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Perspective

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Evo-Devo and an Expanding Evolutionary Synthesis: A Genetic Theory of Morphological Evolution

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Biologists have long sought to understand which genes and what kinds of changes in their sequences are responsible for the evolution of morphological diversity. Here, I outline eight principles derived from molecular and evolutionary developmental biology and review recent studies of species divergence that have led to a genetic theory of morphological evolution, which states that (1) form evolves largely by altering the expression of functionally conserved proteins, and (2) such changes largely occur through mutations in the *cis*-regulatory sequences of pleiotropic developmental regulatory loci and of the target genes within the vast networks they control.

Introduction

Biologists have long recognized that to understand the process of evolution we need to understand the roles of genes in development. However, at the time of the "Modern Synthesis" of evolutionary theory that drew together various disciplines including genetics, paleontology, and systematics, very little could be said about the effects of genes on development, let alone on the evolution of form. Despite Huxley's acknowledgment, embryology and developmental genetics played no part in forging the Modern Synthesis (Gilbert et al., 1996) and little role in the fabric of evolutionary biology for many decades thereafter.

The situation started to change twenty-five years ago with the discovery of the homeobox. This coding sequence was first identified in several loci involved in segment formation and identity in the fruit fly *Drosophila* (McGinnis et al., 1984; Scott and Weiner, 1984) and then was found in clusters of related genes (*Hox*) in vertebrates and other animals (McGinnis et al., 1984; Graham et al., 1989; Duboule and Dollé, 1989). These discoveries forced developmental geneticists to confront evolutionary concepts and evolutionary biologists to confront a new source of unforeseen and penetrating genetic insights into the generation and diversification of animal form.

As the dust has settled on some of the earlier surprises, the brief history of evolutionary developmental biology (evo-devo) has begun to inspire some reflection on the contributions of this discipline to overall evolutionary theory. Not surprisingly, the opinions offered range from "evolution cannot be understood without understanding the evolution of development... A revolutionary synthesis of developmental biology and evolu-

...a study of the effects of genes during development is as essential for an understanding of evolution as are the study of mutation and that of selection.

—Julian Huxley, *Evolution: The Modern Synthesis (1942)*

tion is in progress" (Raff, 2000) to "Let's be honest: the much-hyped discipline of evolutionary developmental biology...hasn't quite lived up to expectations—at least, not if one were expecting a revolution in biology" (Richardson, 2003). Still others have drawn an analogy between current enthusiasm for ideas in evo-devo and the theory of punctuated equilibrium as a cautionary tale of an area of research that "has claimed to offer unique and revolutionary insights into the evolutionary process" but of which "little now remains" (Hoekstra and Coyne, 2007).

But do questions posed about evo-devo and evolutionary theory matter to anyone besides the specialists and a few future historians? I think the answers matter very much. By "theory" here, I mean "structures of ideas that explain and interpret facts" (from Gould, 1994). Without theories to organize and interpret facts, without the power of general explanations, we are left with just piles of case studies. Moreover, we are without the frameworks that enable us to make *predictions* about any particular case. Here, I will take the position that we have learned enough about the function, regulation, and history of the genes controlling animal development to formulate a general theory of how form evolves and to make predictions about the genetic path of morphological evolution—predictions that are now being fulfilled for a variety of traits and genes in diverse taxa.

My goals in this article are three-fold. First, to review key principles, many from molecular developmental biology, that laid the foundation for a new understanding of the evolution of form. Second, to articulate a genetic theory of morphological evolution that has emerged from a large body of empirical studies. And third, to explain how evo-devo, by providing long-

sought explanations of the causal links between gene muta-tion, development, and the evolution of form, has filled a major gap in and expanded upon the evolutionary synthesis.

Before I delve into the specific findings from developmental genetics and evo-devo, it is important to establish some his-torical perspective through a brief exploration of the roots of some key ideas about the relationship between the evolution of molecules and form.

The Evolution of Molecules and Morphology: Surprises and Paradoxes

The relationship between the evolution of molecules and mor-phology has been fertile ground for both controversy and major advances in evolutionary biology. On several occasions, evolu-tionary biologists have been surprised by molecular data that did not conform to expectations. In each case, wrestling with new data has led to new conceptual insights.

The first instance was in the early 1960s when initial com-parisons of globins and cytochromes from different species revealed that proteins changed over time but without any apparent effect on function. These observations did not fit the expectation that all evolutionary change was adaptive and led to the proposal that most mutations fixed between spe-cies were functionally neutral or nearly neutral and evolved by genetic drift.

Emile Zuckerkandl, one of the pioneers of molecular evo-lutionary biology, reasoned that phenotypes could change by altering the timing or rate of protein synthesis (Zuckerkandl and Pauling, 1965). He also made the prescient suggestion that there may be an important mechanistic distinction between the evolution of organismal form, which he proposed may depend more on changes in DNA than on alterations in protein expres-sion, and the evolution of function, which would result from physicochemical changes in proteins (Zuckerkandl, 1968).

The second major surprise was the similarity of proteins from species that looked and behaved as differently as, for example, chimps and humans. Mary-Claire King and Allan Wilson under-scored the apparent paradox that presented and the chal-lenge "to explain how species which have such substantially similar genes can differ so substantially in anatomy…" (King and Wilson, 1975). They, like Zuckerkandl and others (Britten and Davidson, 1971), suggested that the evolution of anatomy occurred more by changing gene regulation than by changing protein sequences.

The testing of these proposals, however, had to wait for the genetic revolution in developmental biology and a series of dis-coveries that began to unfold in 1984, when evolutionary biolo-gists were again confronted with molecular data that did not fit prior expectations—namely that the disparate body forms and structures of long-diverged members of the animal kingdom were governed by very similar sets of genes.

To get a sense of the sea change brought about by these discoveries—a "before" picture as a prelude to the "after" pic-ture that will constitute the rest of this article—revealing snap-shots are provided by two book reviews that appeared back to back in the March 1984 issue of *Cell* (published just weeks before the first report of the homeobox). In his review of a new book on embryology, developmental biologist Gunther

Stent noted the lack of general conclusions and ascribed the "turbid" state of embryology as a consequence of the his-tory and complexity of the process (Stent, 1984). He wrote, "embryologists are confronted with an ensemble of unique phenomena...we cannot expect to discover a general theory of development, rather we are faced with a near infinitude of particulars, which have to be sorted out case by case." Next came Peter Lawrence's review of a symposium volume from a meeting on "Development and Evolution" (Lawrence, 1984). His assessment was blunt: "To discuss usefully the interface between two subjects—like evolution and development—one depends on a deep understanding of both. Unfortunately our knowledge of these fields is poor and the result, in the book, is a great deal of pretentious twaddle..." Lawrence pointed out how participants were divided over the potential value, or lack thereof, of genetic approaches to development as well as the question of whether the genome contained a direct or explicit program for development.

Many times over the next decade or so, generalities of animal development would be found among the particulars of *Drosophila* development in the form of shared regulatory genes. The question of the existence of a genomic program for development was quickly transformed into the pursuit of the components and logic of that program—the genetic regu-latory networks that we now recognize to be the essence of animal development (Davidson, 2006). And, with respect to genetic mechanisms governing the evolution of anatomy, the issue turned logically to how components of these regulatory networks evolve.

The collective impact of that pursuit has been to direct atten-tion away from long-standing ideas about the necessity for gene duplication or protein evolution in the origins of novelty and toward changes in gene regulation, regulatory networks, and regulatory sequences as sources of morphological varia-tion and diversity. Below, I will first focus on eight principles, derived from observations from molecular and evolutionary developmental biology, in order to explain why thinking pro-gressed along these lines. These principles form part of the foundation of a genetic understanding of morphological evolu-tion. Then, I will examine the direct evidence concerning the genetic and molecular basis of morphological divergence and describe a specific, predictive theory.

The Road to a Theory: Eight Principles
(1) Mosaic Pleiotropy
Most proteins regulating development participate in multiple, independent developmental processes and the formation and patterning of morphologically disparate body structures.

King and Wilson reached their seminal hypothesis by com-paring the very similar genes of two different species. Here, I will illustrate one of the seminal insights from developmental genetics, and perhaps the most critical observations in terms of implications for the evolution of anatomy, by considering the identical genes of two different forms of the same species.

Take, as an example, the larval and adult forms of the fruit fly *Drosophila melanogaster*. They are more distinct in terms of morphology and ecology than the adult forms of any pair of sister species. Yet, we now know of hundreds of gene products

that, although identical in sequence in the developing larval and adult forms, shape the development of these markedly different body morphologies.

Probing a bit deeper into the development of individual structures, we know of many gene products that, although identical in different tissues or body parts, shape the distinct appearance of each part. For example, the *Drosophila* Decapentaplegic (Dpp) signaling protein shapes embryonic dorsoventral axis polarity, epidermal patterning, gut morphogenesis, and the patterning of wings, legs, and other appendages (Gelbart, 1989).

The same is true for most signaling proteins and transcription factors, components of the "genetic toolkit" that regulate animal development. For instance, the *Sonic hedgehog* protein shapes digit number and polarity, floor plate development, cerebellum development, feather bud formation, and other processes in chickens (McMahon et al., 2003). Each set of structures is obviously much more divergent within individual species than are the corresponding homologous structures of different species.

Proteins that serve multiple roles are said to be pleiotropic (from the Greek meaning "many ways"). Most proteins that regulate development exhibit what is termed "mosaic pleiotropism" (Hadorn, 1961) in that they function independently in different cell types, germ layers, body parts, and developmental stages. This extent of pleiotropism was a surprise as there was no particular reason to expect that the formation and patterning of such different structures would or could involve the same protein.

However, once the mosaic pleiotropism of toolkit proteins is fully appreciated, important evolutionary implications clearly follow. First, because the exact same protein can shape the development of remarkably different body parts, one may infer that the same protein can therefore shape smaller differences in the anatomy of the same part between species. Second, mutations that alter the function or activity of such proteins are likely to have widespread and potentially many negative effects on development and fitness, and their coding sequences may thus be under considerable evolutionary constraints. The existence of these constraints has been borne out by decades of developmental genetic studies, but the remarkable degree of constraint was entirely unforeseen until revealed by comparative functional analyses (see points 2, 3, 4). Third, the use of the same gene in shaping many entirely different traits suggests that in the course of evolution, gene function somehow expanded without gene duplication. This inference, now well confirmed, is contrary to once prevailing ideas concerning the origin of new gene functions (see point 5). Furthermore, it raises the question of how the multifunctionality of these loci evolved. Tacit answers to that question also came from molecular developmental genetics (see points 7, 8).

(2) Ancestral Genetic Complexity
Morphologically disparate and long-diverged animal taxa share similar toolkits of body-building and body-patterning genes.

The best known discoveries of evo-devo are those concerning the presence of homeobox-containing *Hox* genes and *Hox* gene clusters in flies, vertebrates, and most animal phyla. The similarities in animal genetic toolkits extend to a large number and wide variety of transcription factors and components of signaling pathways. The phylogenetic distribution of toolkit genes suggests that a fairly complete modern toolkit was in place in the last common ancestor of bilaterians, prior to the Cambrian period. Furthermore, various signaling pathways have also been found to be much better represented in early-branching animals than their anatomical complexity would suggest. For example, 11 of 12 Wnt gene families known from vertebrates are found in cnidarians (Kusserow et al., 2005), and six of the major bilaterian signaling pathways are represented in sponges (Nichols et al., 2006). The extensive sequence conservation in animal toolkit proteins indicates that considerable functional constraints have operated on many orthologous proteins for more than 500 million years of animal diversification.

(3) Functional Equivalence of Distant Orthologs and Paralogs
Many animal toolkit proteins, despite over 1 billion years of independent evolution in different lineages, often exhibit functionally equivalent activities in vivo when substituted for one another. These observations indicate that the biochemical properties of these proteins and their interactions with receptors, cofactors, etc. have diverged little over vast expanses of time.

Even more striking than their sequence conservation, a wide variety of toolkit proteins are functionally equivalent when substituted for homologous proteins (orthologs and paralogs) in divergent taxa. This line of experimentation began with tests of vertebrate Hox proteins introduced into flies (Malicki et al., 1990;McGinnis et al., 1990). Perhaps the best-known example is the ability of the mouse Pax-6 protein to induce ommatidium formation in *Drosophila*, just like the *Drosophila* Pax-6 (Eyeless) protein (Halder et al., 1995).

Another striking example of functional equivalence is the ability of a cnidarian Achaete-Scute homolog (CnASH) to induce formation of sensory organs in *Drosophila*, just as the *Drosophila* homologs do (Grens et al., 1995). Furthermore, despite over 1 billion years of independent evolution, the CnASH protein forms a heterodimer with the endogenous *Drosophila* binding partner Daughterless, and these dimers bind sequence specifically to sites in target gene-regulatory elements.

What makes these results so surprising and notable is that the function of any transcription factor or ligand is dependent upon interaction with other endogenous proteins—transcription factors, coactivators, corepressors, and parts of the transcriptional machinery in the case of transcription factors, cell-surface receptors in the case of ligands. One might expect that genetic drift or the coadaptation of proteins within lineages would lead to functional incompatibilities among proteins from different taxa, especially those separated by over 1 billion years of independent evolution. Yet, many proteins are so conserved that they can interact and function together with other proteins from long-diverged taxa. These results have been more the rule than the exception for transcription factors (I will discuss informative exceptions later). Numerous studies of eukaryotic transcription demonstrate that the basic transcriptional machinery, many coactivators, corepressors, chromatin-remodeling complexes, and the protein motifs through which transcription factors interact with them, are often very well conserved.
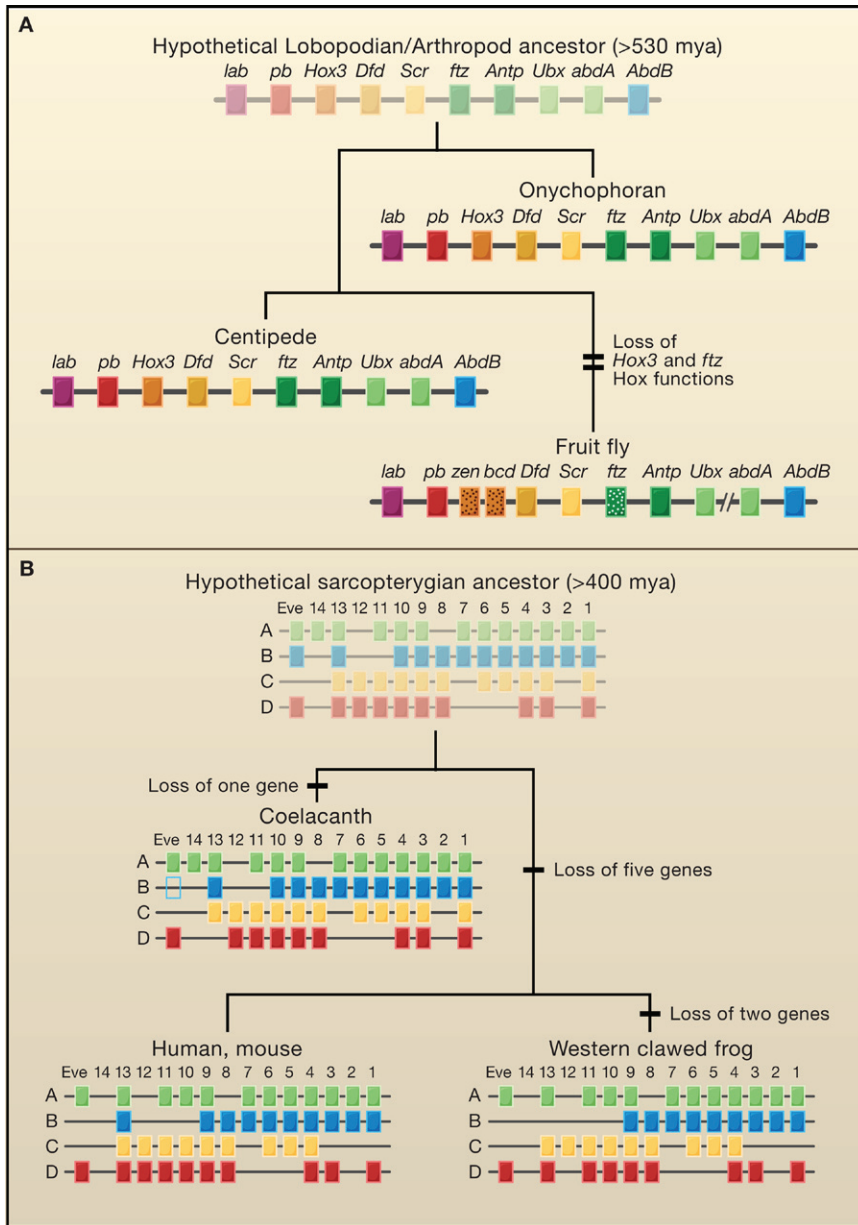
Figure 1. Ancestral Complexity of *Hox* Clusters and the Lack of *Hox* Gene Duplications in Arthropods and Chordates
(A) Based upon the *Hox* gene complements of onychophora and arthropods, a minimum of ten *Hox* genes must have existed in the common ancestor of lobopodians and arthropods. No new *Hox* genes arose in centipedes or insects while the *Hox3* and *ftz* genes were co-opted into new functions in certain insects (stippling).
(B) No new *Hox* genes are known to have evolved since the divergence of tetrapods from a common sarcopterygian ancestor shared with coelacanths. Rather, gene loss has occurred in several lineages. (Figure adapted from Hoegg and Meyer, 2005.)

In addition to their functional equivalence, various toolkit proteins are involved in the development of morphologically disparate structures with similar functions. The association of the Pax-6 protein with eye development throughout the animal kingdom (Gehring and Ikeo, 1999), of several transcription factors required for cardiac tissue development in flies, vertebrates, and other animals (Olson, 2006), of Distal-less/Dlx protein expression in the development of a variety of animal appendages (Panganiban et al., 1997), and other examples have prompted reexamination of the concept of biological homology (Wagner, 2007).

The deployment of homologous transcription factors in similar roles reflects that some parts of genetic regulatory networks (GRNs) present in a common ancestor were conserved in descendant lineages. The existence of common regulatory inputs acting in a similar manner in the development of structures that are not directly related by common ancestry (that is, not homologous) has been referred to as "deep homology" (Shubin et al., 1997). Some highly conserved GRNs have been identified (Davidson, 2006). Because the different structures of long-diverged animals were thought to have arisen independently, the similar roles of orthologous toolkit proteins in their development has prompted reevaluation of their once-presumed independent origins.
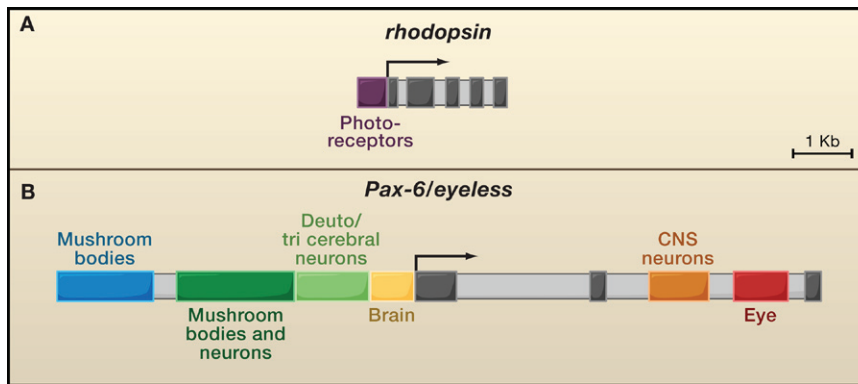
One situation where functional changes within transcription factors might be expected would be among paralogous proteins that have evolved by gene duplication and divergence. However, the functional equivalence of various sets of paralogs has also been well documented (for example, Fitzgerald et al., 1993; Li and Noll, 1994), although, as expected, there are some notable exceptions that I will also discuss later (Zhao and Potter, 2001, 2002).

(4) Deep Homology
The formation and differentiation of many structures such as eyes, limbs, and hearts—so morphologically divergent among different phyla that they were long thought to have evolved completely independently—are governed by similar sets of genes and some deeply conserved genetic regulatory circuits.

(5) Infrequent Toolkit Gene Duplication
Duplications within several prominent toolkit gene families have been surprisingly rare in the course of animal diversification relative to duplications of other gene families. These observations indicate that gene duplication is not a necessary ingredient for morphological novelty, as once assumed, and there is evidence that duplications of some toolkit genes are actually selected against because of their effects on gene dosage-sensitive developmental processes.

From the earliest days of molecular evolutionary biology, the roles for new genes in the origins of new functions has been a pervasive and widely accepted idea. Ohno (1970) claimed that gene redundancy was crucial to novelty and that "allelic mutations of already existing gene loci cannot account for major changes in evolution." Kimura and Ohta (1974) agreed that "gene duplication must always precede the emergence of a gene having a new function." The expectations were no different for the evolution of anatomy. In a classic article in developmental genetics, Lewis proposed that the increased segmental diversity of insects relative to arthropod ancestors was the result of an increase in the number of segment identity-determining *Hox* genes, including the evolution of a haltere-promoting gene to shape this distinctly dipteran trait (Lewis, 1978). This intuitive idea turned out, however, to be incorrect. The common ancestor of all arthropods actually had two more *Hox* genes than do certain modern insects, and the "haltere-promoting" Ultrabithorax gene predated the origin of halteres by more than 300 million years as well as the origin of the entire arthropod phylum (Grenier et al., 1997) (Figure 1A).

Similar observations apply to the history of tetrapod *Hox* genes. Mice and humans share the same set of 39 *Hox* genes, and we do not know of a single new *Hox* gene duplication in a mammalian lineage. Rather, it is clear that since their divergence some 400 million years ago from the last common ancestor shared with coelacanths, tetrapods have actually lost several *Hox* genes (Hoegg and Meyer, 2005) (Figure 1B). The stability of *Hox* gene number in arthropods and tetrapods and the central roles of these genes in the development and evolution of structures such as halteres and limbs is clear evidence that, contrary to expectations, gene duplication is not a requirement for new gene function and that major changes in evolution have occurred through mutations in already existing gene loci.

The relative paucity of such toolkit gene duplications, compared with many other gene families over similar timescales, may indicate that duplicates of some toolkit genes may not be neutral and may be selected against to some degree. One reasonable explanation may be that aneuploidy of regulatory genes is not well tolerated because of dosage effects on genetic regulatory circuits and the development of one or more of the many individual traits each gene may affect. Reduced dosage, for example, of certain *Drosophila Hox*

genes or the Dpp signaling protein or of the vertebrate *Pax-6* gene, *Hox* genes, cardiac transcription factor genes, and the *Sonic hedgehog* gene adversely affect certain traits. Extra doses of transcription factors or of signaling proteins that act in a concentration-dependent manner may also be deleterious. This has been demonstrated for *Pax-6* (Schedl et al., 1996) and other regulators (Arron et al., 2006). In contrast, aneuploidy for other protein families that are not mosaically pleiotropic may be neutral and well tolerated (for example, the opsins, see Cooper et al., 2007).

In summary, duplication of toolkit genes has certainly occurred but abundant comparative data now reveal that duplication is neither necessary nor has it been frequent enough to account for the continuous diversification of animal anatomy over the past several hundred million years. Attention has therefore been focused instead on how changes in the regulation of toolkit genes or their targets are associated with morphological divergence and on how such changes arise.

(6) Heterotopy
Changes in the spatial regulation of toolkit genes and the genes they regulate are associated with morphological divergence.

A vast body of data has accumulated that has linked differences in where toolkit genes are expressed, or where the genes they regulate are expressed, with morphological differences between animals at various taxonomic levels (Carroll et al., 2005). Most studies have analyzed situations in which the spatial location of a developmental process (for example, the making of a limb, the formation of a pigmentation pattern, the development of epithelial appendages, etc.) has been altered. The classical term for such spatial changes in development is heterotopy (from Haeckel; Hall, 2003; changes in the timing of a process are known as heterochrony). The close correspondence between heterotopic shifts in gene expression, development, and morphology, combined with the known roles of these genes in model taxa, have provided compelling evidence that changes in morphology generally result from changes in the spatiotemporal regulation of gene expression during development.

Explanations for the evolution of anatomy have thus focused on the genetic and molecular mechanisms underlying the evolution of spatial gene regulation. And the keys to understanding spatial gene regulation are the architectures of gene-regulatory regions and transcriptional networks.

(7) Modularity of cis-Regulatory Elements

Large, complex, and modular cis-regulatory regions are a distinctive feature of pleiotropic toolkit loci.

The *cis*-regulatory regions of most toolkit loci are larger and more complex than those of loci encoding cell-type-specific proteins engaged in the chemistry of physiological processes. An example of each type of locus will illustrate the point. The *Drosophila* rhodopsin gene family is typical of the latter. Each gene in this family encodes an average-sized protein that is expressed in one or more specific photoreceptor cell types in the compound eye. Detailed experimental studies have shown that the cell-type-specific expression of each gene is dependent upon a single *cis*-regulatory element (CRE) consisting of a few hundred base pairs of noncoding sequences 5′ to the gene promoter (Fortini and Rubin, 1990) (Figure 2A). One protein that binds to this region and is required for proper expression of each rhodopsin gene is the Eyeless/Pax-6 protein (Papatsenko et al., 2001).

In striking contrast to the rhodopsin CRE architecture is the *cis*-regulatory region of the *Eyeless (Ey)* locus, which is required not just for eye development but also for patterning parts of the developing brain and central nervous system. Sequences required for *Ey* expression encompass at least 7 kb of noncoding DNA 5′ and 3′ to the gene and within one intron (Adachi et al., 2003). There are six distinct CREs, averaging about 1 kb in size, that each drive Ey expression in a particular spatial pattern—in the eye, in various lobes and cell types of the developing embryonic, larval, and adult brains, and within the central nervous system (Figure 2B). The complex spatial pattern of *Eyeless* expression, and of all mosaically pleiotropic toolkit genes, is a composite of the independent activities of multiple modular CREs.

The arrays of CREs that independently govern individual toolkit gene expression at different times and places during development presented a new picture of gene organization with three crucial implications for the evolution of form. First, the multiple CREs are plain evidence of how gene function has expanded and diversified without duplication of coding sequences. Second, mutations in one CRE will not affect the function of other CREs or of the protein (that is, they will have fewer or no pleiotropic effects). And third, mutations in coding regions may affect all protein functions (that is, may have the most pleiotropic effects). The multiplicity of toolkit gene CREs accounts for one aspect of their mosaic pleiotropy; the second major contributor is the direct control by toolkit proteins of what we now know to be many individual target genes.

(8) Vast Regulatory Networks

Individual regulatory proteins control scores to hundreds of target gene CREs.

The basic structural unit of a GRN is the functional linkage between a transcription factor and a CRE. A growing body of data on a variety of transcription factors is providing some glimpses into the scale of transcription factor-regulated gene networks, and they are considerably larger than was known until very recently. New techniques are revealing that toolkit transcription factors typically regulate scores to hundreds of individual target genes. This is pleiotropism on an enormous scale that is not yet widely appreciated, and whose evolutionary significance has therefore not yet been grasped.

For example, Stark et al. (2007) estimated that there are, on average, 124 target genes for each of the 67 *Drosophila* transcription factors they analyzed. This figure is exceeded by the number of validated hormone-responsive androgen receptor target genes in just one human cell type (172; Bolton et al., 2007) and the number of signal-regulated STAT3 targets in another cell type (~300; Snyder et al., 2008). Even greater is the number of target genes directly regulated by the *Drosophila* Twist transcription factor during embryogenesis—nearly 500 targets in total, including genes involved in cell proliferation, cell migration, and morphogenesis, as well as nearly one-fourth of all known transcription factor genes (Sandmann et al., 2007).

The evolutionary implications of such vast transcription factor-regulated networks are crucial and inescapable. First, these networks are themselves products of evolution. Each transcription factor-CRE linkage requires that specific factor-binding site sequences exist in the CREs of each target gene, and usually multiple copies thereof. The evolution of these networks has therefore involved the cumulative evolution of many binding sites for each transcription factor. The scale of the Twist-regulated network, for example, means that, in the course of evolution, some 500 linkages have evolved between Twist and different CREs. That required a lot of *cis*-regulatory mutations. Second, it is obvious why mutations that would alter Twist DNA-binding specificity or that of other transcription factors are catastrophic (they could break all linkages) and thus why the evolution of protein function is constrained. And third, these snapshots of transcription factor-regulated networks help us to anticipate possible genetic paths for the evolution of anatomy. Namely, they suggest that linkages within GRNs are added or subtracted by the modification of CREs within target genes. Because mutations in individual CREs will directly alter the expression of only one gene, such changes can selectively affect individual morphological features.

Formulating and Testing a Genetic Theory of Morphological Evolution

Given that development is controlled by GRNs, it follows that the evolution of development and form is due to changes within GRNs. Thus, a central quest of evo-devo is to decipher how the GRNs and their components evolve. With respect to the genetic path of the evolution of form, I have said as much about what is not expected to be linked often to the process (protein evolution and gene duplication) as I have about what has been correlated (spatial changes in gene expression) or is inferred to be involved (CRE evolution). To formulate general genetic explanations—a predictive theory—about the evolution of form, these facts and inferences have to be distilled into hypotheses and tested against direct empirical data.

The cis-Regulatory Hypothesis

The genetic path of evolutionary change is governed by what is functionally possible (in terms of mutational effects), what is probable (in terms of the frequency of different kinds of mutational events), and what is permissible (by natural selection). Any genetic theory of morphological evolution must take all three parameters into account.

The key hypothesis to emerge from the first handful of case studies linking spatial shifts in *Hox* and limb-patterning gene expression to the divergence of animal body patterns (Carroll et al., 1994, 1995; Warren et al., 1994; Averof and Akam, 1995; Burke et al., 1995) concerned the role of *cis*-regulatory sequence mutations. In light of the evidence at the time—the absence of new *Hox* gene duplications in arthropods and tetrapods (low frequency), the extreme conservation of homeodomains and toolkit protein functional equivalency (change is not permissible), and the potential for mutations in one CRE to selectively alter just one feature of gene expression (more permissible)—mutations in CREs of existing genes were proposed to be a more common and a continuous source of morphological variation compared with the evolution of new genes (Carroll, 1995).

Although a logical inference, there was no direct functional support for this proposal for several years. Furthermore, this hypothesis was based on the examination of body plan-level characters and consideration of a small number of genes that sat atop genetic regulatory hierarchies. It also presumed that the constraints on these and other toolkit genes were such that orthologous proteins did not change in meaningful ways. To ascertain whether *cis*-regulatory mutations have been the predominant genetic path of morphological evolution, or not, required that direct functional evidence be gathered concerning a variety of traits and involving a spectrum of toolkit genes in various taxa.

We now know that morphological change and GRN evolution can and has involved other genetic mechanisms besides CRE evolution. However, when we look in greater detail at cases where functional changes in transcription factors have taken place, we will see that an extensive amount of CRE evolution was essential to GRN evolution. Therefore, I am going to develop the case for CRE evolution as the predominant mechanism underlying the evolution of form.

In the analysis that follows, it is important to bear in mind that the issue is not decided simply by counting up case studies demonstrating instances of functional CRE or coding sequence changes in morphological divergence. In order to identify general trends among the particulars of case studies and to ascertain conditions under which certain genetic paths are more or less likely, consideration must also be given to the nature of the trait changes analyzed (for example, heterotopic or not), the properties of the genes studied (for example, mosaically pleiotropic or not), the architecture of the GRNs a given gene is part of, and the evolutionary timescales and taxonomic scales being compared.

A major role of CRE evolution in the evolution of form emerges from these considerations and several facts obtained from direct genetic and molecular analyses of morphological divergence. First, CRE sequence changes are sufficient to account for the evolutionary divergence of traits and gene regulation among populations, species, and higher taxa. Second, CRE evolution is necessary for the rewiring of regulatory networks. Third, gene duplication and coding changes alone are insufficient to rewire regulatory networks. And fourth, CRE variation and divergence are detectable over much shorter timescales and taxonomic distances than are functional differences in transcription factors or new toolkit gene duplications. When all of these factors are weighed, a predictive theory of the genetic path of morphological evolution emerges.

The Sufficiency of CRE Evolution

More than two dozen case studies of evolutionary changes in morphological traits have been attributed to changes in gene CREs. A rapidly growing number of genetic analyses of trait divergence have demonstrated evolutionary changes at loci for which functional coding changes have been ruled out and functional *cis*-regulatory sequence changes have been implicated (Stern, 1998; Sucena et al., 2003; Shapiro et al., 2004, 2006; Colosimo et al., 2005; Steiner et al., 2007; Miller et al., 2007) or directly demonstrated at the molecular level (for example, see Wang and Chamberlin, 2002; Wittkopp et al., 2002; Prud'homme et al., 2006; McGregor et al., 2007, Jeong et al., 2008). These include instances where the gain or loss of binding sites for highly conserved transcription factors has altered CRE function and target gene regulation (Gompel et al., 2005, Jeong et al., 2006). These examples of *cis*-regulatory divergence involve a variety of loci encoding transcription factors, signaling ligands, and pigmentation enzymes in fruit flies, mice, and fish.

What all of these studies share in common are the nature of trait divergence (they are all heterotopic changes), the properties of the loci involved (they each exhibit mosaic pleiotropy), and that the divergences have arisen between closely related populations or species. In fact, there are no known cases of naturally occurring functional changes in coding sequences of mosaically pleiotropic loci among closely related species.

Most significantly, these studies reveal that CRE evolution is sufficient to account for changes in gene regulation within and between closely related species. Given that differences at higher taxonomic levels are the product of the accumulation of species-level divergences, then, in principle, CRE evolution is sufficient to account for the rewiring of regulatory networks at all taxonomic levels (for higher taxonomic examples, see Belting et al., 1998; Zinzen et al., 2006; Hinman et al., 2007; Hinman and Davidson, 2007; Cretekos et al., 2008).

The Necessity for CRE Evolution in GRN Evolution

There are several cases of the nonequivalence of *Hox* or *Hox*-related orthologs or paralogs that have been linked to functional changes in proteins. These include the gain of a cofactor interaction motif and the loss of another in the transition of the Fushi Tarazu (Ftz) protein from a homeotic to a segmentation role in certain insects (Löhr and Pick, 2005); the evolution of the *bicoid* (*bcd*) gene from a *Hox3*-type ancestral gene and its novel role as a maternal anterior determinant (Stauber et al., 2002); the evolution of activity-regulating motifs within insect and crustacean Ultrabithorax proteins associated with limb repression (Grenier and Carroll, 2000; Ronshaugen et al., 2002; Galant and Carroll, 2002); and the functional evolution of the HoxA11 and HoxA13 proteins associated with the evolution of the eutherian female reproductive tract (Zhao and Potter, 2001; Lynch et al., 2004).

At first glance, these examples might appear to refute the predominance of CRE evolution. However, we can't focus solely on the functional changes in a given transcription factor in weighing the relative contribution of coding changes to the
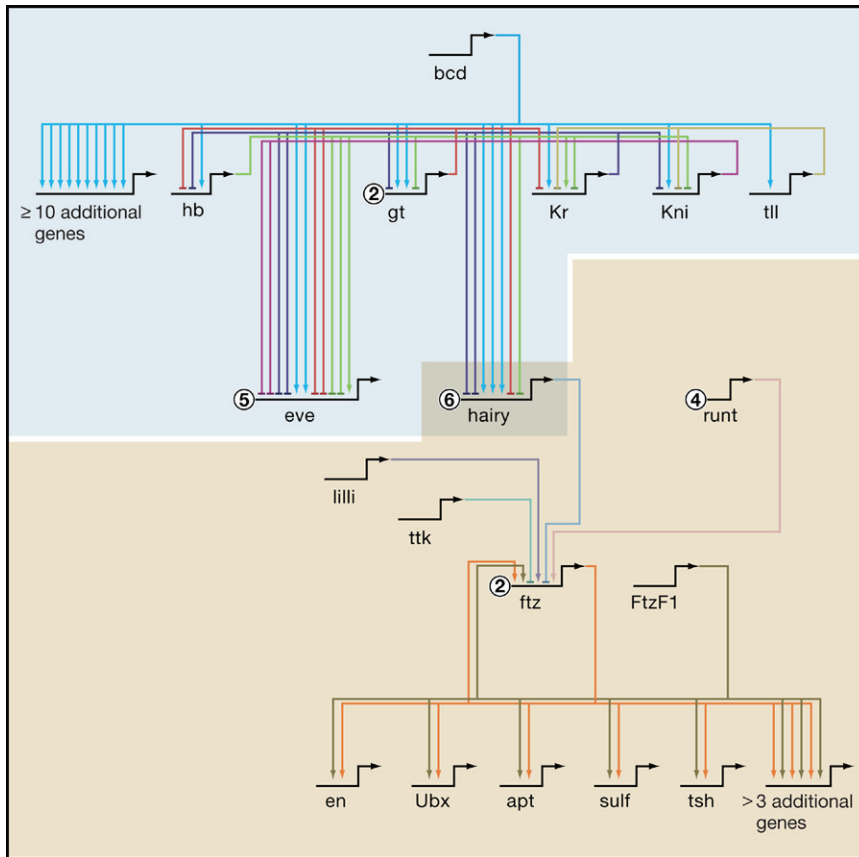
A partial view of the genetic regulatory network (GRN) controlling segmentation in *Drosophila*. Highlighted are neighborhoods surrounding two key loci: (1) the *bcd* gene encoding the Bicoid (bcd) transcription factor (light blue) and (2) the homeobox gene *Fushi Tarazu (ftz)* encoding the Ftz transcription factor (peach). Direct, validated linkages between transcription factors are shown as arrows for positive regulators and crosshatched lines for repressors. Not all inputs are shown. The number of distinct CREs of certain loci are shown in circles. The evolution of the novel Bcd protein (a *Hox3* paralog) was accompanied by the evolution of at least 21 downstream CREs with linkages to additional combinations of inputs. The evolution of the Ftz-FtzF1 interaction and pair-rule function was accompanied by the evolution of CREs in at least ten downstream CREs and two CREs of the *ftz* locus.

and an autoregulatory element that is regulated by Ftz-FtzF1 (Han et al., 1998) (Figure 3). Altogether, in just the immediate "neighborhood" of *ftz* (at the locus and downstream) in the GRN, a conservative estimate is that at least a dozen CREs have evolved in conjunction with Ftz acquiring its role as a pair-rule gene (Figure 3). Each CRE requires linkage to a minimum of 2–4 transcription factors. Thus, the evolution of one new cofactor interaction motif has been accompanied by at least two dozen new transcription factor-CRE linkages in the GRN.

The case of the *bicoid* gene, an upstream component of the same segmentation GRN, presents a similar story. The Bicoid protein possesses a distinct DNA-binding preference from that of its Hox relatives, but the gain of that activity is not meaningful without taking into consideration the downstream CREs upon which Bcd acts. Bcd exerts its effects during a brief window of development by regulating at least 21 target CREs that contain clusters of Bcd-binding sites (Ochoa-Espinosa et al., 2005) (Figure 3). These include both gap gene and pair-rule stripe CREs, which are in turn regulated by combinations of gap and pair-rule proteins (see Nasiadka et al., 2002 for review). At least 40 and perhaps more than 80 regulatory linkages have evolved in the immediate Bcd neighborhood of the segmentation GRN (Figure 3).

Similarly, the evolution of a novel Ultrabithorax activity-regulating motif was impotent without the concomitant and/or subsequent evolution of target CREs. In fact, the novel QA motif in Ultrabithorax is not required for limb repression in flies (Hittinger et al., 2005), and the repression of just one single limb target gene CRE required the evolution of six different transcription factor linkages (Gebelein et al., 2004).

A comparable picture of the large number of CRE linkages accompanying the evolution of new transcription factor interactions is emerging from the analysis of fungal GRNs (Tuch et al., 2008). Thus, the structure and logic of GRNs is such that

evolution of form. It is critical to appreciate that protein evolution is not sufficient to establish novel linkages within GRNs. CREs must evolve in downstream target genes, and often in upstream genes and at the locus as well, for GRN function to evolve. Understanding the relative contribution of new motifs or genes to GRNs requires examination of the numerous changes in GRNs that have accompanied such events and the timescales and taxonomic scales over which these events are detectable.

For example, the Ftz protein is part of an extensive and well-studied GRN that establishes the segmental organization of the *Drosophila* embryo. The novel Ftz protein motif enabled it to interact more strongly with the Ftz-F1 cofactor (Löhr and Pick, 2005), but loci that are regulated by the Ftz-FtzF1 complex possess specific binding sites for both transcription factors in their CREs. Recent genomic surveys have revealed at least ten direct target gene CREs that are regulated by Ftz-FtzF1 during a short phase of early *Drosophila* development (Figure 3) (Bowler et al., 2006; L. Pick, personal communication). Furthermore, in order for Ftz to act as a pair-rule regulator, its expression evolved to be regulated in a novel seven-striped pattern. This spatial pattern is generated through two separate CREs of the *ftz* locus: a "zebra" element" that is directly regulated by two other pair-rule proteins, the well-conserved and mosaically pleiotropic Hairy and Runt transcription factors, and at least two other proteins (see Vanderzwan-Butler et al., 2007 and references therein)

even when coding changes in regulatory proteins arise, the evolution of the GRNs to which they belong involves far more numerous changes in linkages to CREs.

The known cases of coding sequence evolution must also be weighed against the vast timescales and taxonomic distances involved, and in light of the prevalence of functional equivalence. For instance, the insect-onychophora divergence dates to more than 500 million years ago as does the divergence of the *Hoxa9-13* genes. Some divergence in activity over such timescales should not be so surprising. Rather, it is the frequent observation of functional equivalence that is the more remarkable.

In summary, if we picture a cladogram of diversifying animal lineages over any timescale and plot upon it the relative frequency of toolkit gene duplications, functional protein changes, and the gain, loss, and modification of regulatory linkages in GRNs via CREs, the category of CRE changes outnumbers the other events. CRE evolution is an essential ingredient of GRN evolution and hence is the predominant mechanism underlying the evolution of development and form.

Exceptions and Rules

Are there any exceptions to the necessity of CRE evolution and the insufficiency of protein evolution in morphological divergence? There are, and consideration of the exceptions has helped to define a general rule about the evolution of form.

Obvious and common differences among vertebrates include scale, plumage, or fur colors. Coding changes at several loci have been associated with differences in coloration. These include numerous instances of fur and plumage changes associated with evolution of the Melanocortin-1 receptor (MC1R; see Mundy, 2005 for review); the independent evolution of albinism in different cave fish populations due to coding mutations in the *Oca2* gene (Protas et al., 2006); and differences in human skin pigmentation associated with coding variation at Oca2 and the solute transporter gene *SLC24A5* (Lamason et al., 2005). How can these apparent exceptions be explained?

A potential answer is mosaic pleiotropism. Stern (2000) and I (Carroll, 2005) have argued that the most important determinant of what kinds of mutations are permissible under natural selection is the potential pleiotropic effects of individual mutations. Those mutations with greater pleiotropic effects are expected to have more deleterious effects on fitness and should thus be a less frequent source of variation and divergence than those mutations with fewer or no pleiotropic effects. For mosaically pleiotropic loci, functional mutations in coding regions are expected to affect multiple traits and are less likely to be tolerated, but for loci that are not mosaically pleiotropic, coding region mutations may be better tolerated.

All three pigmentation loci appear not to be mosaically pleiotropic. The roles of these proteins appear to be restricted to pigment-producing melanocytes and there is no evidence that their expression or function are governed independently in different populations of melanocytes (there is no evidence yet for multiple discrete CREs). Thus, coding mutations in these loci appear to have minimal, if any, effects on characters other than pigmentation. Furthermore, the changes in traits in these circumstances are not heterotopic, the spatial distributions of melanocytes are unaffected, and the mutations do not gener-

ate new patterns, rather they affect pigment production in all melanocytes and only alter the color of extant patterns.

By taking into account the lesser constraints upon minimally pleiotropic genes, a general "rule" has been formulated for the genetic path of morphological evolution that pertains to most toolkit loci. The rule is that when two conditions exist: (1) a protein plays multiple roles in development, and mutations in the coding sequence are known or likely to have pleiotropic effects and (2) the locus contains multiple CREs, then regulatory sequence evolution is the more likely mode of genetic and morphological change than is coding sequence evolution (Carroll, 2005). This rule has recently been shown to be predictive in a number of case studies of morphological divergence involving heterotopic changes in pigmentation and *cis*-regulatory changes in mosaically pleiotropic loci (Steiner et al., 2007; Miller et al., 2007; Jeong et al., 2008).

How Do CREs Evolve?

The growing evidence for the role of mutations in CREs in the evolution of GRNs and animal form shift the questions concerning the genetic path of morphological evolution from the reasons *why* CRE evolution predominates to the matter of *how* CREs actually evolve. The details are important. Regulatory sequences have different properties and are under different constraints than coding sequences (Wray, 2007). These are still early days for the analysis of metazoan CRE divergence, but recent studies have revealed several mechanisms through which linkages in GRNs may be gained, lost, or modified through the evolution of CREs.

(1) Co-option of New Transcription Factor Inputs by Mutations in Existing CREs. New regulatory linkages and new gene expression patterns have been shown to arise through mutations in existing CREs that create new binding sites for transcription factors (Wang and Chamberlin, 2002; Gompel et al., 2005).

(2) Co-option of Transposable Elements (TEs) as New CREs. One of the general questions concerning CRE evolution is how do new CREs arise? Do they evolve from scratch from naive DNA sequence, or do they evolve from pre-existing elements? It has recently been shown that there are thousands of TE insertions near developmentally regulated human genes and that the TE-derived sequences are under strong purifying selection (Lowe et al., 2007). Some of these TE sequences are functional CREs (Bejerano et al., 2006) that contain binding sites for and represent a substantial fraction of CREs regulated by a specific transcription factor (Wang et al., 2007). These findings indicate that the enormous numbers of TEs in many animal genomes are also potential sources of new functional (or nearly functional) CREs, an idea first put forward several decades ago (Britten and Davidson, 1971).

(3) Loss of Transcriptional Inputs in Existing CREs. A simple, and certainly frequent, means of rewiring GRNs is via the mutational loss of transcription factor binding sites in CREs. Such events have been documented, for example, in a Hox-regulated network (Jeong et al., 2006) and in GRNs for the T-brain transcription factor in echinoderms (Hinman and Davidson, 2007).

(4) Remodeling of CREs. In addition to the qualitative gain or loss of regulatory linkages, the strength of regulatory linkages can also be modified by changing the number, affinity, or topol-

ogy of transcription factor binding sites within a CRE. These changes can alter the output of a CRE such that the pattern (Zinzen et al., 2006) or level of gene expression is altered.

Several issues are as yet largely unexplored. Foremost among them are the population genetics of CRE variation and divergence. The properties of CREs present some unique considerations with respect to the frequency of potentially functional mutations. Most importantly, CREs present a wide target area for relevant mutations (Stern, 2000). There appears to be significant latitude as to where mutations or insertions may have regulatory effects. CRE function can evolve in the same direction by the gain of activator binding sites or the loss of repressor binding sites and vice versa. In addition, the spacing and orientation of sites affects CRE activity such that small insertions or deletions of sequence between transcription factor binding sites can have substantial functional effects.

Evolutionary Theory and Synthesis

What does evo-devo have to show for the past 25 years? How do its contributions to evolutionary thought measure up to those of other theories and disciplines? Is there a new synthesis afoot? I have presented the case for a genetic theory of morphological evolution that can be condensed into two statements: (1) form evolves largely by altering the expression of functionally conserved proteins; and (2) such changes largely occur through mutations in the *cis*-regulatory regions of mosaically pleiotropic developmental regulatory genes and of target genes within the vast regulatory networks they control.

It is important to underscore that this theory is a statement about tendencies not absolutes, asserting that most, not all, pertinent mutations are regulatory. It is also important to underscore that this is a specific theory about the evolution of animal form, and not a general statement about evolutionary adaptation (some authors have conflated the two, e.g., Hoekstra and Coyne, 2007). I don't believe that any comparable genetic theory of adaptation is forthcoming, as all types of molecular changes clearly contribute substantially to organismal adaptation (Carroll, 2006).

While restricted to form, nevertheless this theory has a synthetic character. It considers the nature of mutations and their effects on fitness, applies across taxonomic and time scales, and offers a causal explanation that links changes in genes to alterations in development and the evolution of form. It thus has important implications for and many fruitful areas of overlap with other disciplines such as population genetics and paleontology.

There can be no doubt that if the facts and insights of evo-devo were available to Huxley, embryology would have been a cornerstone of his Modern Synthesis, and so evo-devo is today a key element of a more complete, expanded evolutionary synthesis.

REFERENCES

Adachi, Y., Hauck, B., Clements, J., Kawauchi, H., Kurusu, M., Totani, Y., Kang, Y.Y., Eggert, T., Walldorf, U., Furukubo-Tokunaga, K., et al. (2003). Conserved *cis*-regulated modules mediate complex neural expression patterns of the eyeless gene in the *Drosophila* brain. Mech. Dev. *120*, 1113–1126.

Arron, J.R., Winslow, M.M., Polleri, A., Chang, C.P., Wu, H., Gao, X., Neilson, J.R., Chen, L., Heit, J.J., Kim, S.K., et al. (2006). NFAT dysregulation by increased dosage of *DSCR1* and *DYRK1A* on chromosome 21. Nature *441*, 595–600.

Averof, M., and Akam, M. (1995). *Hox* genes and the diversification of insect and crustacean body plans. Nature *376*, 420–423.

Bejerano, G., Lowe, C.B., Ahituv, N., King, B., Siepel, A., Salama, S.R., Rubin, E.M., Kent, W.J., and Haussler, D. (2006). A distal enhancer and an ultraconserved exon are derived from a novel retroposon. Nature *441*, 87–90.

Belting, H.G., Shashikant, C.S., and Ruddle, F.H. (1998). Modification of expression and *cis*-regulation of *Hoxc8* in the evolution of diverged axial morphology. Proc. Natl. Acad. Sci. USA *95*, 2355–2360.

Bolton, E.C., So, A.Y., Chaivorapol, C., Haqq, C.M., Li, H., and Yamamoto, K.R. (2007). Cell- and gene-specific regulation of primary target genes by the androgen receptor. Genes Dev. *21*, 2005–2017.

Bowler, T., Kosman, D., Licht, J.D., and Pick, L. (2006). Computational Identification of Ftz/Ftz-F1 downstream target genes. Dev. Biol. *299*, 78–90.

Britten, R.J., and Davidson, E.H. (1971). Repetitive and non-repetitive DNA sequences and a speculation on the origins of evolutionary novelty. Q. Rev. Biol. *46*, 111–138.

Burke, A.C., Nelson, C.E., Morgan, B.A., and Tabin, C. (1995). *Hox* genes and the evolution of vertebrate axial morphology. Development *121*, 333–346.

Carroll, S.B. (1995). Homeotic genes and the evolution of arthropods and chordates. Nature *376*, 479–485.

Carroll, S.B. (2005). Evolution at two levels: on genes and form. PLoS Biol. *3*, e245. 10.1371/journal.pbio.0030245.

Carroll, S.B. (2006). The Making of the Fittest (New York: W.W. Norton and Company).

Carroll, S.B., Gates, J., Keys, D.N., Paddock, S.W., Panganiban, G.E., Selegue, J.E., and Williams, J.A. (1994). Pattern formation and eyespot determination in butterfly wings. Science *265*, 109–114.

Carroll, S.B., Weatherbee, S.D., and Langeland, J.A. (1995). Homeotic genes and the regulation and evolution of insect wing number. Nature *375*, 58–61.

Carroll, S.B., Grenier, J.K., and Weatherbee, S.D. (2005). From DNA to Diversity: Molecular Genetics and the Evolution of Animal Design, Second Edition (Malden, MA: Blackwell Publishing).

Colosimo, P.F., Hosemann, K.E., Balabhadra, S., Villarreal, G., Jr., Dickson, M., Grimwood, J., Schmutz, J., Myers, R.M., Schulter, D., and Kingsley, D.M. (2005). Widespread parallel evolution in sticklebacks by repeated fixation of Ectodysplasin alleles. Science *307*, 1928–1933.

Cooper, G.M., Nickerson, D.A., and Eichler, E.E. (2007). Mutational and selective effects on copy-number variants in the human genome. Nat. Genet. *39* (*Suppl.*), S22–S29.

Cretekos, C.J., Wang, Y., Green, E.D., Program, N.C.S., Martin, J.F., Rasweiler J.J., 4th, and Behringer, R.R. (2008). Regulatory divergence modifies limb length between mammals. Genes Dev. *22*, 141–151.

Davidson, E.H. (2006). The Regulatory Genome: Gene Regulatory Networks in Development and Evolution (Oxford: Academic Press).

Duboule, D., and Dollé, P. (1989). The structural and functional organization of the murine *HOX* gene family resembles that of *Drosophila* homeotic genes. EMBO J. *8*, 1497–1505.

Fitzgerald, K., Wilkinson, H.A., and Greenwald, I. (1993). *glp-1* can substitute for *lin-12* in specifying cell fate decisions in *Caenorhabditis elegans*.

Development *119*, 1019–1027.

Fortini, M.E., and Rubin, G.M. (1990). Analysis of *cis*-acting requirements of the *Rh3* and *Rh4* genes reveals a bipartite organization to rhodopsin in *Drosophila melanogaster.* Genes Dev. *4*, 444–463.

Galant, R., and Carroll, S.B. (2002). Evolution of a transcriptional repression domain in an insect *Hox* protein. Nature *415*, 910–913.

Gebelein, B., McKay, D.J., and Mann, R.S. (2004). Direct integration of *Hox* and segmentation gene inputs during *Drosophila* development. Nature *431*, 653–659.

Gehring, W.J., and Ikeo, K. (1999). *Pax 6*: mastering eye morphogenesis and eye evolution. Trends Genet. *15*, 371–377.

Gelbart, W.M. (1989). The decapentaplegic gene: a TGF-beta homologue controlling pattern formation in *Drosophila.* Development *107* (*Suppl.*), 65–74.

Gilbert, S.F., Opitz, J.M., and Raff, R.A. (1996). Resynthesizing evolutionary and developmental biology. Dev. Biol. *173*, 357–372.

Gompel, N., Prud'homme, B., Wittkopp, P.J., Kassner, V.A., and Carroll, S.B. (2005). Chance caught on the wing: *cis*-regulatory evolution and the origin of pigment patterns in *Drosophila.* Nature *433*, 481–487.

Gould, S.J. (1994). Hen's Teeth and Horse's Toes (New York: W.W. Norton & Company).

Graham, A., Papalopulu, N., and Krumlauf, R. (1989). The murine and *Drosophila* homeobox gene complexes have common features of organization and expression. Cell *57*, 367–378.

Grenier, J.K., and Carroll, S.B. (2000). Functional evolution of the Ultrabithorax protein. Proc. Natl. Acad. Sci. USA *97*, 704–709.

Grenier, J.K., Garber, T.L., Warren, R., Whitington, P.M., and Carroll, S.B. (1997). Evolution of the entire arthropod *Hox* gene set predated the origin and radiation of the *onychophoran/arthropod* clade. Curr. Biol. *7*, 547–553.

Grens, A., Mason, E., Marsh, J.L., and Bode, H.R. (1995). Evolutionary conservation of a cell fate specification gene: the *Hydra achaete-scute* homolog has proneural activity in *Drosophila.* Development *121*, 4027–4035.

Hadorn, E. (1961). Developmental Genetics and Lethal Factors (London: Methuen & Co Ltd, John Wiley & Sons, Inc).

Halder, G., Callaerts, P., and Gehring, W.J. (1995). Induction of ectopic eyes by targeted expression of the eyeless gene in *Drosophila.* Science *267*, 1788–1792.

Hall, B.K. (2003). Evo-Devo: evolutionary development mechanisms. Int. J. Biol. Sci. *47*, 491–495.

Han, W., Yu, Y., Su, K., Kohanski, R.A., and Pick, L. (1998). A binding site for multiple transcriptional activators in the *fushi tarazu* proximal enhancer is essential for gene expression *in vivo*. Mol. Cell. Biol. *1998*, 3384–3394.

Hinman, V.F., and Davidson, E.H. (2007). Evolutionary plasticity of developmental gene regulatory network architecture. Proc. Natl. Acad. Sci. USA *104*, 19404–19409.

Hinman, V.F., Nguyen, A., and Davidson, E.H. (2007). Caught in the evolutionary act: precise *cis*-regulatory basis of difference in the organization of gene networks of sea stars and sea urchins. Dev. Biol. *312*, 584–595.

Hittinger, C.T., Stern, D.L., and Carroll, S.B. (2005). Pleiotropic functions of a conserved insect-specific *Hox* peptide motif. Development *132*, 5261–5270.

Hoegg, S., and Meyer, A. (2005). *Hox* clusters as models for vertebrate genome evolution. Trends Genet. *21*, 421–424.

Hoekstra, H.E., and Coyne, J.A. (2007). The locus of evolution: evo devo and the genetics of adaptation. Evolution *61*, 995–1016.

Jeong, S., Rokas, A., and Carroll, S.B. (2006). Regulation of body pigmentation by the Abdominal-B Hox protein and its gain and loss in *Drosophila* evolution. Cell *125*, 1387–1399.

Jeong, S., Rebeiz, M., Andolfatto, P., Werner, T., True, J., and Carroll, S.B. (2008). The evolution of gene regulation underlies a morphological difference between two *Drosophila* sister species. Cell *132*, 783–793.

Kimura, M., and Ohta, T. (1974). On some principles governing molecular evolution. Proc. Natl. Acad. Sci. USA *71*, 2848–2852.

King, M., and Wilson, A.C. (1975). Evolution at two levels in humans and chimpanzees. Science *188*, 107–116.

Kusserow, A., Pang, K., Sturm, C., Hrouda, M., Lentfer, J., Schmidt, H.A., Technau, U., van Haeseler, A., Hobmayer, B., Martindale, M.Q., et al. (2005). Unexpected complexity of the *Wnt* gene family in a sea anemone. Nature *433*, 156–160.

Lamason, R.L., Mohideen, M.A., Mest, J.R., Wong, A.C., Norton, H.L., Aros, M.C., Jurynec, M.J., Mao, X., Humphreville, V.R., Humbert, J.E., et al. (2005). SLC24A5, a putative cation exchanger, affects pigmentation in zebrafish and humans. Science *310*, 1782–1786.

Lawrence, P.A. (1984). Unpinioned opinions. Cell *36*, 570–571.

Lewis, E.B. (1978). A gene complex controlling segmentation in *Drosophila.* Nature *276*, 565–570.

Li, X., and Noll, M. (1994). Evolution of distinct developmental functions of three *Drosophila* genes by acquisition of different *cis*-regulated regions. Nature *367*, 83–87.

Löhr, U., and Pick, L. (2005). Cofactor-interaction motifs and the cooption of a homeotic *Hox* protein into the segmentation pathway of *Drosophila melanogaster.* Curr. Biol. *15*, 643–649.

Lowe, C.B., Bejerano, G., and Haussler, D. (2007). Thousands of human mobile element fragments undergo strong purifying selection near developmental genes. Proc. Natl. Acad. Sci. USA *104*, 8005–8010.

Lynch, V.J., Roth, J.J., Takahashi, K., Dunn, C.W., Nonaka, D.F., Stopper, G.F., and Wagner, G.P. (2004). Adaptive evolution of *HoxA-11* and *Hox-13* at the origin of the uterus in mammals. Proc. Biol. Sci. *271*, 2201–2207.

Malicki, J., Schughart, K., and McGinnis, W. (1990). Mouse *Hox*-2.2 specifies thoracic segmental identity in *Drosophila* embryos and larvae. Cell *63*, 961–967.

McGinnis, N., Kuziora, M.A., and McGinnis, W. (1990). Human *Hox*-4.2 and *Drosophila* deformed encode similar regulatory specificities in *Drosophila* embryos and larvae. Cell *63*, 969–976.

McGinnis, W., Garber, R.L., Wirz, J., Kuroiwa, A., and Gehring, W.J. (1984). A homologous protein-coding sequence in *Drosophila* homeotic genes and its conservation in other metazoans. Cell *37*, 403–408.

McGregor, A.P., Orgogozo, V., Delon, I., Zanet, J., Srinivasan, D.G., Payre, F., and Stern, D.L. (2007). Morphological evolution through multiple *cis*-regulatory mutations at a single gene. Nature *448*, 587–590.

McMahon, A.P., Ingham, P.W., and Tabin, C.J. (2003). Developmental roles and clinical significance of hedgehog signaling. Curr. Top. Dev. Biol. *53*, 1–114.

Miller, C.T., Beleza, S., Pollen, A.A., Schluter, D., Kittles, R.A., Shriver, M.D., and Kingsley, D.M. (2007). *Cis*-Regulatory changes in *Kit* ligand expression and parallel evolution of pigmentation in sticklebacks and humans. Cell *131*, 1179–1189.

Mundy, N.I. (2005). A window on the genetics of evolution: MC1R and plumage colouration in birds. Proc. Biol. Sci. *272*, 1633–1640.

Nasiadka, A., Dietrich, B.H., and Krause, H.M. (2002). Anterior-posterior patterning in the *Drosophila* embryo. In Advances in Developmental Biology and Biochemistry, Volume 12, M.L. Depamphilis, ed. (Amsterdam: Elsevier) pp. 156–204.

Nichols, S.A., Dirks, W., Pearse, J.S., and King, N. (2006). Early evolution of animal cell signaling and adhesion genes. Proc. Natl. Acad. Sci. USA *103*, 12451–12456.

Ochoa-Espinosa, A., Yucel, G., Kaplan, L., Pare, A., Pura, N., Oberstein, A., Papatsenko, D., and Small, S. (2005). The role of binding site cluster strength

in Bicoid-dependent patterning in *Drosophila*. Proc. Natl. Acad. Sci. USA *102*, 4960–4965.

Ohno, S. (1970). Evolution by Gene Duplication (Berlin: Springer-Verlag).

Olson, E.N. (2006). Gene regulatory networks in the evolution and development of the heart. Science *313*, 1922–1927.

Panganiban, G., Irvine, S.M., Lowe, C., Roehl, H., Corley, L.S., Sherbon, B., Grenier, J.K., Fallon, J.F., Kimble, J., Walker, M., et al. (1997). The origin and evolution of animal appendages. Proc. Natl. Acad. Sci. USA *94*, 5162–5166.

Papatsenko, D., Nazina, A., and Desplan, C. (2001). A conserved regulatory element present in all *Drosophila rhodopsin* genes mediates *Pax6* functions and participates in the fine-tuning of cell-specific expression. Mech. Dev. *101*, 143–153.

Protas, M.E., Hersey, C., Kochanek, D., Zhou, Y., Wilkens, H., Jeffery, W.R., Zon, L.I., Borowsky, R., and Tabin, C.J. (2006). Genetic analysis of cavefish reveals molecular convergence in the evolution of albinism. Nat. Genet. *38*, 107–111.

Prud'homme, B., Gompel, N., Rokas, A., Kassner, V.A., Williams, T.M., Yeh, S.D., True, J.R., and Carroll, S.B. (2006). Repeated morphological evolution through *cis*-regulatory changes in a pleiotropic gene. Nature *440*, 1001–1002.

Raff, R.A. (2000). Evo-devo: the evolution of a new discipline. Nat. Rev. Gen. *1*, 74–79.

Richardson, M. (2003). A naturalist's evo-devo. Nat. Genet. *34*, 351.

Ronshaugen, M., McGinnis, N., and McGinnis, W. (2002). *Hox* protein mutation and macroevolution of the insect body plan. Nature *415*, 914–917.

Sandmann, T., Grardot, C., Brehme, M., Tongprasit, W., Stole, V., and Furlong, E.E. (2007). A core transcriptional network for early mesoderm development in *Drosophila melanogaster*. Genes Dev. *21*, 436–449.

Schedl, A., Ross, A., Lee, M., Engelkamp, D., Rashbass, P., van Heyningen, V., and Hastie, N.D. (1996). Influence of *PAX6* gene dosage on development: overexpression causes severe eye abnormalities. Cell *86*, 71–82.

Scott, M.P., and Weiner, A.J. (1984). Structural relationships among genes that control development: sequence homology between the *Antennapedia*, *Ultrabithorax*, and *fushi tarazu* loci of *Drosophila*. Proc. Natl. Acad. Sci. USA *81*, 4115–4119.

Shapiro, M.D., Marks, M.E., Peichel, C.L., Blackman, B.K., Nereng, K.S., Jonsson, B., Schulter, D., and Kingsley, D.M. (2004). Genetic and developmental basis of evolutionary pelvic reduction in threespine sticklebacks. Nature *428*, 717–723.

Shapiro, M.D., Bell, M.A., and Kingsley, D.M. (2006). Parallel genetic origins of pelvic reduction in vertebrates. Proc. Natl. Acad. Sci. USA *103*, 13753–13758.

Shubin, N., Tabin, C.J., and Carroll, S.B. (1997). Fossils, genes and the evolution of animal limbs. Nature *388*, 639–648.

Snyder, M., Huang, X.Y., and Zhang, J.J. (2008). Identification of novel direct *STAT3* target genes for control of growth and differentiation. J. Biol. Chem. *283*, 3791–3798.

Stark, A., Lin, M.F., Kheradpour, P., Pedersen, J.S., Parts, L., Carlson, J.W., Crosby, M.A., Rasmussen, M.D., Roy, S., Deoras, A.N., et al. (2007). Discovery of functional elements in 12 *Drosophila* genomes using evolutionary signatures. Nature *450*, 219–232.

Stauber, M., Prell, A., and Schmidt-Ott, U. (2002). A single *Hox3* gene with composite *bicoid* and *zerknüllt* expression characteristics in non-*Cyclorrhaphan* flies. Proc. Natl. Acad. Sci. USA *99*, 274–279.

Steiner, C.C., Weber, J.N., and Hoekstra, H.E. (2007). Adaptive variation in beach mice produced by two interacting pigmentation genes. PLoS Biol. *5*, e219. 10.1371/journal.pbio.0050219.

Stent, G.S. (1984). From probability to molecular biology. Cell *36*, 567–570.

Stern, D.L. (1998). A role of Ultrabithorax in morphological differences between *Drosophila* species. Nature *396*, 463–466.

Stern, D.L. (2000). Evolutionary developmental biology and the problem of variation. Evolution *54*, 1079–1097.

Sucena, E., Delon, I., Jones, I., Payre, F., and Stern, D.L. (2003). Regulatory evolution of *shavenbaby/ovo* underlies multiple cases of morphological parallelism. Nature *424*, 935–938.

Tuch, B.B., Galgoczy, D.J., Hernday, A.D., Li, H., and Johnson, A.D. (2008). The evolution of combinatorial gene regulation in fungi. PLoS Biol. *6*, e38. 10.1371/journal.pbio.0060038.

Vanderzwan-Butler, C.J., Prazak, L.M., and Gergen, J.P. (2007). The HMG-box protein Lilliputian is required for *runt*-dependent activation of the pari-rule gene *fushi-tarazu*. Dev. Biol. *301*, 350–360.

Wagner, G.P. (2007). The developmental genetics of homology. Nat. Rev. Genet. *8*, 473–479.

Wang, T., Zeng, J., Lowe, C.B., Sellers, R.G., Salama, S.R., Yang, M., Burgess, S.M., Brachmann, R.K., and Haussler, D. (2007). Species-specific endogenous retroviruses shape the transcriptional network of the human tumor suppressor protein *p53*. Proc. Natl. Acad. Sci. USA *104*, 18613–18618.

Wang, X., and Chamberlin, H.M. (2002). Multiple regulatory changes contribute to the evolution of the *Caenorhabditis lin-48* ovo gene. Genes Dev. *16*, 2345–2349.

Warren, R.W., Nagy, L., Selegue, J., Gates, J., and Carroll, S.B. (1994). Evolution of homeotic gene regulation and function in flies and butterflies. Nature *372*, 458–461.

Wittkopp, P.J., Vaccaro, K., and Carroll, S.B. (2002). Evolution of *yellow* gene regulation and pigmentation in *Drosophila*. Curr. Biol. *12*, 1547–1556.

Wray, G.A. (2007). The evolutionary significance of *cis*-regulatory mutations. Nat. Rev. Genet. *8*, 206–216.

Zhao, Y., and Potter, S.S. (2001). Functional specificity of the *Hoxa13* homeobox. Development *128*, 3197–3207.

Zhao, Y., and Potter, S.S. (2002). Functional comparison of the *Hoxa 4*, *Hoxa 10*, and *Hoxa 11* homeoboxes. Dev. Biol. *244*, 21–36.

Zinzen, R.P., Cande, J., Ronshaugen, M., Papatsenko, D., and Levine, M. (2006). Evolution of the ventral midline in insect embryos. Dev. Cell *11*, 895–902.

Zuckerkandl, E. (1968). Hemoglobins, Haeckel's "Biogenic Law," and molecular aspects of development. In Structural chemistry and molecular biology, A. Rich and N. Davidson, eds. (San Francisco, CA: W.H. Freeman), pp. 256–274.

Zuckerkandl, E., and Pauling, L. (1965). Evolutionary divergence and convergence in proteins. In Evolving Genes and Proteins, V. Bryson and H. Vogel, eds. (New York: Academic Press), pp. 97–166.