Unraveling the Tangled Skein: The Evolution of Transcriptional Regulatory Networks in Development

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Abstract

The molecular and genetic basis for the evolution of anatomical diversity is a major question that has inspired evolutionary and developmental biologists for decades. Because morphology takes form during development, a true comprehension of how anatomical structures evolve requires an understanding of the evolutionary events that alter developmental genetic programs. Vast gene regulatory networks (GRNs) that connect transcription factors to their target regulatory sequences control gene expression in time and space and therefore determine the tissue-specific genetic programs that shape morphological structures. In recent years, many new examples have greatly advanced our understanding of the genetic alterations that modify GRNs to generate newly evolved morphologies. Here, we review several aspects of GRN evolution, including their deep preservation, their mechanisms of alteration, and how they originate to generate novel developmental programs.

1. WHAT ARE GENE REGULATORY NETWORKS, AND HOW DO THEY CONTROL DEVELOPMENT?

Internal node: a gene within a GRN that can control the expression of other genes; these genes often encode transcription factors and members of signaling pathways

Terminal node: a gene that is activated at the terminus of a GRN; these genes typically have no role in regulating gene expression and instead influence final cell type differentiation

Although trees (38) and landscapes (160) are widely used metaphors in evolutionary biology, networks serve as a driving analogy to conceptualize developmental genetics (39). Some of the first uses of the network metaphor were introduced to help visualize the context-dependent regulation of enzyme expression in bacteria (60). In higher eukaryotic systems, perhaps the clearest articulation was a seminal review by Britten & Davidson (23). At their core, gene regulatory networks (GRNs) drive development through differential gene expression: Despite identical DNA in every cell, only a subset of the genes in a genome are activated at any one time or place during development. This is exemplified by the specific expression of transcription factors in broad zones of the *Drosophila* embryo (142) and the corresponding absence or disruption of these regions in mutant embryos that lack these factors (108). Such transcription factors coordinate the transcription of many genes: As combinations of particular factors are expressed in restricted patterns, downstream genes are consequently activated, which eventually leads to the activation or repression of genes that are directly responsible for conferring cellular behavior, such as growth, migration, shape, adhesion, and elasticity. These subordinate genes of the network are generally thought to represent the network's periphery—they are usually unable to further influence transcriptional events in the network. Therefore, although nearly every transcription factor is an internal node, they ultimately regulate the activity of terminal nodes (**Figure 1**).

1.1. Each Gene in a Network Is Connected Through Its *Cis*-Regulatory Transcriptional Control Sequences

To participate in a particular GRN, a gene must contain a *cis*-regulatory apparatus that binds transcription factors of the network to activate expression in the zone of interest. In general, the recruitment of a single transcription factor is insufficient to initiate the downstream expression of target genes: Activator synergy dictates a requirement for more than one activating transcription

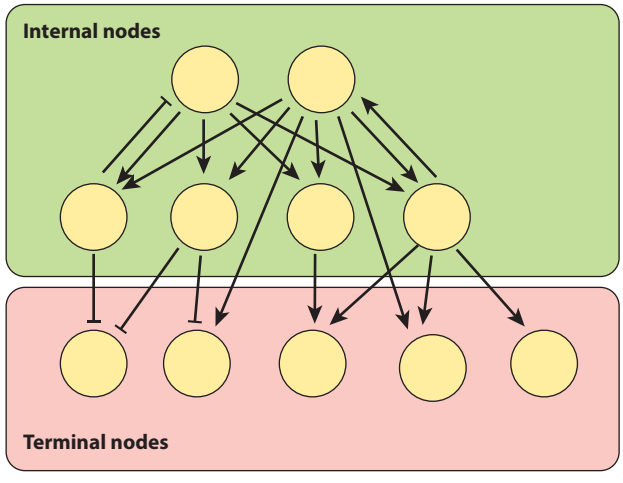


Figure 1

Abstract gene regulatory network depicting internal and terminal nodes. Each yellow circle is a gene that either activates (*arrow*) or represses (*horizontal bar*) its targets. Internal nodes (*green shaded area*) affect the expression of other nodes, whereas terminal nodes (*pink shaded area*) do not.

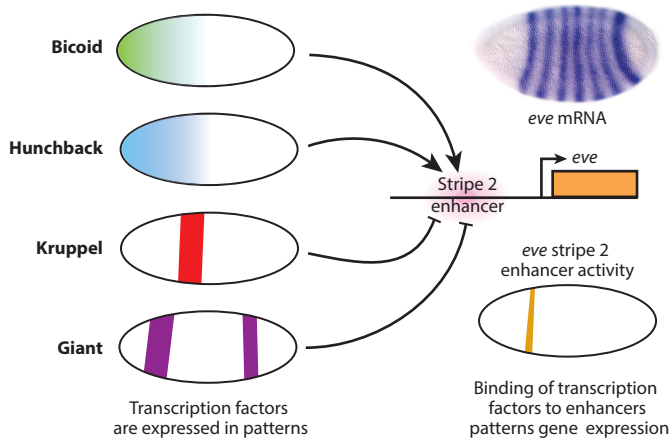


Figure 2

Spatially patterned transcription factors act at enhancers to sculpt downstream gene expression. (*Left*) The gap genes encode transcription factors that define broad zones of the early *Drosophila* embryo, in part through their regulation of the *even-skipped* (*eve*) gene regulatory region. (*Right*) The *eve* gene is expressed in seven stripes in the early *Drosophila* embryo. The stripe 2 enhancer of *eve* is regulated by maternal and gap gene transcription factors, which activate and repress the enhancer to generate a thin stripe of expression at a precise register along the embryo.

factor to bind. These factors bind to a special type of transcriptional regulatory sequence, variably referred to as enhancers, *cis*-regulatory modules, or *cis*-regulatory elements. Enhancers are generally 200–1,000 base pairs in length and contain multiple short stretches of DNA to which particular transcription factors specifically dock. Multiple protein-DNA complexes comprise a combinatorial logic of activators and repressors that sculpt a limited pattern of expression (84). The pattern of gene expression is dictated by the spatial distribution of the transcription factors that bind the enhancer. Hence, deciding as a committee, multiple transcription factors present in a particular cell type cooperate to activate target enhancer sequences and drive expression of subordinate genes of the network.

One of the first examples of an enhancer that was demonstrated to integrate multiple positive- and negative-acting transcription factor binding sites regulates the expression of the *even-skipped* (*eve*) gene in the early *Drosophila* embryo (140) (**Figure 2**). This gene encodes a transcription factor expressed in seven stripes that functions to set up segmented zones of the embryo (92). Each of several distinct modules of regulatory DNA in the noncoding sequences that surround the *eve* coding unit drives one or two of the seven stripes of *eve* expression (65). The stripe 2 enhancer contains binding sites for spatially restricted activators and repressors that combinatorially function to generate a discrete pattern of activation in the second stripe (**Figure 2**). Because individual modules require the docking of multiple activating transcription factors in close proximity on the DNA, they are thought to act relatively independently, or exhibit modularity: The action of one enhancer is generally assumed to not interfere with the function of other, nonoverlapping modules.

Enhancer: a regulatory DNA sequence to which transcription factors bind to regulate the expression of a target gene; also called a *cis*-regulatory module or *cis*-regulatory element

Combinatorial logic: the control of gene expression by the binding of different transcription factors that each provide an input, determining the precise dynamics of gene expression

Modularity: a property of regulatory sequences that allows them to be altered independently, resulting in few pleiotropic consequences for other activities of a given gene

1.2. Connecting Nodes with Genetic and Biochemical Tests of Network Hierarchy

Several experimental methods have been used to establish the hierarchical relationships between genes that form our picture of GRNs (**Table 1**). These range from simple tests of genetic epistasis

Table 1 Methods for inferring regulatory connections within gene regulatory networks

Assay	Description of method	Direct or indirect linkage
Genetic test	A target gene's expression is measured in a background for which a regulating gene's function has been manipulated (using an existing genetic mutant or by CRISPR/Cas9 knockout, or knockdown by RNA interference or morpholino treatment).	Indirect
DNA-binding assay	A small portion of the regulatory region is shown to bind the factor in a gel-shift assay.	Direct
Chromatin immunoprecipitation	Whether a region of interest binds a factor in vivo is determined by measuring the recovery of the bound region upon formaldehyde fixation and antibody pull-down for a particular DNA-binding factor.	Direct
Reporter assay mutation	A mutation is introduced that disrupts the binding of a factor in a reporter assay, which then shows the predicted effect on reporter gene expression (e.g., increase or decrease, expansion or contraction).	Direct

(9) to the direct biochemical confirmation that a transcription factor directly binds to a given segment of regulatory DNA (140). A classical approach is to monitor the expression of a potential downstream gene in an animal that is a mutant or otherwise deficient in another gene in the network (30, 125). Such genetic tests can establish that one gene lies upstream of another in the GRN but cannot distinguish whether the interaction is direct (i.e., the factor in question binds to the enhancer of the downstream gene) or indirect (e.g., the factor activates one or more downstream genes that ultimately regulate the gene in question). To determine whether a relationship in a network is direct, one must demonstrate the direct binding of that factor to the regulatory sequence responsible for the given pattern of gene expression. This can be done through a gel-shift assay in vitro or through in vivo approaches such as chromatin immunoprecipitation. The demonstration of direct binding is usually complemented by a functional reporter assay, in which the identified binding site is mutated and shown to affect the tissue-specific activation of the regulatory sequence. However, although these tests are the gold standard for drawing direct connections in gene regulatory hierarchies of model organisms (e.g., fly, mouse, nematode, or sea urchin), such tests are labor intensive and are not always possible, especially in an evolutionary context that involves nonmodel species.

Gel-shift assay: a technique that measures the mobility of a DNA probe to infer whether a transcription factor binds a particular sequence; also called an electrophoretic mobility shift assay

Chromatin immunoprecipitation: an in vivo assay to determine transcription factor occupancy on regulatory DNA

Reporter assay: an in vivo test in which an enhancer is fused to a reporter gene such as GFP or lacZ, the expression of which is monitored when transgenically introduced

***Trans*-regulatory landscape:** the content of regulatory genes present in a cell, cell type, or territory of the embryo

1.3. Integration of Multiple Gene Regulatory Network Tiers Can Generate Complex and Dynamic Logical Outputs

Gene expression, especially during early development, can be highly dynamic: Transcripts can appear, recede, and reappear in different territories, all within a few cell divisions. Enhancer modules must therefore interpret a *trans*-regulatory landscape that itself may be highly dynamic in order to provide a complex temporal and spatial output of expression. One example of how such precise execution of dynamic expression is encoded in regulatory DNA derives from the sea urchin *foxa* gene locus (43). This gene is first expressed within the domain that is fated to become endomesoderm (i.e., both endoderm and nonskeletogenic mesoderm), but within a 2-h window, expression is precisely extinguished from future nonskeletogenic mesoderm cells while persisting exclusively in the outer tier of cells fated to become endoderm (116) (**Figure 3*a***).

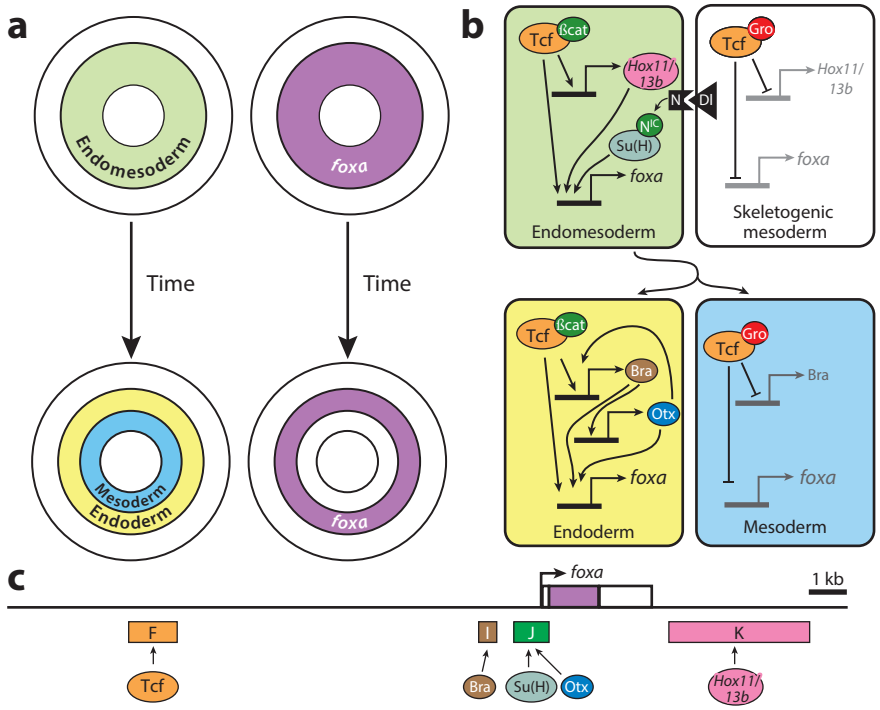


Figure 3

Dynamic regulation of *foxa* by multiple enhancer modules. (*a*) Two vegetal views of a sea urchin embryo. (*Left*) The endomesoderm segregates into the endoderm and mesoderm during development. (*Right*) *foxa* mRNA is first expressed throughout the endomesoderm and is subsequently maintained in the endoderm. (*b*) The gene regulatory network architecture that controls *foxa* expression during endoderm development. A Notch-Delta signal from the skeletogenic mesoderm activates the Su(H) transcription factor. (*c*) Schematic of the *foxa* locus, detailing the relative positions of four modules that integrate diverse inputs in the endomesoderm network to control dynamic *foxa* expression. Abbreviations: βcat, β-catenin; Bra, Brachyury; Dl, Delta; Gro, Groucho; N, Notch; N^{IC}, Notch intracellular domain; Su(H), Suppressor of Hairless.

Shortly thereafter, expression levels in this territory markedly increase (43). This change in gene expression is necessary for the segregation of endoderm and mesoderm in the sea urchin larva, and Foxa is a critical driver of the GRN for endoderm formation (116, 136). Genetic tests that perturbed endomesoderm formation using morpholinos delineated the GRN that regulates *foxa* expression (115, 136) (**Figure 3*b***). The Tcf-β-catenin complex, the Notch effector Suppressor of Hairless [Su(H)], and the homeobox factor Hox11/13b initiate *foxa* expression throughout the endomesoderm lineage. Just one cell division later, signals from the adjacent micromeres function to clear nuclear β-catenin from only the innermost tier of endomesoderm cells (133). This results in the recruitment of the Groucho repressor to Tcf, which inactivates *foxa* in the inner cells, which subsequently adopt a nonskeletogenic mesoderm fate. Thus, the TCF binding sites that first functioned to activate *foxa* expression then repress *foxa* expression as nuclear β-catenin is cleared.

The *cis*-regulatory apparatus that implements this dynamic pattern of expression is spread among multiple modules distributed across 20 kb surrounding the *foxa* locus. Employing a bacterial artificial chromosome vector system to untangle the regulatory control region of this gene, de-Leon & Davidson (43) characterized four modules termed F, I, J, and K in the upstream and downstream regions of *foxa* (**Figure 3*c***). Although each module executes a particular function, they

control expression by interacting with the basal promoter in combination; thus, module F responds to Tcf, module J to Su(H), and module K to Hox11/13b. As Tcf associates with the Groucho corepressor, module F then acts as a toggle switch, repressing *foxa*. In the presumptive endoderm, module K then also receives an input from Otx, while module I is activated by Brachyury. These new inputs function to ramp up *foxa* expression. This system illustrates how, beyond encoding multiple tissue-specific domains of expression, modules can cooperate to control highly dynamic expression in a single territory.

Pleiotropic: having multiple, independent functions; regulatory genes frequently affect the expression of multiple other genes at multiple times and places and are therefore especially pleiotropic

1.4. Casting Developmental Evolution in Terms of Network Biology

The intricately organized nature of GRNs naturally inspires the question of how their complexity evolves: How do new phenotypes manifest through the alteration of networks? How do new networks arise? Indeed, because development is proximately controlled at the upper tiers by the spatial restriction of regulatory genes such as those encoding transcription factors and signaling pathway components, this framework lends itself naturally to the study of evolutionary development. Every mutation occurs somewhere in a GRN: If a change occurs in a transcription factor that patterns the early embryo, then this change modifies the upper tiers of a network. One would expect the resulting change in the *trans*-regulatory landscape to alter many subordinate genes in the GRN, potentially causing drastic phenotypic differences. If the change resides in a gene that has no downstream regulatory connections (a terminal node), such as actin or myosin, then one would expect that although there may be phenotypic consequences, the effects on the network would be minimal compared with those of internal nodes. Because the early phases of development depend so critically on the establishment of specific expression patterns, evolutionary change often occurs in the *cis*-regulatory sequences that generate patterned gene expression. Such mutations change the expression of the gene in question, having relatively few pleiotropic effects (i.e., phenotypic consequences in other tissues). In the examples that follow, we discuss the deep evolutionary conservation of network components and circuits (Section 2), alterations at many levels of existing networks (Section 3), and the origins of networks that generate completely new developmental structures (Section 4).

2. DEEP CONSERVATION OF NETWORK COMPONENTS AND INDIVIDUAL LINKAGES

Ever since the first comparisons of gene coding sequences across animal phyla, it has been appreciated that many of the genes that pattern development are highly conserved. The small number of developmental signaling pathways that generate differences in cell fate are, for the most part, conserved across the animal kingdom (57, 117). A core set of conserved transcription factors that sit at the termini of these signaling pathways make signal-regulated decisions at enhancers (13). The major classes of transcription factor are highly conserved across animal phyla, appearing in even the most primitive of animals (141). Hence, the "developmental toolkit" (29) that builds the internal nodes of GRNs is deeply conserved in sequence. As discussed below, these factors also often tend to maintain conserved roles during development.

2.1. Conserved Roles of Developmental Patterning Genes in Evolutionarily Distant Homologous Processes

One of the first, and perhaps still best-characterized, examples illustrating the unexpected conservation of developmental gene function derives from studies of the Hox gene complex. The

Hox transcription factors pattern body axial identity and are organized into gene clusters in a very similar way across bilaterians, such that this gene complex was once described as the Rosetta Stone of animal pattern formation (139). However, many other examples of deeply conserved functional roles exist for genes of the developmental toolkit. The transcription factor Distal-less is expressed within the appendages of flies, mice, and everything in between (111). A conserved role for the Pax6/Eyeless transcription factor was characterized in vertebrate and invertebrate eye formation (124). The brain-patterning proneural transcription factors related to *Drosophila* Achaete-Scute are expressed in and required for neuronal development across metazoa (72). These parallels have several implications for the evolution of GRNs. First, they suggest that when a key role for a transcription factor evolves, it is often preserved. Second, they suggest that, because transcription factors maintain conserved roles in GRNs, their DNA-binding specificities tend to evolve slowly or (as discussed in Section 3) maintain ancestral functionality. Finally, the deep conservation of collections of transcription factors in certain developmental processes suggests that connections between regulatory factors often remain conserved within GRNs.

A large number of experiments in the 1980s and 1990s established that many of the genes of the developmental toolkit are functionally equivalent and have been minimally altered during evolution. Tests of functional equivalence generally involve the misexpression of a toolkit gene in a distantly related species, followed by a comparison with the phenotype generated by the endogenous species' gene. Surprisingly, many of these tests were successful. A key example of this was the expression of the mouse *Pax6* gene in *Drosophila*, which can generate ectopic eyes resembling those induced by its *Drosophila* homolog *eyeless* (63). Although many examples of striking functional equivalence exist for developmental toolkit transcription factors (61, 62, 64, 93, 153), exceptions to this rule nevertheless exist (33, 53, 132). In Section 3, we discuss some established cases of transcription factor protein evolution.

Network topology: the shape or structure of a GRN defined by the regulatory connections, or edges, between nodes

Subcircuit: a set of several genes, or nodes, within a GRN that have regulatory interconnections; such genes may execute some particular, discrete function

Positive feedback loop: a subcircuit in which the genes positively and mutually regulate the expression of other genes in the subcircuit

2.2. The Deep Preservation of Gene Regulatory Network Subcircuits

Given that many of the transcription factors of the developmental toolkit have maintained similar DNA-binding specificities and functional roles in patterning organs or specifying particular cell types, one key question is to what degree this conservation extends to the underlying GRN. If protein-DNA linkages are conserved either at the DNA sequence or functional level, one could infer which portions of the GRN have been maintained and which parts have changed (1). These studies must first determine whether orthologous genes are connected to each other by identical arrangements of regulatory linkages in distant taxa and, if so, elucidate which developmental processes they control.

The GRN for endomesoderm specification in the sea urchin is one of the most extensively studied networks, naturally forming a platform for comparison of GRN conservation and divergence (115, 116). Early studies of the evolutionary preservation of GRNs determined whether a similar network topology could be detected in a distantly related echinoderm, the sea star (67, 97). Sea urchins and sea stars are representatives of two classes of the phylum Echinodermata, which diverged approximately 400 million years ago (118). This comparison revealed that certain portions of the network (subcircuits) are indeed quite similar, in spite of the long span of time over which such linkages must have been maintained (67, 97). For instance, a subcircuit composed of four orthologous transcription factors (Gata4/5/6, Otx, Blimp1, and Foxa) is highly similar in both taxa, as is another subcircuit of four factors that direct mesoderm specification (97). Strikingly, in both instances, the orthologous transcription factors were engaged in a positive feedback loop—e.g., Otx regulates the expression of *gata4/5/6*, and Gata4/5/6 in turn regulates the expression of *otx*. The positive feedback loop is a commonly observed network motif present in many biological

and nonbiological systems that maintains activity at a node for an extended period (5). During development, one can envision that such positive regulatory feedback acts to reinforce gene expression states, particularly if the inputs are early and transient. Thus, the conserved function may be to initiate and then maintain the specification of cell types.

Phylogenetic footprinting: the use of sequence conservation to identify important binding sites within regulatory DNA

Studies of the GRN for the heart show an even deeper ancestry of conservation in network topology (37). The NK-2 transcription factor—encoded by a gene named *tinman* in *Drosophila*, because of the lack of a heart in these mutants (19)—shares a key position in the heart specification network of *Drosophila* and vertebrates (78, 85). In addition, the MEF2 transcription factor has an ancestral role in the specification of muscle cell identity, a role that was employed during the evolution of the heart (17). The vertebrate GATA4/5 and the homologous *Drosophila* Pannier factors also serve similar roles in these networks (6, 76, 105). These striking similarities in heart and endomesoderm GRNs, which arose 500 and 700 million years ago, respectively, suggest that once these connections were established, their loss would be developmentally catastrophic, and thus highly unlikely. Davidson (40) had dubbed these subcircuits kernels, by analogy with the part of a computer's operating system that is so crucial to the machine's function that it operates in a protected memory space.

2.3. Deep Conservation of Individual Transcription Factor Binding Site Sequences

Recent studies have shown that, above and beyond a deeply preserved topology of certain GRNs, the connections between network components sometimes remain unchanged at the level of the DNA sequences that bind individual transcription factors. Phylogenetic footprinting is frequently employed to find important parts of regulatory elements (147). If a sequence serves an important transcription factor binding function, then the logic follows that it may have withstood the erosion of evolutionary change, and thus its sequence would be conserved. This property of regulatory sequences has been successfully exploited to find many functional binding sites in regulatory DNA (126, 162). Over the past ten years, striking examples of sequence conservation that have delved progressively deeper into metazoan history have emerged.

Shortly after the generation of vertebrate genome sequences, a number of studies documented the existence of ultraconserved regions—stretches of DNA 100–200 base pairs in length that share an extraordinary level of sequence conservation across the genomes of rodents and humans, sometimes extending all the way to sequences in the genomes of fish (15, 114). In vivo reporter assays confirmed that many of these conserved elements represent transcriptional regulatory sequences (151), and yet, paradoxically, mice engineered to lack some of these segments lacked obvious phenotypic consequences, possibly because of functional redundancy in the tested genomic regions (3). Ultraconservation has proven to be a quite useful characteristic for inferring important evolutionary events (see Section 3.4). However, some studies have shown that regulatory sequence conservation among fish and humans may be only the tip of the iceberg.

For a small handful of cases, deep regulatory sequence conservation has been observed at the root of the bilaterian lineage (conserved between protostomes and deuterostomes). Two examples of this phenomenon have been documented for network connections in the nervous system GRN between the Notch signaling pathway, effectors of the Hairy/Enhancer of Split (HES) transcription factor family, and the proneural transcription factors that specify neuronal fates (127, 129). First, a binding site matching a stringent ten-nucleotide definition for the Notch-regulated HES factors is present upstream of both human and fly proneural genes of both the *atonal* and *achaete-scute* families (129). The invariant presence of this site close to the promoter and conservation among alignable species allowed the authors to infer that this binding site most likely arose in the

ancestral bilaterian proneural gene. Furthermore, a different study showed that the HES genes that target the proneural factors are themselves involved in a deeply conserved circuit consisting of a rigidly spaced pair of binding sites for the Notch-regulated Su(H) transcription factor, combined with sites for the proneural factors present in both protostome and deuterostome lineages (127). Second, perhaps the most striking example of deep conservation of individual transcription factor binding sites is a conserved noncoding region present in the human *SoxB2* gene locus, which is recognizable in the cnidarian *Nematostella vectensis* (134). This remarkably conserved sequence was tested for regulatory activity in an impressive array of transgenic organisms (zebrafish, sea urchin, and *Drosophila*), and the common theme observed for each organism was that this DNA sequence drives reporter expression in the nervous system.

Hence, from the gross anatomy of network topology, to sequence conservation, and even to individual binding sites encoded in the DNA, many aspects of GRN network architecture are deeply conserved. As discussed in the next section, the identification of these relationships provides a powerful platform for inferring major events during GRN evolution, particularly in the human lineage.

Microevolutionary change: evolutionary change occurring within a population or between very recently diverged species

Macroevolutionary change: evolutionary change occurring at or beyond the species level

Intraspecific variation: variation within a population of individuals of the same species

3. THE EVOLUTIONARY MODIFICATION OF GENE REGULATORY NETWORKS

Although the deep conservation of the developmental toolkit genes and their connections within metazoan networks highlight a remarkable degree of stability in some aspects of GRN architecture, these examples leave open the question of how GRNs change to generate diverse phenotypes. Here, we illustrate examples in which different parts of a GRN's structure have been modified. These range from changes to the expression of terminal nodes to the alteration of high-level factors in the network—changing the patterns of expression as well as modifying DNA-binding specificity itself. We draw from examples that include microevolutionary changes within populations or between closely related species as well as macroevolutionary divergence that occurred in the distant past.

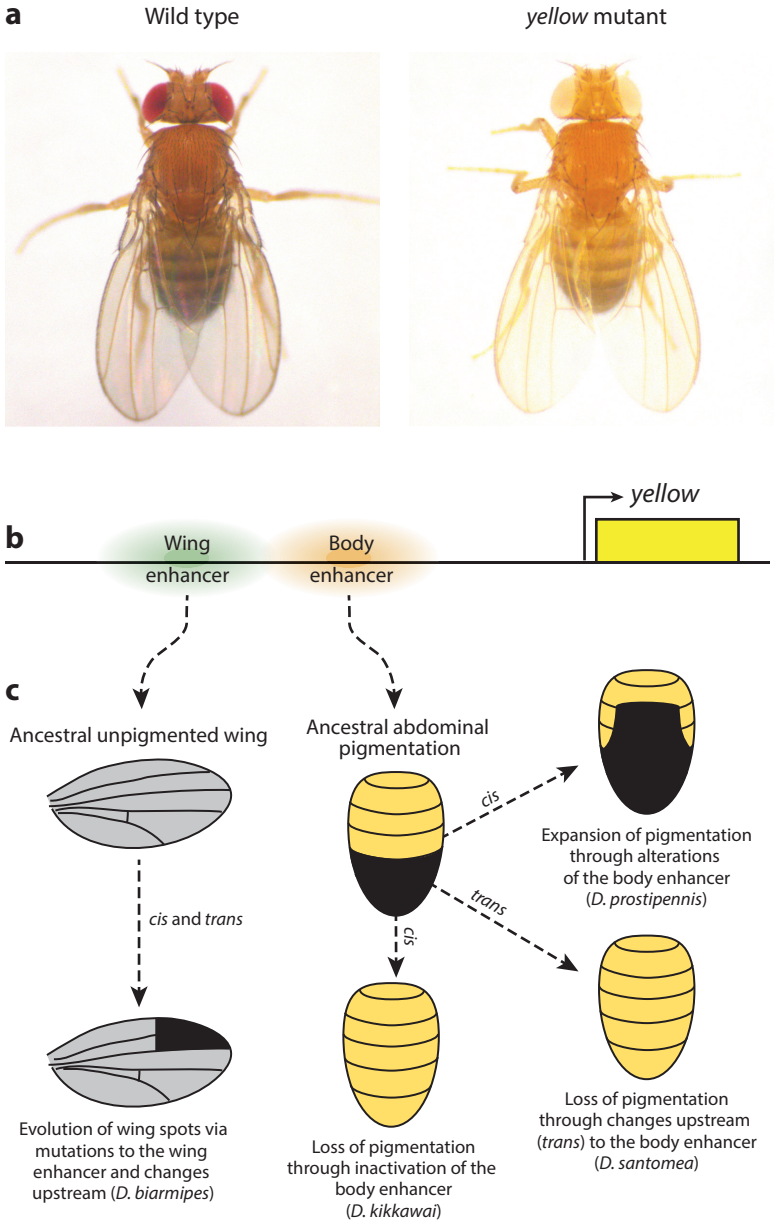
3.1. Modification of *Cis*-Regulatory Elements at the Periphery of Gene Regulatory Networks

One model system in which GRN evolution has been extensively studied is centered on the rapidly evolving pigment patterns that adorn the body parts of *Drosophila* species (156). With more than 1,500 species in the genus *Drosophila*, there is ample variation in pigment patterning, including multiple examples of intraspecific variation (79, 107, 121, 148) and several cases of variation between closely related species that can be crossed for genetic mapping (28, 87, 158, 161). Many of the genes in the melanin synthesis pathway were discovered as some of the first genetic markers that occurred as spontaneous mutants in the fly room of Thomas Hunt Morgan approximately a century ago (21, 22, 106). Several of these enzymes are patterned in expression, correlating with the spatial patterns of pigments they are required to produce (70, 152, 157).

Perhaps the most well-studied gene of the *Drosophila* pigmentation system is *yellow*, named for its coloration phenotype (106). Mutants for this gene exhibit a body-wide lightening of pigmentation on their abdomens, bristles, and wings (**Figure 4*a***). Wing and abdomen expression of *yellow* is encoded by two separable enhancers in the gene's upstream regulatory region (58) (**Figure 4*b***). This regulatory architecture also highlights where mutations underlying evolutionary shifts in *yellow* expression have occurred. Differences in gene expression may be caused by mutations to the regulatory apparatus of the gene in question (a *cis* change) or by alterations in upstream regulators (a *trans* change) (155). A simple way to distinguish between *cis* and *trans*

mechanisms is to compare the activities of the given regulatory regions in a reporter assay within a common genetic background (130). If differences in expression are recapitulated by the enhancer sequences, then one can conclude that changes arose in *cis*. By contrast, *trans*-regulatory changes would result in an absent or poor recapitulation of the species-specific expression pattern.

Tests of the wing and abdominal enhancers of *yellow* have revealed a range of evolutionary shifts in these regulatory sequences (**Figure 4*c***). For example, mutations inactivating the abdominal enhancer were established in *Drosophila kikkawai* (71), whereas its inactivity in *Drosophila santomea*



was attributed to changes upstream (70, 71). These changes are not limited to instances of trait loss; the expansion of abdominal pigmentation was associated with *cis* changes in the abdominal enhancer of *yellow* (110). A stunning case of trait gain was observed in the wing spots of *Drosophila biarmipes*, which have been attributed to a complex assembly of changes in *cis* (59) combined with a *trans* change that altered the expression of the transcription factor Distal-less (7). It is important to note that modification of *yellow* expression alone cannot account for any of these phenotypes (for example, in the absence of *yellow* gene function, strong patterns of pigmentation still form). However, these cases have provided key examples illustrating *cis*-regulatory mutations contributing to a morphological phenotype.

3.2. Altering the *Trans*-Regulatory Landscape by Changes in *Cis* Regulation

The operational definition of a terminal node would dictate that changes to node function (or expression) result in a minimally modified GRN that differs only at the state of the gene that was changed. When higher tiers in the GRN are modified, ripples of change are predicted to emanate throughout the network. Although this is likely to be quite pleiotropic, many examples of this phenomenon exist. Perhaps the simplest type of change is the modification to the expression pattern or timing of a transcription factor or signaling pathway ligand. Examples include the loss of armor plates and spines in sticklebacks (31, 35, 137), the loss of trichomes in *Drosophila* larvae (51, 52, 99, 145), and the diversification of the wing spots mentioned in Section 3.1 (7).

One of the most striking cases illustrating changes in the spatial patterning of the *trans* landscape comes from work in butterfly mimicry. The coloration patterns of closely related species of butterfly display an enormous degree of variation, exemplified by the elaborate differences between *Heliconius* butterflies (**Figure 5**). Early genetic studies in several *Heliconius* species suggested that a relatively small number of loci play a substantial role in wing pattern variation (73, 150). In different regions, distinct species will converge on similar pigmentation patterns to share the burden of educating predators. Through a remarkable set of mapping analyses, genomics, and developmental work, we now know that much of this phenotypic variation is caused by differences in expression that have evolved from changes in the extensive *cis*-regulatory apparatus of the same *trans* regulators present in the butterfly wing. Of three regions that control the bulk of color-pattern variation, the *D* and *Sd* loci have been the most extensively studied.

Mapping of the *D* locus revealed a common genomic position for the gene that causes drastic differences in red color patterns in subspecies of *Heliconius melpomene* and *Heliconius erato* (14, 112).

Figure 4

Examples of alterations to a terminal gene regulatory network node, the pigment-promoting enzyme gene *yellow*. (*a*) Pigmentation phenotypes of a wild-type *Drosophila melanogaster* male (*left*), showing dark pigmentation of the posterior abdomen, bristles, and wings, and a male mutant for the *yellow* gene (*right*), showing a lightening of the abdomen, bristles, and wings. The imaged mutant also has a mutated *white* gene, causing a loss of red eye color. (*b*) Schematic of the *yellow* gene, highlighting the relative positions of wing and body enhancers, which have been altered in *cis* and *trans* during the evolution of pigment phenotypes. (*c*) Modifications to the wing and body elements of the *yellow* gene. (*Left*) A combination of mutations to the wing enhancer along with upstream factors that regulate its activity underlies the transition from an ancestrally uniformly gray wing (*top*) to the dark spot of pigmentation present in *Drosophila biarmipes* (*bottom*). (*Right*) Three independent alterations of the body enhancer accompanied the expansion and loss of ancestral male-specific pigmentation. In *Drosophila kikkawai*, the enhancer was inactivated by *cis* changes, whereas a similar loss in *Drosophila santomea* occurred entirely through changes in upstream factors. The expansion of pigmentation in *Drosophila prostipennis* was traced to changes within the body element.

Figure 5

An example of Müllerian mimicry among *Heliconius* species. *Heliconius erato* (*top*) and *Heliconius melpomene* (*bottom*) have evolved comimic phenotypes to share the burden of educating predators about their unpalatability.

An elegant microarray analysis in which pigmented portions of red-morph wings were compared with those of unpigmented subspecies revealed that within the *D* locus genomic region, the *optix* gene was uniquely upregulated in red-colored regions (131). Refined mapping data using naturally occurring hybrids also implicated *optix* as the causal locus for controlling red pigment patterns (131). An expansive comparison of *optix* expression among different *H. erato* morphs and other diverse red morphs uncovered a remarkable correlation between its mRNA pattern and phenotype. Notwithstanding ~25 million years of divergence, sequences of the *optix* coding region differed only at synonymous sites, eliminating the possibility that amino acid coding sequence variants might contribute to this phenotype. Taken together with association studies in hybrid zones that genetically pinpointed variation to *optix*, these results indicate that *cis*-regulatory changes at *optix* account for a large diversity of patterns of *optix* expression (131), downstream of which dozens of genes involved in synthesizing red pigment patterns have been altered in expression (66). A survey of Optix expression across the nymphalid clade that contains *Heliconius* revealed a complex and interesting history of its association with diverse types of modified wing scales, differing in color and morphology (95).

The *Sd* locus controls the size, position, and shape of forewing color bands by changing the black regions of the wings, thus acting like a shutter to control where color appears. Mapping of the *Sd* locus implicated the *WntA* gene, which encodes a secreted signaling molecule (96). Clever experiments using chemical treatment to modify Wnt signaling further supported a role for *WntA* in the *Sd* phenotype. As with *optix*, the relevant variation appears to lie within the *cis*-regulatory DNA of *WntA*, and the expression of *WntA* is correlated with the black domains in various *Heliconius* butterflies (96). Remarkably, this role of *WntA* is not restricted to *Heliconius* butterflies, as it also appears to be responsible for similar variation in black patterns in distantly related butterflies (54). How the diverse genetic programs driven by *optix* and *WntA* originated remains an open and quite interesting question.

The Hox loci provide another important example of evolutionary changes to the expression of highly conserved genes. As mentioned above, Hox genes control anterior-posterior patterning throughout the bilateria, and the overall structure and function of the Hox gene complex has been well conserved through more than 500 million years of evolution. Nevertheless, changes in Hox gene expression do underlie several examples of morphological evolution. Some of these involve later functions of Hox genes in fine tuning specific morphological features associated with particular body regions. For example, at the microevolutionary scale, changes in the levels

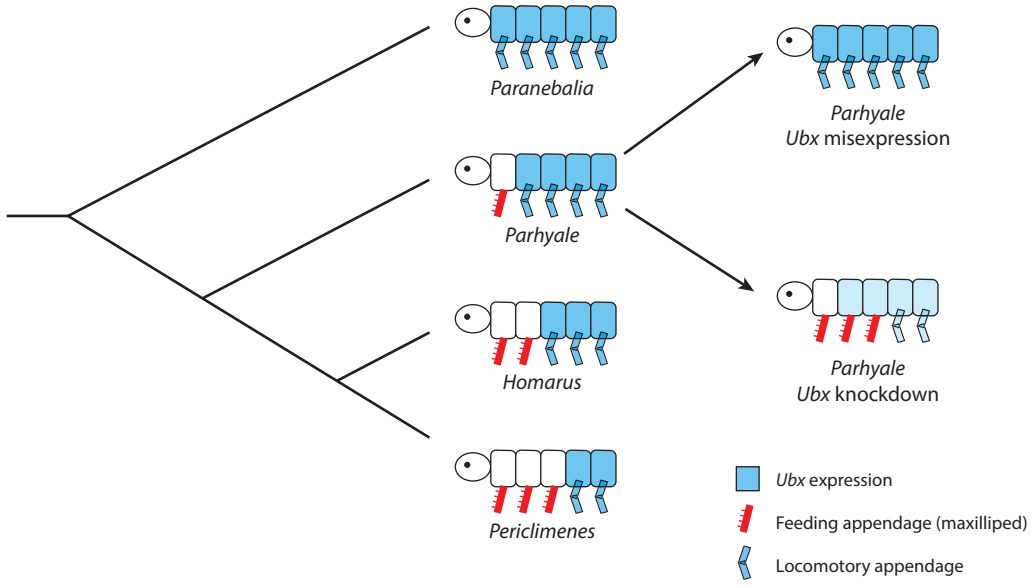


Figure 6

Diversification of crustacean limb patterns caused by changes in *Ubx* expression. (*Left*) The anterior boundary of *Ubx* expression (*blue*) correlates with the morphological transition between thoracic locomotory appendages (*blue*) and maxilliped feeding appendages (*red*) in several species of crustaceans. (*Right*) RNA interference experiments in *Parhyale* that reduce *Ubx* function transform locomotory appendages into maxillipeds. By contrast, *Ubx* misexpression results in the conversion of maxillipeds into locomotory appendages.

of *Ultrabithorax* (*Ubx*) expression underlie differences in the pattern of hairs on the T2 legs of closely related *Drosophila* species (143). On the other hand, large shifts in the initial expression domains of Hox genes correlate with macroevolutionary changes in body plans. For example, in crustaceans, the anterior border of *Ubx* expression can reside at the first, second, third, or fourth thoracic segment, depending on the species (**Figure 6**). This expression correlates with the morphological boundary between feeding and locomotory appendages in these animals (8). Further evidence for the functional significance of these shifts in expression was provided by gain- and loss-of-function studies in the crustacean genus *Parhyale*, which showed that *Ubx* regulates the distinction between feeding and locomotory appendage identity (86, 113) (**Figure 6**). For example, knockdown of *Parhyale Ubx* transforms posterior locomotory appendages to the anterior feeding appendage identity (86). Although it is not known for certain that these changes are due to mutations in the *cis*-regulatory elements of crustacean *Ubx*, these results illustrate how even the most highly conserved toolkit genes can play a key role in the evolution of drastic morphological features.

3.3. Diversification of Transcription Factor Function at the Protein-Coding Level

As discussed above, there is an abundance of direct evidence demonstrating that a significant source of evolutionary variation in GRNs resides within enhancers. However, a growing number of examples indicate how transcription factor–coding regions can also acquire mutations that modify their downstream GRNs. Theoretical support for the predominance of regulatory DNA mutations derives from the inherent modularity exhibited by enhancers, which is thought to limit

pleiotropy (40, 159). However, transcription factor proteins can also be modular, and therefore, it has been argued, they can also evolve in ways that reduce pleiotropic effects (91). The modulation of protein function arises from the interaction domains that can independently execute their functions. For instance, transcription factors may have multiple DNA-binding domains as well as multiple protein-protein interaction domains. The DNA-binding domains function to direct the protein to specific binding sites in enhancers to affect the transcription of the target gene. The protein interaction domains bind other proteins to modify the mode of gene regulation. For instance, these domains can interact with obligate dominant repressors such as Groucho, cofactors that enhance binding and transcriptional control, or proteins that modify the factor posttranslationally to regulate its location, stability, or activity.

Position weight matrix (PWM): a matrix of a transcription factor's binding specificity based on multiple sequences known to bind the factor

There are several ways that proteins can evolve changes in the use of these modules to circumvent pleiotropy so that only a subset of their function is affected. The first source of variation comes from gene rearrangement processes that can shuffle the domains so that orthologs in different taxa have variations in the types and numbers of domains. Newly acquired domains can then direct new GRN functions while leaving the original functions intact. For example, a comprehensive analysis of chordate genomes revealed extensive domain shuffling (74). In particular, the authors found that some transcription factors acquired new transactivation domains and that these appeared to be linked to the evolution of vertebrate-specific characters (74).

Newly acquired domains may also execute their functions in only some spatiotemporal contexts if they facilitate binding of an interacting partner that is restricted in expression. Hence, not only can proteins evolve new domains that act independently of other domains, but these new functions may apply to only a subset of the protein's function in time and space. One particularly interesting example derives from studies of HoxA11 during the development and evolution of the placenta in mammals (20, 91). HoxA11 represses the expression of pregnancy-related genes. During the evolution of placental mammals, HoxA11 acquired the ability to bind the FoxO1a transcription factor. In endometrial stromal cells, which express FoxO1a, HoxA11 interacts to activate rather than repress pregnancy-related genes.

Another mechanism through which protein domains may act in only some fraction of their spatiotemporal environments is through context-dependent splicing—i.e., alternative splicing events that occur in particular times and places in the embryo. A study comparing genome-wide splicing events in three primates showed that these events differ between lineages and sexes, thereby demonstrating that splicing regulation is context specific and changing rapidly (18). The functional consequences of context-specific splicing are highlighted by the *doublesex* gene in *Drosophila*, which is differently spliced in males and females, and these splice forms differently regulate downstream genes (56, 154) to direct sex-specific GRNs.

Another, entirely unexpected source of modularity has also been uncovered. Recent technological advances have permitted a sensitive and high-throughput assessment of the binding preferences of transcription factors (16). In these experiments, proteins are allowed to bind to DNA microarrays of all possible 8-, 10-, or 12-mer sequences. The motifs can be collated into a position weight matrix (PWM), which represents the preference for each base at each position within the motif. This analysis has revealed that many transcription factors not only have a primary preference PWM but also exhibit preferences for an additional, secondary PWM that cannot be explained by the primary PWM owing to nucleotide interdependencies (11). Interestingly, when paralogous transcription factors are analyzed, their primary PWMs are quite similar, but they often differ in their preference for a secondary PWM (11).

A critical finding from an evolutionary perspective is that orthologous transcription factors can also evolve altered preferences for secondary PWMs in different species. Cheatle Jarvela et al. (32) showed that orthologous Tbox transcription factors from mouse, sea urchin, and sea star

share a highly similar primary PWM but have derived different preferences for a secondary, low-affinity motif. This demonstrates that a single DNA-binding domain can evolve preferences for alternative DNA-binding motifs without altering their preferences for their ancestral primary motif. The next challenge is to understand the types of GRNs that are controlled by enhancers using low-affinity, secondary motifs. This will reveal the types of developmental processes that are under the regulation of these more pliable aspects of the protein's function.

3.4. Inferring Evolutionary Events Within Human and Primate Gene Regulatory Networks

The question of how developmental networks evolve naturally leads to the topic of how these studies apply to human evolution: What were the genetic changes accounting for humans' unparalleled mental capabilities, manual dexterity, and bipedalism? Indeed, early comparisons of human protein sequences to those of primates motivated the idea that changes in gene regulation might underlie much of human uniqueness (75). This sentiment was only further bolstered by the realization that the human genome contains less than half the number of genes than was widely anticipated (12). Although it is technically challenging to experimentally validate changes within human developmental GRNs, several studies have mounted convincing cases for a number of changes in the human lineage. Because of experimental limitations, these studies have required the clever use of computational techniques and experimentation in nonprimate models.

One line of inquiry depends on the identification of highly conserved sequences among vertebrates that have been drastically altered in humans (119, 122). Because these accelerated regions show extreme alterations of highly conserved noncoding elements, it stands to reason that they would represent functional changes in the human lineage, possibly affecting gene regulation. The first documented example, *human accelerated region 1* (*HAR1*), revealed a significantly altered noncoding RNA gene whose expression was quite similar in both human and macaque brain (120). Although this represents a change in RNA secondary structure rather than gene expression, *HAR1* is expressed in a region of the human neocortex that has been greatly elaborated along the human lineage. A second example, *human accelerated conserved noncoding sequence 1* (*HACNS1*), mapped to a noncoding region that drives expression in the vertebrate limb (123). Comparisons of regulatory sequence function in mouse reporter assays revealed that the human version of this regulatory sequence was much more active than any other primate reporter, extending into the thumb. Hence, one of the genes adjacent to this sequence may have evolved an expanded domain of expression in the thumb, possibly contributing to human adaptations of the hand.

Although it is difficult to verify the phenotypic consequences of accelerated evolution of *HAR1* and *HACNS1*, studies examining the loss of conserved noncoding regions may provide more straightforward examples of changes underlying human uniqueness. McLean et al. (100) systematically identified 510 human-specific deletions that occurred within conserved noncoding elements (hCONDELs). From this set, the authors provided compelling evidence for two examples, showing that the chimp and mouse sequences deleted in the human lineage are active regulatory elements. One of these hCONDELs occurred within the androgen receptor, deleting an enhancer active in penile spines. The androgen receptor confers responsiveness to circulating androgens, and this hormone signaling was previously known to be required for penile spine formation. The deletion of this spine enhancer correlates very well with the absence of these androgen-dependent structures in humans compared with those in many other mammals.

The other hCONDEL identified a change that may have contributed to increased brain size (100). *GADD45G* is a tumor suppressor gene known to inhibit cell cycle progression and induce apoptosis. The loss of this enhancer, active in the subventricular zone, may have led to decreased

GADD45G expression, relaxing negative regulation on the growth of the human neocortex. Although such findings seem more clear cut than the examples listed above for accelerated regions, there are many open questions concerning these case studies. For example, several conserved noncoding sequences have been deleted in the mouse with no apparent phenotypic consequence (3). Thus, without data concerning the expression status of these genes during human development, these changes may have been silent because of redundancy with other elements in these genes.

Several studies of human-specific changes have focused instead on tissues for which gene expression, activity, and even chromatin state can be measured, such as skin fibroblasts (138) or circulating blood (149). One prime example compared expression of glucose transporters in the human and chimp brain and muscle tissue, revealing a potential reduction in muscle expression of *SLC2A4* that was accompanied by an increase in brain activity of *SLC2A1* in the human lineage (49). These expression changes may have tipped the energy balance between muscle and brain tissues to support the more energetically demanding needs of the human brain. Both genes have an excess of substitutions within their 5′ untranslated regions, a signature suggesting positive selection in a potential regulatory region.

To disentangle correlation from causation for any study of GRN evolution, the ultimate form of evidence should establish the sufficiency of implicated genetic changes to generate the evolved phenotype in question. This type of evidence is generally available only for a small number of model organisms that have tools for transgenesis or homologous recombination. This type of experimental support is especially problematic for studies of human GRN evolution, in that the closest model organism in which such studies are possible may be so different that the results of placing a humanized allele will be meaningless. In a vanishingly small number of cases, such experiments have been attempted. Of these, perhaps the greatest amount of information is available for the *FOXP2* gene.

FOXP2 encodes a forkhead domain transcription factor, and mutations in this gene were identified in individuals with speech disorders that stem from a lack of coordinated face and mouth movements (81). Stimulated by the finding that variation in *FOXP2* could affect one's ability to speak, a comparative sequencing analysis of humans and several primates revealed an excess of changes to its amino acid sequence in the human lineage, a sign of positive selection (48). Intriguingly, these changes predated the divergence of Neanderthals from modern humans (80). To the extent that differences can be distinguished, *FOXP2*/*Foxp2* expression in human and mouse brains is quite similar (82).

To test the potential functional implications of the amino acid changes in *FOXP2*, Enard et al. (47) engineered a humanized mouse by introducing two human-specific amino acids into the endogenous mouse gene. These changes resulted in increases in dendrite length, depressions in dopamine levels, and an alteration in ultrasonic vocalization in humanized mice compared with controls. Recently, behavioral assays have shown that, compared with wild-type individuals, the humanized *FOXP2* mice have a slight increase in their ability to learn how to use spatial cues to obtain rewards and are more dynamic in transitioning between reward systems (135). The GRN-level effects of the amino acid changes at *FOXP2* have been more recalcitrant to functional characterization. Transcriptome profiling approaches revealed very few major changes in gene expression, which could be due to these amino acid changes affecting FOXP2 function in just a few cell types (135).

Although the amino acid changes at *FOXP2* may be the most detailed example of GRN-level change in the human lineage, the applicability of the mouse model to human evolution must be interpreted with caution. Some evidence suggests that *FOXP2* is subject to long-distance regulation with elements perhaps 3 Mb away from the promoter (2). Furthermore, *cis*-regulatory mutations along the human lineage have been implicated in changes to *FOXP2* expression as well (94). *FOXP2*

is currently the best available example, but it likely represents one of a vast number of changes involved in human brain evolution. Thousands of genes exhibit differential brain expression among primates (10), no small number of which may contribute to humans' exquisite mental capabilities. As more information and increasingly inventive ways to probe the phenotypic consequences of mutations specific to the human lineage arise, the role of *FOXP2* relative to these other changes will become apparent.

4. NETWORK ORIGINATION

Perhaps the biggest challenge in understanding how networks evolve is the problem of understanding novelty: How do completely new structures that have no obvious precursor originate? In particular, this is a complex problem because of the difficulty in elucidating evolutionary events in the distant past, where the most obvious of novelties such as appendages, eyes, or the turtle's shell arose.

4.1. Co-Option of the Appendage-Patterning Network During Beetle Horn Evolution

One system that has gained much traction is the origination of beetle horns, which have long been appreciated as a novelty of particular adaptive significance. Beetles are one of the most successful groups of species, and thousands of types of beetles bear horns that project from various parts of the body (46). Used by males as weaponry in the struggle to find mates, this sexually selected structure can increase the chances of a male's success in obtaining and preserving mates (101). At the molecular level, this structure is a perfect exemplar of GRN co-option—the redeployment of an existing network to evolve a new structure.

Across the arthropods, the genes that participate in appendage formation are highly conserved (77, 111). The ectopic deployment of top-level regulators of this network is sufficient to induce additional appendage outgrowths (26), suggesting that, when deployed at the right place and time, this program could be co-opted to generate novel structures. Analysis of the appendage battery in developing horns has demonstrated the expression of several genes in this network during development (102, 104). Indeed, the *Distal-less* gene, associated broadly with appendages in many species of protostomes and deuterostomes (111), is expressed in both pronotal and head horns (102). The deployment of these genes in the novel context of the horn suggests that a conserved downstream program for proximo-distal-axis formation is activated in the developing horn, causing proximo-distal-axis-responsive enhancers to be redeployed in the novel setting.

An emerging theme of this research is the realization that not all genes of the network appear to contribute to the morphology of the horn. Whereas the proximo-distal-axis genes *Distal-less* and *homothorax* exhibit altered horn morphology upon RNA interference (RNAi) knockdown, the *dachshund* gene failed to show a horn phenotype, even though RNAi conditions generated phenotypes in other tissues (103). This finding suggests that perhaps not all co-opted portions of the network make developmental contributions to the morphological novelty.

4.2. Co-Option of the Echinoderm Larval Skeleton

Although many examples of GRN co-option have been uncovered among groups of arthropods, this phenomenon has also been implicated in other developmental systems, supporting the concept that network co-option may be a universal evolutionary phenomenon. One such example is the echinoderm larval skeleton. Sea urchin larvae develop long, biomineralized skeletal rods that

provide the characteristic pyramidal shape to their plutei larvae. Representatives of outgroup species of echinoderm (e.g., crinoids and sea stars) do not make larval skeletons. However, all echinoderms make a skeleton during their adult stages, as this is a defining feature of the phylum. Gao & Davidson (55) investigated whether the GRN for the larval skeleton could have been co-opted from an ancestral GRN that directed the formation of adult skeletogenesis. They showed that many transcription factors, signaling molecules, and terminally acting genes involved in the formation of the biomineral are indeed coexpressed during both the development of the larval skeleton and the formation of the adult skeleton. Because the GRN for the development of the larval skeleton has been characterized in extraordinary detail (109), the authors were able to show that genes with overlapping expression patterns map to regions of the GRN acting downstream of initial specification processes. Importantly, some orthologs of these genes were also coexpressed in the developing skeleton of the adult sea star, suggesting that this adult skeletogenic GRN is basal. Recently, McCauley et al. (98) showed that the sea cucumber, which is a sister taxon to the sea urchin, also expresses some (but not all) of these genes during the formation of its much reduced larval skeleton. This suggests that, upon co-option, there has been further remodeling of the GRN to modify skeletal morphology.

Transposable element (TE): a self-replicating element that can insert itself into the genome

4.3. Where Do Nodes in the Network Come From?

Two major themes unify the examples of novelties described above. First, new morphological structures often arise from new and unique expression patterns of ancestral genes rather than from the evolution of new genes. Second, the ancestral genes associated with such novelties are often transcription factors and signaling pathway ligands that are predicted to alter many subordinate genes in their respective networks. In some of these cases, the network that has been co-opted can be reasonably inferred (e.g., beetle horn co-option of the proximo-distal-axis specification network), but in many cases the downstream network's identity remains unknown. Hence, these two properties also raise two emerging questions about how the GRNs underlying these novel morphological structures evolve: How do genes evolve novel expression patterns that place them into new networks? And how does network co-option proceed at the level of individual genes and the genome? Recent advances provide a rich picture of the molecular mechanisms by which new expression patterns evolve.

The question of how expression patterns arise can be rephrased in terms of how enhancers originate or evolve new activities at the molecular level. Several mechanisms have been hypothesized to underlie the evolution of new enhancer activities. The simplest is *trans*-regulatory modification, in which a preexisting enhancer is activated in a new location because its upstream regulators have been deployed in this new tissue. The studies of beetle horn and sea urchin larval skeleton co-option imply that dozens if not hundreds of preexisting enhancers are activated in the novel site of GRN deployment.

In addition to *trans*-regulatory co-options, multiple mechanisms by which novel enhancer activities have arisen through changes in *cis* have been documented (**Figure 7**). These mechanisms typically differ by the type of ancestral information that was present before the evolution of the novelty. For example, many studies have implicated transposable elements (TEs) in the origination of enhancer sequences (50). As much as two-thirds of the human genome is composed of repetitive sequences derived from transposons (41). Indeed, many characterized enhancers overlap TEs and display striking patterns of conservation within the TE sequence, suggesting that purifying selection has acted on these regions (15, 88). During the evolution of pregnancy, hundreds of genes expressed in the endometrial stromal cells became associated with nearby transposon insertions that alter stromal cell gene activity (90).

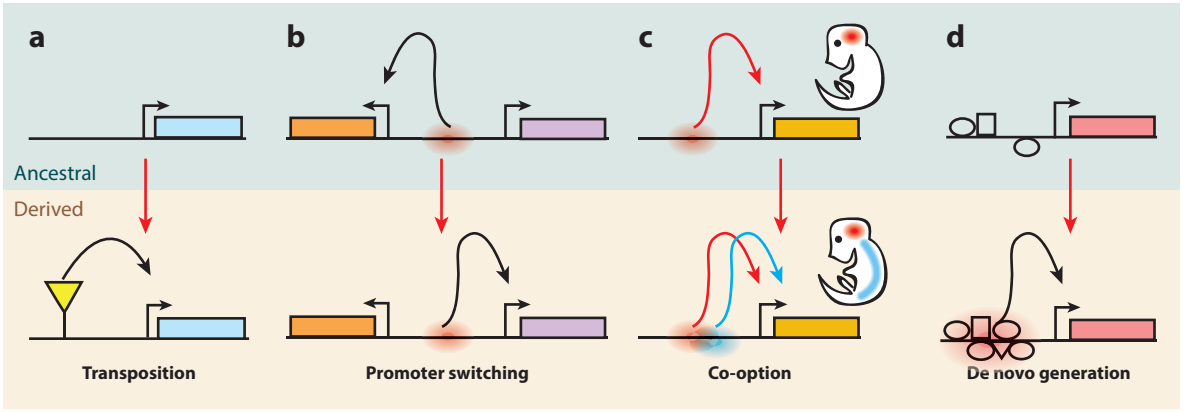


Figure 7

Models for enhancer origination. (*a*) Transposition: The insertion of a transposable element that contains functional enhancer sequences may activate the adjacent gene. (*b*) Promoter switching: A preexisting enhancer may evolve changes in the control of enhancer-promoter specificity that cause it to interact with an alternate promoter. (*c*) Co-option: The alteration of a preexisting enhancer may co-opt it to drive a new expression pattern in an additional tissue. (*d*) De novo generation: The evolution of new binding sites in otherwise nonfunctional DNA may result in the de novo generation of an enhancer sequence. Adapted from Reference 128.

However, of the many cases of TE exaptation, very few are subject to functional analysis in vivo (42). In one detailed example, the neuronal enhancers of the vertebrate *Pomc* gene derived from TEs but clearly replaced an ancestral enhancer, which was likely lost after the TE insertion gained enhancer activity (44). Furthermore, current examples of TE exaptation that have been linked to phenotypes are associated with increased abundance, rather with than the evolution of expression that was previously absent. In the case of *teosinte branched 1*, a combination of two transposons increased expression in domesticated maize (144). Similarly, pesticide resistance in *Drosophila* is associated with increased expression of *Cyp6G* in the gut caused by a transposon insertion that is sufficient to drive gut expression (34). Thus, although it is entirely possible that many genes have gained new expression patterns via TE exaptation, examples that show clearly novel expression domains are currently needed

Another potential mechanism driving the evolution of new expression patterns is to simply alter enhancer-promoter specificity (**Figure 7*b***). Enhancers are subject to tight control of their associations with promoters, often exclusively interacting with only a single promoter. Insulator elements restrict enhancers from interacting with the wrong promoter (24), whereas promoter tethering elements facilitate interactions with specific target promoters (4, 25). A famous example that illustrates the exquisite control of promoter specificity is the limb enhancer of *Sonic hedgehog*, which is located ~1 Mb upstream of its promoter, in the intron of a gene two genes away (83). Evolution of enhancer-promoter control sequences may cause an adjacent gene to adopt the activity of the nearby enhancer. In practice, chromosomal rearrangement has been documented to drive such promoter-switching events. In the *tinman* gene complex of the beetle *Tribolium castaneum*, a chromosomal inversion caused a change in an enhancer's position relative to an insulator sequence, which allowed the *C15* gene to adopt an enhancer from the *ladybird* gene (27). The *Rose-comb* locus of the domesticated chicken offers a phenotypically relevant example of chromosome rearrangement leading to novel expression (69). In this case, the *MNR2* gene, encoding a homeodomain transcription factor, is located 3 kb away from the edge of a large-scale (7 Mb) chromosomal inversion in animals bearing the *Rose-comb* phenotype. Placement of this transcription factor in this new regulatory environment is associated with the novel embryonic

Exaptation: the reuse or co-option of an ancestral gene, feature, or trait for a new usage (for example, a transposable element that evolves a function as an enhancer)

expression of *MNR2* in the developing comb mesenchyme, suggesting that the inversion placed *MNR2* in close proximity to a comb mesenchyme enhancer (69).

Shadow enhancer: a second enhancer that encodes a specificity that is identical to another enhancer of the same gene but generally farther away from the promoter

One potential pitfall of the promoter-switching mechanism is pleiotropy—interaction with a new promoter may alter the ancestral pattern of the original target gene of the enhancer. However, the wide prevalence of shadow enhancers, in which a single expression pattern is encoded by multiple separable enhancer elements (68), may alleviate the potentially pleiotropic effects of such promoter-switching events. In this case, the loss of one enhancer caused by an alteration of enhancer-promoter specificity or chromosomal rearrangement may have little effect on the overall expression of the ancestral gene.

A potentially powerful mechanism to generate novel expression domains is the reuse or co-option of individual ancestral enhancers (**Figure 7*c***). In this case, the preexisting enhancer already contains a dozen or more binding sites for upstream transcription factors. Because most transcription factors are expressed in multiple tissues during development, any instance of a transcription factor binding site could lead to ectopic activation in a new setting that contains the upstream factor. Hence, most enhancers may be merely a few mutations away from generating a novel activity in a new tissue. A study of a novel expression pattern of the *Nep1* gene in the *D. santomea* optical lobe revealed a co-option event that arose over the 400,000 years separating *D. santomea* from its sister species *D. yakuba* (128). In this case, short stretches of sequence within this enhancer were shown to affect function in both the novel and ancestral expression activities. This demonstrates how individual transcription factor binding sites can be reused to generate new patterns of expression. In a genome-wide survey of enhancer-associated chromatin marks in human, macaque, and mouse limbs, Cotney et al. (36) found that a large fraction (18%) of enhancers gained in the human lineage showed evidence of ancestral enhancer-associated marks in other tissues. Hence, co-option may be a driving mechanism for the generation of many enhancers in the human genome.

Finally, enhancers may simply evolve from nonfunctional sequences that contain chance binding sites. A study of pseudogenized exons in zebrafish established that sequences previously used as exons exhibit enhancer activity, suggesting that upon pseudogenization, a regulatory role evolved to generate novel patterns of gene expression (45). Although this mechanism may require more steps than we generally think is likely, it may nevertheless be more prevalent than anticipated. For example, several studies have indicated that some enhancers rapidly turn over binding sites, evolving new sites to replace old ones to maintain a conserved output (89, 146). This indicates that the evolution of a complex enhancer containing many new binding sites is perhaps not so implausible. However, more examples are needed, and the identification of clear instances of de novo generation is difficult, as it requires evidence that the ancestral sequence contained no activity in any tissue anywhere in the developing organism or adult.

4.4. Influences of Enhancer Origination Mechanisms on Network Shape

It may be that the mechanisms at work during the evolution of enhancer activities dictate the shape and hierarchical relationships within a GRN. For example, if a network evolved through the widespread exaptation of a TE, as proposed for mammalian pregnancy (90), the resulting network may be flat and wide, with very little stratification. On the other hand, models of wholesale network co-option posit that the hundreds of genes deployed in a new cell type result from a small number of changes in the upper tiers of the GRN that cause many subordinate genes to be expressed. A variety of mechanisms may lie at the heart of these changes that initiate network co-option: promoter switching, de novo enhancer evolution, transposition, or co-option of preexisting binding sites within an enhancer. Given the preexisting structure of a co-opted network, it may be more stratified than a network rewired by TE exaptation.

However, the case of GRN co-option via the expansion of a preexisting enhancer's activity to a new location poses an interesting conundrum. If a preexisting enhancer evolves a new expression pattern in a new territory because of its preexisting inputs, then this would, in a sense, lead to the novel setting becoming more similar to the enhancer's ancestral setting. If the ancestral setting matches that of the GRN that is being redeployed, then this raises the questions of how the novelty can differ from the structure encoded by the ancestral network and how such co-opted networks can become specialized while mitigating pleiotropic effects on other tissues. Perhaps one underappreciated solution to this problem is that the derived networks always exist in the context of a new cell type or tissue that implements its own preexisting network. Little is known about how new networks interact with the preexisting networks.

5. CONCLUDING REMARKS

The last decade has seen great strides in our understanding of GRN structure and function, paving the way for a clearer definition of how GRNs evolve to generate morphological phenotypes. Below, we briefly discuss some of the challenges for the future in achieving a deeper understanding of GRN evolution, origination, and conservation.

The deep conservation of regulatory factors in developmental GRNs was one of the observations that motivated the hypothesis that regulatory sequence evolution may drive morphological diversification. However, such findings also led to the discovery that many subcircuits within GRNs may have a deep ancestry that has been preserved since their origination in early metazoans. The systematic identification of these ancient linkages will reveal the core circuits of GRNs that are recalcitrant to change and broadly apply to a large number of species (including humans). A key challenge in this endeavor is the relatively small number of GRNs that have been mapped in any species, which is further compounded by the need to map such GRNs in multiple species so that similarities and differences can be assessed. A second major task is to explore how deeply conserved regulatory sequences, such as those found in *SoxB2*, are employed in different organisms. Why are they preserved for so long? And what principles dictate their redeployment to other developmental settings?

Alongside deep conservation, some of the most interesting aspects of GRN biology are the parts that are changing. We now have several initial examples of GRN evolution, and there are many conceptual advances to be made in this arena. The ubiquity of *cis*-regulatory mutations that alter morphological phenotypes is becoming increasingly accepted, and a major challenge now is to obtain a clearer picture of how these mutations affect transcription factor binding, and the logical operations that determine transcriptional output. Only a small number of enhancer sequences are well studied in any one species, and a mutation to such an element may create or destroy the binding of a small handful of the hundreds of transcription factors present in the metazoan genome. It is fair to say that any case study of enhancer evolution is likely to be missing important details concerning which factors are bound in vivo. This is an area that will improve with the state of the art of regulatory biology in model organisms.

For alterations of transcription factor coding sequences, we are now at the point where likely changes have been implied, but very few detailed examples of their direct phenotypic consequences exist. Progress in this respect has been hampered to some degree by the focus of these studies on macroevolutionary changes. It may well be easier to detect a difference in coding sequence function over a longer evolutionary distance, but the trade-off is that it then becomes difficult to form a before-and-after picture of the phenotypic consequences that these changes engendered. We believe that this area of GRN biology is ripe for intensive experimental treatment.

Perhaps one of the most exciting aspects of GRN evolution is an area that will require much experimental attention: the origination of GRNs and novel morphological phenotypes. Although all of the current examples rest on the tried and true inferences based on comparative analyses of gene expression, examples that probe the circuits themselves are needed. We need examples in which we can point to the causative changes and the downstream enhancers that were activated during the incipient stages of network co-option. Systems in which the network co-option event can be recapitulated through gene misexpression studies would allow us to probe which connections were required to generate the novel phenotype, as opposed to connections that lacked phenotypic consequences (e.g., *dachshund* in the beetle horn). How sloppy is the origination of a novel structure? And after its establishment, how were connections refined, altered, or pruned? Answers to these questions will illuminate the origins of morphological complexity, one of the biggest challenges in the understanding of evolution.

SUMMARY POINTS

1. Gene regulatory networks (GRNs) lie at the heart of animal development, controlling which subset of the genome is activated in time and space to generate a tissue's physical properties.
2. Most transcription factor families and developmental signaling pathways are conserved across the animal kingdom.
3. The GRNs for many organ systems have a deep metazoan ancestry, with some individual transcription factor binding sites remaining conserved for hundreds of millions of years.
4. The alteration of transcriptional regulatory sequences (*cis*-regulatory evolution) is a commonly observed mechanism for morphological evolution that modifies the pattern and level of a gene's expression.
5. A multitude of mechanisms exist to modify transcription factor function at the coding sequence level.
6. Many human adaptations are thought to have resulted from *cis*-regulatory evolution, and mounting evidence for this mechanism has accumulated in several case studies.
7. The co-option (or reuse) of preexisting GRNs is thought to underlie the generation of novel structures.
8. Several mechanisms for the deployment of genes into new tissues exist, which may dictate a GRN's resulting shape and topology.

FUTURE ISSUES

1. Ancient subcircuits that form the kernels of GRNs need systematic identification and functional characterization.
2. Elucidation of how mutations are translated into differences in transcription factor binding and transcriptional output is a crucial yet understudied facet of connecting noncoding sequence variation to phenotypic consequences.

3. Studies elucidating the phenotypic consequences of coding sequence evolution within GRNs are required in order to understand the impact of drastic changes to a network's internal nodes.
4. Increasingly clever usage of model systems is needed to advance understanding of human evolution.
5. Investigations of morphological novelties in systems amenable to the evaluation of regulatory sequence function will be crucial to obtain a clearer picture of GRN origination.

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Errata

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