is testable. One can expect that it will be possible to determine the extent of gene losses incurred by each phylum in the ancestral developmental tool-kit repertoire by comparative genomics in the near future.

The Genetic Tool-kit

The sequencing of complete genomes allows powerful inferences to be made about the history of life. Consider that if a gene present in humans (or any other chordate) is also found in either an Ecdysozoan or Lophotrochozoan animal, then the inescapable conclusion has to be that this gene was present in Urbilateria as well (Figure 1B). Similarly, if a gene is found in both a cnidarian and a chordate, it must also have been present in Urbilateria (Figure 1B). For example, until recently we believed that chordates had evolved new classes of genes serving as extracellular antagonists of growth factor signaling. This was because both *C. elegans* and *D. melanogaster* lacked the Wnt inhibitors Dickkopf (Dkk) and secreted Frizzled-Related proteins (sFRPs), as well as the BMP antagonist Noggin. A simple Blast search through the genome of a mollusc (the limpet *Lottia gigantea*) reveals that it contains several Dkks and sFRPs, as well as Noggin (J.-L. Plouhinec and E.M.D.R., unpublished data). Therefore, one can conclude that these Lophotrochozoan and chordate genes were present in the Urbilateria genome but were secondarily lost in *C. elegans* and *Drosophila*. Urbilateria may have had in place all of the diverse types of proteins currently used in animal development, and for some phyla it may have been downhill from there, for gene loss constitutes a powerful agent of rapid evolutionary change.

The prominent role of gene loss in evolution has been revealed by studies on the sea anemone *Nematostella vectensis*. It is estimated that cnidarians diverged from the bilateria at least 650 mya from a common ancestor designated "Ureumetazoa" (to distinguish it from the ancestor of all animals or "Urmatazoa," which would include sponges; Kusserow et al., 2005). Cnidarians are diploblastic animals, consisting only of ectoderm and endoderm and lacking the mesodermal layer, yet they contain all of the signaling pathways used by higher

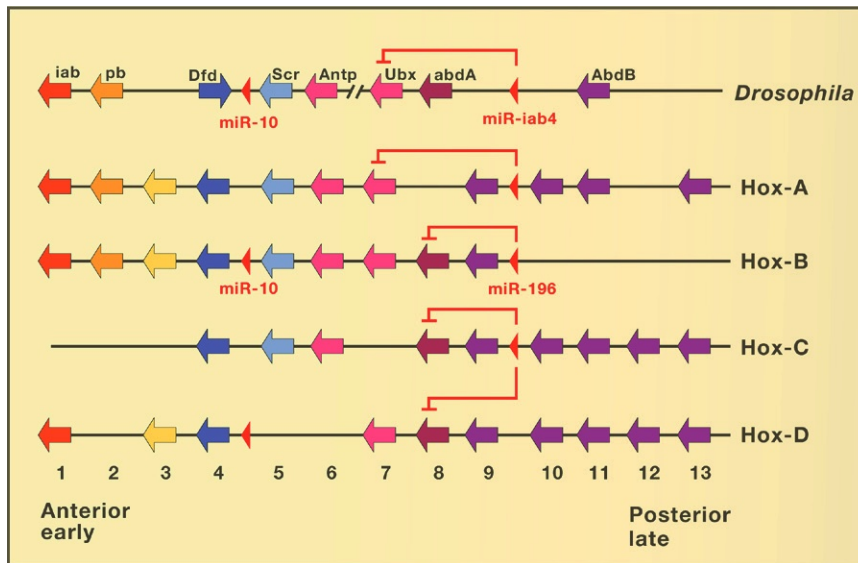


Figure 2. Hox Complexes of *Drosophila* and Mammals

The Hox complex has been duplicated twice in mammalian genomes and comprises 39 genes. Note that microRNA genes, which inhibit translation of more anterior Hox mRNAs, have been conserved between *Drosophila* and humans.

nomenon colinearity and hypothesized that new genes had been recently duplicated in flies and added sequentially to repress more anterior segmental identities in a multiple-legged ancestor that looked like a centipede (Lewis, 1978). When the DNA of the *Antennapedia* Hox gene complex was isolated after years of “chromosome walking” cloning (Scott et al., 1983; Garber et al., 1983), the groups of Matthew Scott and Walter Gehring searched for the hypothetical duplicated regulatory genes of Lewis. Instead, they

discovered a short conserved 180 nucleotide region called the homeobox that encoded a 60 amino acid DNA-binding domain called the homeodomain that became a mother lode for developmental biologists (McGinnis et al., 1984; Scott and Weiner, 1984). Homeobox probes detected related hybridizing bands in many animals, including vertebrates (McGinnis et al., 1984; Carrasco et al., 1984). In the original paper in which we reported the cloning of the vertebrate gene *Hox-C6* (Carrasco et al., 1984), the last sentence in the abstract read: “If the frog gene cloned here eventually turns out to have functions similar to those of the fruit fly genes, it would represent the first development-controlling gene identified in vertebrates.” This wishful thinking eventually proved true.

The cephalochordate amphioxus has a single Hox complex comprising 14 Hox genes (García-Fernández, 2005). In vertebrates, the entire ancestral genome has been duplicated twice (Dehal and Boore, 2005). These whole-genome duplications may have been a key component in the evolutionary success of vertebrates. Mammals contain four Hox complexes (designated A–D) of about 100 kb each with 13 paralogous (duplicated) genes (Figure 2). The complexes follow the rules of colinearity, with genes at one end being expressed earlier and more anteriorly than those at the other end. A mouse or human has a total of 39 Hox genes, as some of the duplicated genes were lost (Figure 2) (Lemons and McGinnis, 2006; Duboule, 2007).

The degree of overall homology and the conservation of regulatory complexity between vertebrate and insect Hox complexes is simply amazing. In vertebrates, a microRNA, miR-196, is found in Hox complexes A, B, and C (Yekta et al., 2004) (Figure 2). In posterior regions of the embryo, miR-196 is transcribed by promoter elements of paralog 10, processed, and bound to the 3′-untranslated region of *Hox-8* mRNAs (Figure 2). *Hox-C8* mRNA and miR-196 have a single mismatch over the 22 nucleotide microRNA sequence, and binding triggers degradation by RNA interference (RNAi). More anterior mRNAs in Hox-7 and Hox-6 paralogous groups also bind to miR-196

animals (Technau et al., 2005). About 2.5% of *Nematostella* proteins were lost by bilateral animals yet have homologs in fungi or plants. The human genome has 13 subfamilies of Wnt signaling proteins that can be recognized by protein sequence similarities (Guder et al., 2006). Remarkably, the sea anemone has 12 of these Wnt subfamilies, which are expressed in distinct bands along the oral/aboral (mouth to foot) axis (Kusserow et al., 2005; Guder et al., 2006). *C. elegans* has a grand total of only five Wnt genes and *Drosophila* has only seven. Thus, one must conclude that deuterostomes retained most of the Wnt gene families present in the cnidarian ancestors of Urbilateria and that Ecdysozoans lost many of them (Figure 1B). These comparative genomic findings suggest that gene losses may have played a fundamental role in the evolution of body plans.

A worthwhile bioinformatics strategy will be to reconstruct all protein classes present in the genome of Urbilateria, in order to determine to what extent the various phyla have retained the complete ancestral repertoire. It could well turn out that none has. In silico reconstructions of hypothetical ancestral genomes have already been carried out in the fungi and proven highly informative (Wapinski et al., 2007). Sequencing a complete genome of at least one species of each of the 35 animal phyla is a perfectly attainable goal with current technology. The ability to retrace the gene duplications, deletions, and mutations that occurred as animals evolved from their ancient ancestors would greatly advance our understanding of the natural history of animal life on earth. Biology is a historical science, and for this reason it will be fascinating to unravel the successive molecular steps by which we evolved into our present human condition.

Hox Complexes and the A-P Axis

E.B. Lewis made the remarkable discovery that genes that controlled the identity of the abdominal segments of the fly (by repressing formation of legs and wings) were clustered in the genome and occupied the same order along the DNA as they were expressed along the A-P body axis. He called this phe-

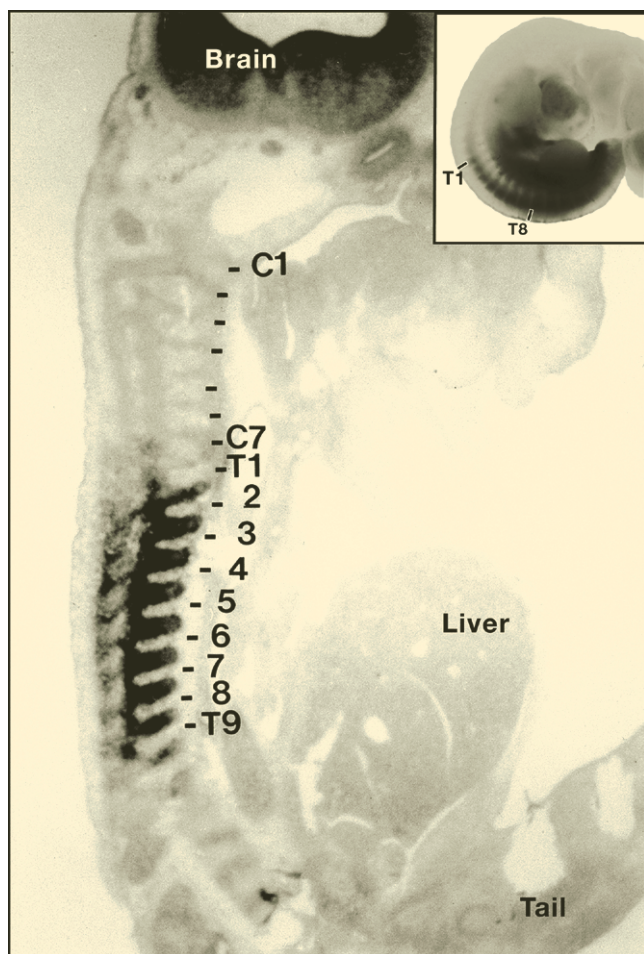


Figure 3. Posterior Repression of *Hox-C6* mRNA Translation in the Mouse Embryo

Translation of *Hox-C6* mRNA is seen in eight thoracic segments of the day 13 mouse embryo but blocked in the tail region, probably through the action of microRNAs. The inset shows that *Hox-C6* mRNA is expressed all the way to the tip of the tail (using a *Hox-C6-lacZ* gene fusion). Note that the anterior border of expression of the *Hox-C6* protein starts in the posterior half of the T1 segment, indicating that the sclerotome has already resegmented (G. Oliver and E.M.D.R., unpublished data; for methods see Oliver et al., 1988).

but with more mismatches, causing translational inhibition instead of mRNA degradation. This translational repression mechanism ensures that anterior Hox proteins are not translated posteriorly, explaining earlier observations that some mouse Hox proteins were not expressed in posterior regions of the embryo, whereas their transcripts were expressed all the way to the tip of the tail (Figure 3). In the *Bithorax* gene complex of *Drosophila*, the *infra-abdominal 4* (*iab-4*) gene (Lewis, 1978) is found at the equivalent location of miR-196 (Figure 2). The *iab-4* gene encodes a microRNA that binds to and inhibits *Ultrabithorax* mRNA translation and is expressed in abdominal segments; when *miR-iab4* is overexpressed in the haltere it causes homeotic transformations into a wing (Ronshaugen et al., 2005). In the *Antennapedia* complex a second microRNA, miR-10, has been mapped between *Deformed* and *Sex combs reduced* and at homologous positions in the mammalian

Hox-B and Hox-D complexes (Figure 2) (Yekta et al., 2004). Translational repression by microRNAs probably explains the enigmatic phenomenon of “posterior dominance” observed in Hox function (Duboule, 2007).

Such an intricate machinery dedicated to specify identities along the A-P axis would, in all probability, not have evolved in the same way twice. For this reason, the inescapable conclusion is that a Hox complex was already functioning in Urbilateria. All arthropods, including centipedes, have a complete set of similar Hox genes (Carroll et al., 2001). Therefore, Lewis’ hypothesis that recently duplicated genes controlled abdominal identity in fruit flies was not correct yet provided the cornerstone of Evo-Devo. Hox complexes have been sequenced in multiple phyla and from this work it can be deduced that the urbilaterian ancestor had a Hox complex of at least seven genes (García-Fernández, 2005).

This conserved regulatory gene network can be used to generate different morphological outcomes. A gene such as *Ubx* can repress wing formation in thoracic segment 3 (T3) of a fruit fly yet activate the formation of beautiful colored patterns in the hindwings of butterflies (Carroll et al., 2001). Homeodomain proteins can act either as activators or repressors of transcription, depending on other transcription factors that bind to the same enhancer complex. Ultimately, the proteins that bind to enhancers are determined by the sequence of the DNA enhancer element to which they bind. By mutating DNA sequences new enhancer elements can be generated that regulate the transcription of genes that determine different morphological outcomes, providing a principal source of variation during evolution (Carroll, 2005).

The discovery of Hox gene complexes led to the fundamental realization that the gene networks that control animal body plans share deep historical genetic homologies. The next big challenge for the Hox field will be to unravel the molecular mechanisms that provide the positional information for the sharp A-P borders of Hox gene expression in vertebrates. There are good candidates to mediate this positional information, such as retinoic acid and FGFs (fibroblast growth factors), but the role of the Wnt A-P gradient remains largely unexplored.

Mechanisms of Segmentation

The body plans of vertebrates and invertebrates are metameric, i.e., comprised of repeated segments. In 1822, the French naturalist Etienne Geoffroy Saint-Hilaire proposed that arthropod segments and mammalian vertebrae were examples of a unity of plan in animal design (Appel, 1987). Insect and vertebrate metamerism share striking similarities in their mode of development. *Drosophila* first forms parasegments, which are subsequently subdivided so that each one forms the posterior half and the anterior half of the adjoining definitive segments (Carroll et al., 2001). In mammals, vertebrae develop from a portion of the somite called the sclerotome. As first described by Remak in 1855, sclerotomes undergo resegmentation, so that the posterior half of one sclerotome and the anterior half of the next one fuse to form the vertebral bodies (Bagnall et al., 1988). This mechanism ensures that the muscles and tendons generated by each somite span adjoining vertebrae, facilitating movement

coordination. In mouse mesoderm, the anterior borders of Hox gene expression coincide initially with somite borders. The consequences of somite resegmentation can be visualized in Figure 3, in which Hox-C6 protein is observed in the posterior half of the T1 sclerotome, whereas its anterior half, derived from the preceding somite, is negative for this Hox gene. The resegmentation of parasegments and somites indicates that, despite profound anatomical differences, a deep homology may exist in the mechanisms of animal segmentation (De Robertis, 1997).

In Nobel Prize-winning work, segmentation in *Drosophila* was dissected genetically and found to be controlled by the sequential activity of three groups of genes, the gap, pair-rule, and segment-polarity genes (Nüsslein-Volhard and Wieschaus, 1980). In vertebrates, segmental somites form in posterior tissue called paraxial mesoderm, through which waves of transcripts sweep each time a somite is formed at the anterior end. Rhythmically cycling genes include many components of the Notch and Wnt pathways (Pourquié, 2003). This vertebrate segmentation clock is driven by Notch signaling (Dale et al., 2003), yet, surprisingly, Notch does not appear to play a main role in *Drosophila* segmentation. However, *Drosophila* represents a minority of the insects in which the embryo develops very rapidly, with all segments developing simultaneously (termed long germ-band development). In most insects (and other arthropods such as spiders), segment formation occurs by a different mechanism called short germ-band development. In these insects, the embryo initially occupies only a short region of the egg that then elongates by proliferation of a posterior growth zone that sequentially generates segments in an anterior to posterior sequence as in chick or mouse embryos (Damen, 2007).

Importantly, in the spider embryo Notch and its ligand Delta have been shown to be expressed in the posterior growth zone in a dynamic way (probably cyclic but so far hard to demonstrate conclusively), and RNAi inhibition demonstrated that spider segmentation has an absolute requirement for Notch signaling, as in vertebrates (Stollewerk et al., 2003). In addition, studies in zebrafish indicate that *hair1* and *7*, genes that are activated by Notch, are required for the formation of alternate somite boundaries (Henry et al., 2002). This is reminiscent of the pair-rule phenotypes of *Drosophila*. It should be mentioned that in amphioxus researchers have failed to find cycling, leading to the proposal that the paraxial mesoderm clock is a vertebrate innovation (Minguillón et al., 2003). The key experiment still missing in the metamerism field is the demonstration that segmentation genes cycle in protostomes as they do in vertebrates. This is no doubt being actively investigated in several systems. Therefore, although it is still premature to conclude that Urbilateria was segmented, the plot has thickened over the past decade (De Robertis, 1997).

Conserved Pathways of Cell-Cell Communication

Cells are the building blocks of animals, and the diverse animal forms arise from the way cells arrange themselves and proliferate with respect to each other. Cells use a surprisingly small number of cell-cell signaling pathways to

communicate with each other (e.g., Weinberg, 2006). These pathways transduce signals from the external medium to turn genes on and off in the nucleus. The principal signaling pathways are: (1) receptor tyrosine kinases (RTKs), such as FGF, EGF (epidermal growth factor), IGF (insulin-like growth factor), and insulin and ephrin receptors; (2) receptor serine/threonine kinases such as TGF- β (transforming growth factor β), Activin, Nodal, and BMP (bone morphogenetic proteins) receptors; (3) Wnt growth factors, which signal through LRP6 (lipoprotein-receptor related protein 6) and Frizzled receptors; (4) Hedgehog proteins, which signal through the Patched and Smoothed transmembrane proteins; (5) Notch, a membrane receptor that is activated by membrane-bound ligands from adjoining cells such as Delta, Serrate, and Jagged; (6) G protein-coupled receptors (GPCRs), also known as 7-transmembrane serpentine receptors, which transduce a multitude of small molecule and polypeptide signals such as odorants, adrenalin, histamine, prostaglandins, chemokines, and gonadotrophins; and (7) nuclear hormone receptors, which are transcription factors that are activated by hydrophobic ligands such as steroid hormones, thyroid hormone, and retinoic acid. We now view the BMP pathway as the key regulator of D-V patterning, but it probably started as a simple conversation between two cells. However, once it was adopted by urbilaterian ancestors to pattern one of the body axes, this aspect of its function became pre-eminent, at least in the eyes of developmental biologists. Probably most of the other signaling pathways became equally indispensable by networking with each other to generate essential body structures.

Each signaling pathway is used many times at different stages of development and consists not only of the growth factors and receptors indicated above but also of additional extracellular and intracellular regulators. Many of these signaling pathways, such as RTKs, were already present in single-cell choanoflagellate ancestors (King et al., 2003), and all of them were present in sea anemones, which diverged from bilaterians at least 650 mya (Technau et al., 2005). This poses the question of what is the evolutionary significance of these deep gene homologies. Gould (2002) compared this dilemma to that of an archeologist that finds an ancient brick. A brick says very little about the building it came from. Finding a Corinthian column, however, gives much more information about how the building might have looked and about its lineage. A single conserved gene resembles a brick, but discovering an exquisitely intricate signaling network like that of D-V patterning resembles finding a Corinthian column, or perhaps an architectural blueprint.

The Chordin-BMP Pathway and the D-V Axis

A gradient of D-V positional information regulates the subdivision of the embryo into tissue types. For example, in the chordate body plan the ectoderm is subdivided into CNS, neural crest, and epidermis, and the mesoderm into notochord, somite, and intermediate (kidney) and lateral (body wall) mesoderm. In *Xenopus* and zebrafish, these cell differentiation decisions are mediated by a conserved extracellular pathway involving ventral BMPs and their antagonist

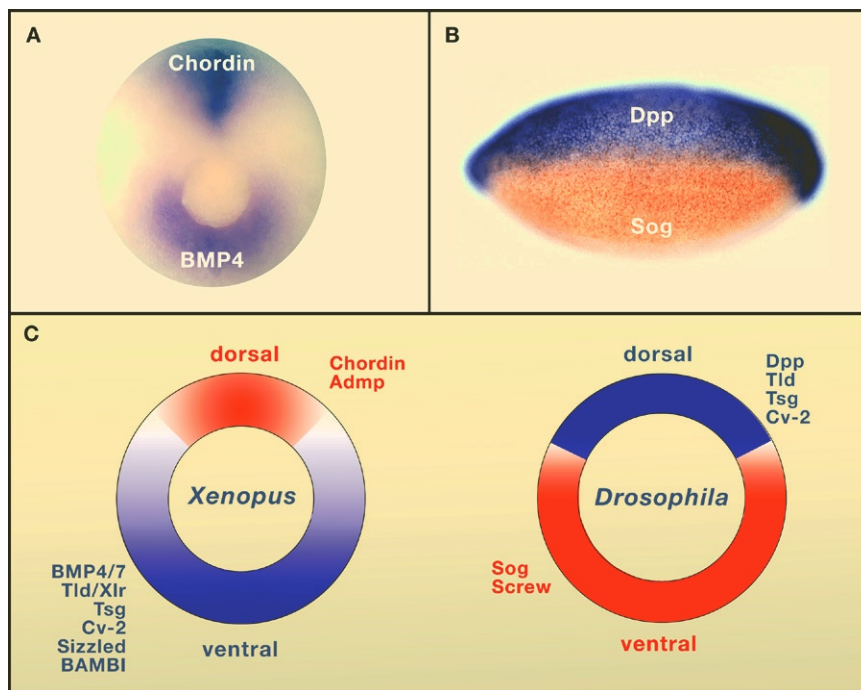


Figure 4. The Conserved Chordin-BMP Signaling Network

Although the Chordin-BMP signaling network is conserved, there has been a D-V axis inversion from *Drosophila* to *Xenopus*.

(A) In *Xenopus*, Chordin is expressed on the dorsal side and BMP4 at the opposite ventral pole (image courtesy of Hojoon X. Lee).

(B) In *Drosophila*, Dpp is dorsal (blue) and Sog is ventral (in brown) in the ectoderm (Image courtesy of Ethan Bier and reproduced from François et al., 1994, Genes Dev. 8, 2602–2616, with permission from Cold Spring Harbor Laboratory Press, copyright 1994).

(C) A network of conserved secreted proteins mediates D-V body patterning in *Xenopus* and *Drosophila*.

Chordin expressed in the dorsal organizer region (reviewed in De Robertis, 2006; Little and Mullins 2006). As shown in Figure 4, in *Drosophila* the same molecular machinery is utilized, except that the homologs are called Dpp (Decapentaplegic) and Sog (Short gastrulation) and an inversion of the axis takes place (O'Connor et al., 2006 and references therein; Appel, 1987). The Chordin-BMP developmental gene network controls D-V pattern in a range of organisms such as spiders (Akiyama-Oda and Oda, 2006), hemichordates (Lowe et al., 2006), and amphioxus (Yu et al., 2007) and therefore was ancestral to bilateral animals.

An elaborate biochemical pathway of extracellular protein-protein interactions mediates the formation and maintenance of a D-V gradient of BMP signaling during the gastrula stage (Figure 4C). Chordin is a secreted BMP antagonist expressed in the dorsal (low-BMP) region of the *Xenopus* gastrula. ADMP (Anti-Dorsalizing Morphogenetic Protein) is a BMP-like protein that is counterintuitively secreted in the same low-BMP region as Chordin. On the ventral side, BMP4 expression becomes localized at mid-gastrula to a ventral center located opposite of the Chordin-secreting dorsal pole (Figure 4A). Ventral center genes are activated by high BMP signals. BMP receptors transduce the signal by phosphorylating the carboxyl terminus of a transcription factor called Smad1, which in turn activates transcription of several secreted proteins in the ventral side (Figure 4C). These are: (1) BMP4 and BMP7; (2) Xolloid-related (Tolloid), a zinc metalloproteinase that cleaves Chordin at two specific sites and allows inactive BMPs in Chordin/BMP complexes to signal; (3) Sizzled/Ogon, an sFRP that functions as a competitive inhibitor of the Tolloid proteinase; (4) Crossveinless-2 (Cv-2), a Chordin-like secreted protein; (5) BAMBI (BMP and activin membrane-bound inhibitor), a naturally occurring domi-

nant-negative BMP receptor lacking the intracellular catalytic domain; and (6) Twisted-gastrulation (Tsg), a protein that binds both to Chordin, making it a better antagonist, and to BMP, facilitating its signaling (De Robertis, 2006; Little and Mullins 2006). Homologous proteins are expressed during *Drosophila* D-V patterning (Figure 4C;

O'Connor et al., 2006), except that BAMBI and sFRPs have been lost in *Drosophila* (but are present in some Lophotrochozoan animals). Such an intricate biochemical mechanism is most unlikely to have evolved independently twice in evolution, and the inescapable conclusion is that the Chordin-BMP pathway patterned the urbilaterian gastrula.

Why is such a complex network of extracellular proteins required to generate a simple gradient of BMP activity? The answer probably lies in the self-regulatory nature of animal development. If an early embryo of a frog, chick, or cricket is cut in half, well-proportioned twins can be formed. In *Xenopus*, this robust self-regulation results from communication between the dorsal and ventral poles of the embryo over long distances (De Robertis, 2006). The key step is the cleavage of Sog/Chordin by Tolloid, which serves as a sink for the Dpp/BMP/ADMP ligands. Self-regulation results from the dorsal and ventral signaling centers being under opposite transcriptional control: if BMP levels are lowered, the production of ADMP is increased, whereas at high BMP levels the expression of the BMP feedback inhibitors BAMBI, Cv-2, and Sizzled serve to dampen the signal (Figure 5) (Reversade and De Robertis, 2005). There is molecular evidence for a similar communication between the dorsal and ventral sides of the early embryo in hemichordates (Lowe et al., 2006), amphioxus (Yu et al., 2007), and spiders (Akiyama-Oda and Oda, 2006).

Smad1 is a transcription factor that regulates the activity of hundreds of downstream target genes. The Chordin-BMP biochemical pathway is extracellular and therefore simultaneously regulates all of these target genes. In such a hard-wired global system, the mixing and matching of individual components on particular enhancer DNA-binding sites is no longer the critical step. The D-V patterning system is highly dependent on physi-

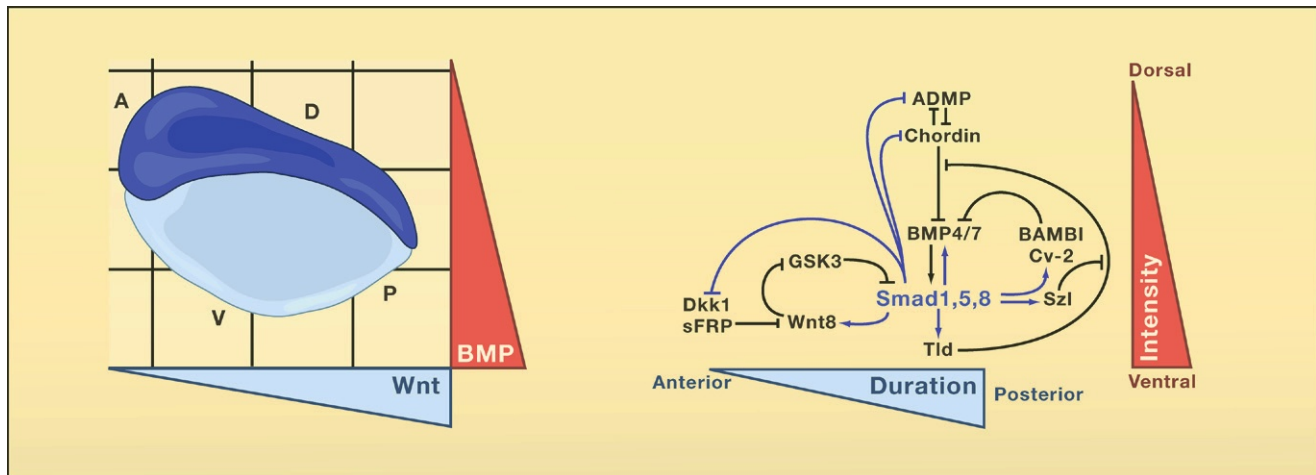


Figure 5. A-P and D-V Integration in the Embryonic Morphogenetic Field

Shown is a model of the embryonic morphogenetic field in which a Cartesian System of A-P (Wnt) and D-V (BMP) gradients are integrated at the level of phosphorylation of the transcription factors Smads 1, 5, and 8. Note that in this self-regulating model the BMP gradient provides the intensity, but the Wnt gradient controls the duration of the Smad 1, 5, 8, signal. Direct protein-protein interactions are shown in black, and transcriptional activity of Smads 1, 5, and 8 are in blue.

cochemical properties such as protein diffusion rates, dissociation constants, the catalytic activity of the Tolloid proteinase, and the affinity of its inhibitor Sizzled, all of which have been measured to some degree (Lee et al., 2006; O'Connor et al., 2006). Evolutionary adaptations in this extracellular signaling system are likely to have involved mutations that affect these properties.

Integrating A-P and D-V Patterning

When identical twins are produced, either experimentally or naturally, the A-P and D-V axes are perfectly integrated in the resulting embryos. We are now beginning to understand how this remarkable regulatory feat may be achieved. The A-P and D-V axes provide a global embryonic positional system of Cartesian coordinates that determine where the various organs of the body form in later development (Figure 5). The principal A-P morphogenetic gradient is thought to be provided by Wnt signals, which are maximal at the posterior blastopore in *Xenopus* and amphioxus embryos (Niehrs, 2004; Yu et al., 2007). In planarians, A-P specification is also regulated by Wnt, as inhibition of the canonical Wnt pathway by RNAi causes the ectopic regeneration of head structures (Gurley et al., 2007; Petersen and Reddien, 2007).

Recent work from our laboratory indicates that the Smad1 transcription factor may serve as a platform to integrate the A-P (Wnt) and D-V (BMP) patterning gradients in *Xenopus* (Fuentelba et al., 2007). Smad1, in addition to being activated by BMP, receives inhibitory phosphorylations by MAPK (mitogen-activated protein kinase, an enzyme activated by RTKs) and GSK3 (glycogen synthase kinase 3) (Sapkota et al., 2007; Fuentelba et al., 2007). When both these sites are phosphorylated Smad1 is degraded, terminating the BMP signal. Because GSK3 activity is inhibited by Wnt signaling, Wnt causes the duration of the BMP signal to increase (Fuentelba et al., 2007). In this model, the D-V and A-P gradients would become integrated at the level of

Smad1, with BMP regulating the intensity and Wnt the duration of its transcriptional activity in the nucleus (Figure 5). It is tempting to speculate that the simple addition or removal of phosphorylation sites in Smad1 could greatly impact the integration of the initial coordinates of the D-V and A-P body plan during evolution.

The Ancestry of the Bilaterian CNS

The CNS forms dorsally in chordates and ventrally in protostomes (Figure 1A) in regions in which the BMP gradient is low, suggesting that Urbilateria had a differentiated CNS that was inverted during evolution (De Robertis and Sasai, 1996). However, Lowe et al. (2006) recently discovered that in the hemichordate *Saccoglossus kowalevskii*—which has a diffuse intraepidermal nerve net—neural differentiation is independent of BMP signaling (other proteins such as FGF can induce neural tissue in many organisms), whereas the hemichordate overall D-V body pattern is still established by the conserved Chordin-BMP axis. This raised the issue of whether the bilaterian ancestor had a centralized CNS separate from the epidermis or a diffuse one (Lowe et al., 2006). This debate stimulated new work by the Arendt group, which revealed that annelids share deep homologies with vertebrates, such that entire molecular fingerprints have been conserved in D-V CNS neuronal cell type patterning (Denes et al., 2007). Amazingly, annelids even have neurosecretory cells that secrete Vasopressin/Oxytocin/Neurophysin prohormone and are homologous to cells in the zebrafish hypothalamus. These specialized neurons differentiate specifically in regions of the brain that coexpress *rx* (*retina homeobox*), *vax* (*ventral anterior homeobox*), *nkx2.1* (*Nirenberg and Kim homeobox 2.1*), and *miR-7* in both organisms (Tessmar-Raible et al., 2007). In *Drosophila*, the overall D-V arrangement of neuronal cell types in the CNS is regulated by a gradient of BMP signals (Mizutani et al., 2006), as is also the case in zebrafish (Little and Mullins, 2006). In addition,

as is now well established, the *Pax6* gene is an upstream regulator of the development of eyes in all organisms (Gehring, 1998; Arendt and Wittbrodt, 2001). Taken together, these new results support the view that hemichordates lost neural centralization secondarily, and that Urbilateria had an organized CNS with remarkably elaborate neuronal cell types that were inherited by its descendants.

The Genetics of Evolutionary Adaptation

If the genetic tool-kit was so conserved, how is it that natural selection generated such a rich variety of species? One of the most important recent advances in evolutionary biology has been the ability to identify the actual mutations that provided the variation on which selective pressure acted upon in natural animal populations (reviewed by Hoekstra and Coyne, 2007). Adaptive mutations can be classified as (1) *cis*-regulatory, (2) structural, (3) duplications, and (4) gene deletions. Let us consider them in turn.

cis-Regulatory Mutations

cis-regulatory mutations probably provide the variation for most of the novelties in body form in animal species (Carroll et al., 2001; Davidson, 2006). This is achieved by bringing together combinations of transcription factors on DNA regulatory regions—called enhancers—that control the spatial and temporal expression of genes. Tissue-specific enhancer modules can be added or deleted, avoiding pleiotropic effects on other regions of the body. Here we can examine only two examples of *cis*-regulatory adaptations: spine loss in sticklebacks and maxilliped variations in crustaceans.

Three-spine stickleback fish populations became isolated in glacial lakes in North America after the last ice age 15,000 years ago. One beneficial adaptation for some populations was to lose the two pelvic girdle spines to avoid predation by dragonfly nymphs that cling to them. Genetic crosses allowed the mapping of the pelvic reduction phenotype to the *Pitx-1* homeobox gene (Shapiro et al., 2004). This adaptive mutation causes a loss of expression of *Pitx-1* exclusively in the pelvic region, preserving the essential developmental role of this gene in other parts of the body. *Pitx-1* provides an interesting example of parallel evolution because similar *cis*-regulatory mutations were selected independently in different stickleback populations. Conversely, the stickleback work implies that the spines were evolutionary novelties obtained through the formation of a new enhancer for the *Pitx-1* gene.

In crustaceans (such as shrimp and lobsters) thoracic limbs have been transformed into feeding appendages called maxillipeds many times in the course of evolution. In each of these independent events, the appearance of maxillipeds correlates with a shift in the border of expression of a Hox gene (Averof and Patel, 1997), although it is not known whether the changes in Hox gene expression provided the initial cause of these homeotic transformations. Shifts in Hox gene expression also occur in vertebrates; for example, in snakes the Hox-C6 protein is expressed in hundreds of vertebrae as thoracic segments multiplied, whereas in the mouse it is expressed only in eight thoracic segments (Cohn and Tickle, 1999; Figure 3). The cases of the stickleback

spines and crustacean maxillipeds lead to the important conclusion that mutations in the *cis*-regulatory elements of development-controlling genes, such as *Pitx-1* and Hox genes, could provide the substrate for the evolutionary selection of body form.

Structural Mutations

Structural mutations affect the sequence of proteins and play an important role in adaptations, as in the examples of hemoglobin and melanic adaptive mutations. As described by Max Perutz in the 1980s, a single amino acid change in globin increases the affinity of hemoglobin for oxygen in high-altitude migrating geese compared to their common low-flying relatives (Hoekstra and Coyne, 2007). Melanism has occurred many times independently and, interestingly, is frequently caused by independent gain-of-function mutations in the 7-transmembrane receptor for melanocortin (Mc1r). The black leopard, black jaguar, melanic birds (whip-tail, skua, and others), beach mice, and lizards all have activating mutations in this receptor (Hoekstra and Coyne, 2007). Conversely, loss-of-function mutations in the melanocortin receptor are found in yellow Labradors and human redheads (Carroll, 2005). This receptor has been naturally selected in many parallel evolutionary events for pigment adaptations because it lacks pleiotropic effects. Another form of structural variation is the swapping of protein domains or modules between structural genes that combine new functions, a well-recognized powerful force during protein family evolution (e.g., <http://pfam.sanger.ac.uk>). In addition, structural protein mutations that preserve viability do occur in development-controlling genes. An excellent example is provided by the Ultrabithorax protein of the crustacean *Artemia salina*, which contains regulatory phosphorylation sites that, when phosphorylated, allow the formation of appendages in the crustacean abdomen. Interestingly, these phosphorylation sites were lost in *Drosophila*, which does not develop limbs in the abdomen (Ronshaugen et al., 2002).

Gene Duplications

Gene duplications are a powerful source of evolutionary variation. The duplicates can be used for new functions without losing the original one (Ohno 1970; Wapinski et al., 2007). Duplications also serve to disentangle intertwined gene networks. For example, if a protein that serves as a subunit in two different protein complexes is duplicated, each paralog can be dedicated to a single one. Such disentangling has occurred repeatedly in the evolution of fungi, for example in the case of a protein subunit of two transcriptional machinery factors known as SAS and TFIID in *S. cerevisiae* (Wapinski et al., 2007). The consequences of gene duplications are also evident in the *Xenopus* D-V patterning system. For example, the ventral gene *BMP4* has a dorsal counterpart in *ADMP*, and each gene has been placed under opposing transcriptional control, permitting the self-regulation of D-V pattern (De Robertis, 2006). Extensive gene expansions can occur during evolution. For example, in the sea urchin genome hundreds of Toll-like receptors and other Leucine-rich-repeat proteins have been duplicated in order to provide diversity to the innate immunity recognition system (Rast et al., 2006). In other cases, it has been sufficient to duplicate exons in

tandem to increase diversity, rather than an entire gene, producing many alternatively spliced proteins from a single gene as in the case of *Dscam*, a homophilic cell repulsion molecule that produces tens of thousands of protein isoforms in insects but not in vertebrates (Wojtowicz et al., 2007).

Gene Deletions

Gene deletions provide a very effective way of rapidly adapting to new ecological niches (Wapinski et al., 2007). Mexican Tetra fish became entrapped multiple independent times in caves within the last million year period. Many cave animals, such as salamanders, shrimp, and fish, adapt to the new environmental conditions by becoming albino and losing their eyes. By backcrossing troglodyte Tetras to their surface river relatives, Protas et al. (2006) demonstrated that the albinism phenotype mapped to independent deletions in the *Oca2* (*ocular* and *cutaneous albinism-2*) gene in different cave populations. This proved that rapid adaptive change can indeed be caused by gene losses in natural animal populations. During evolution, entire stages of development can be lost (e.g., free-swimming marine larvae), segmentation has been lost in nematodes and planarian flatworms (which undoubtedly shared a common segmented protostome ancestor, Figure 1B), and a multitude of genes have been lost (e.g., Dkk, sFRPs, Noggin, and some Wnt genes in *Drosophila* and *C. elegans*). Rapid adaptations due to loss of protein functions come at the cost of constraining future variation and evolutionary outcomes. For example, nematodes are wonderfully adapted to live in their ecological niches, yet once *C. elegans* lost the gene tool-kit required for making eyes or a metameric body, their descendants will not recover these structures ever again.

In sum, several types of mutations, some acting on the function of conserved developmental gene networks, provide the variation on which natural selection acts. In some cases, the same adaptive mutations have been selected repeatedly in evolution. It will be very interesting to investigate the genetic tool-kit losses and duplications within each phylum with respect to the archetypal bilaterian, for this may have provided a key event in the evolution of body plans.

Historical Constraints

In 1959, at the time of the centennial celebrations of the Origin of Species (Darwin, 1859), a modern synthesis between population genetics and evolution became established (chronicled in Gould, 2002). The creative power of natural selection working on random mutations over immense periods of geological time explained the immense variety of life on earth. What we are learning from Evo-Devo is that the source of variation of importance for evolution resides in deeply homologous developmental gene networks shared by all animals. A key question is to what extent these deep genetic homologies discovered by Evo-Devo have channeled, or constrained, the outcomes of evolution (De Robertis and Sasai, 1996; Gould, 2002).

This brings us to the old dilemma of homology and convergence in evolution. Homology refers to two structures arising from an ancestral structure by the action of natural selection on common ancestors. An example of homology could be

the hoof of a horse and the middle digit of the ancestors from which it evolved. It should be kept in mind that once two species separate, the pressures of natural selection will act on separate subjects and at different times. A recurrent theme in evolution is that convergent solutions are found for common functional problems. An example of convergent evolution could be the wings of pterodactyls, birds, or bats, which evolved at different times but represent similar adaptations. Convergence is generally considered to be driven exclusively by functional needs, in this particular example the need for flight. Given the deep homologies among developmental gene networks, natural selection might use variations in these ancestral gene networks repeatedly, following the channel of least resistance. However, in the example given here it is not yet known which developmental networks were mutated to generate these wings.

We usually think of constraints as negative influences that prevent particular changes. However, in evolutionary biology the constraints resulting from the obligatory use of conserved developmental gene networks should also be considered a positive influence, which facilitates effective adaptive responses to the strictures of natural selection. This channeling of selective pressure by the underlying genetic structure is designated parallelism (Gould, 2002). One example of parallelism could be the above-mentioned repeated evolution of maxillipeds in different crustacean species via changes in the borders of Hox gene expression (Averof and Patel, 1997). Given the conserved tool-kit of intertwined networks, we can expect that many morphological solutions currently believed to be caused by convergent evolution will be caused by parallelisms channeled by the natural selection of variations in ancestral developmental gene networks. In other words, many body plans that would be very adaptive might not exist in nature because they cannot be achieved unless compatible with the developmental pathways that generate the required changes in body form.

For this reason, it is useful to reflect on how Urbilateria was constructed. Did it contain the complete genetic tool-kit animals now use? Was it segmented? Were the D-V and A-P axes patterned by BMP/Chordin, Wnt, and Hox genes? Did the gene machinery that generates body form channel the variation on which natural selection works into similar morphological solutions? Just imagine, as an intellectual exercise, that if the answer to all the above open questions were affirmative, animals could have used the same genetic strategy already present in the urbilaterian archetypal ancestor to evolve the elongated metameric structures of a snake, millipede, or earthworm in particular ecological niches. This unity of plan may strike one as most unlikely at present, but it is worth remembering that not so long ago the conventional wisdom was that eyes had arisen independently 40 to 60 times by convergent evolution (Salvini-Plawen and Mayr, 1977; Gehring, 1998).

This is a wonderful time to study evolution. Next year will bring the sesquicentennial of the publication of the Origin of Species (Darwin, 1859)—which also marks 200 years of Charles Darwin's birth—and a fresh round of celebrations. We can expect that the new discipline of Evo-Devo, with its

marriage between developmental biology and Darwinian theory, will play a prominent role in this coming anniversary, having helped uncover the deep historical homologies that provide the underpinnings of animal body plans and anatomical diversity.

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