OPINION

The evolution of hierarchical gene regulatory networks

Douglas H. Erwin and Eric H. Davidson

Abstract | Comparative developmental evidence indicates that reorganizations in developmental gene regulatory networks (GRNs) underlie evolutionary changes in animal morphology, including body plans. We argue here that the nature of the evolutionary alterations that arise from regulatory changes depends on the hierarchical position of the change within a GRN. This concept cannot be accomodated by microevolutionary nor macroevolutionary theory. It will soon be possible to investigate these ideas experimentally, by assessing the effects of GRN changes on morphological evolution.

The molecular biology of developmental gene regulation is generating new mechanistic insights into the evolution of animal morphology. The binding of transcription factors to regulatory DNA sequences controls the spatial and temporal expression of genes in the developing organism, and because each transcription factor provides multiple inputs to other genes, regulatory gene interactions form a network. Developmental gene regulatory networks (GRNs) have a modular structure, or architecture, consisting of sub-circuits — each with a given function that is determined by the set of available transcription factors (the regulatory state). For example, some sub-circuits operate differentiation gene batteries (BOX 1; FIG. 1a), some control morphogenetic function (FIG. 1b), some interpret initial transient spatial cues in the developing organism, and others mediate the regulatory response to external signals. A realistic understanding of how evolution works should reflect the characteristics of developmental GRNs (BOX 1) that underpin morphological evolution at multiple levels[1–4]. By extension, to understand the evolution of the body plan we need to understand the evolution of the genomically encoded regulatory processes that control development. We now have direct evidence that these regulatory processes are generated by the operation of developmental GRNs[2–6].

GRN theory and examples have been the subject of recent reviews[1,7–9]. The GRNs that determine the course of development are those that establish the diverse regulatory states in the spatial domains of the developing animal, as they determine which genes will be active or inactive in each domain. However, differentiation and morphogenesis are the product of the thousands of downstream protein-coding genes that encode functionally specialized proteins, and of the interactions and self-assemblies of these proteins. It is the 'why' of their presence in a given domain of the organism that depends on the GRN.

FIGURE 1c shows that specification GRNs are composed of different kinds of sub-circuit, and provides an introduction for the main thesis of this article: that changes in different types of these components have different phenotypic effects[10]. All such changes result from evolutionary alterations in *cis*-regulatory modules that control expression of the genes of the GRN but, as we argue in this article, their effects are strikingly different depending on where in the GRN architecture these changes occur.

> "Although changes to GRN architecture that are due to *cis*-regulatory change have occurred throughout animal evolution, we argue that their effects are highly dependent on where in the GRN architecture they occur."

GRNs are hierarchical, and some changes have far larger effects than others (BOX 1; FIG. 1c). This, together with the fact that some GRN components can change rapidly whereas others (known as 'kernels') are extremely stable, has important evolutionary consequences. The probability of successful evolutionary change therefore differs across GRN topology. Standard evolutionary models do not accommodate these temporal asymmetries in the patterns of change in GRNs: although it is not generally appreciated, both microevolution and macroevolution assume that the probabilities of different mutations do not vary systematically through time, although their probabilities of success might vary considerably owing to environmental and ecological conditions.

Metazoans have various layers of gene-regulatory control, including chromatin modification and the action of microRNAs. We focus on the crucial role of the conservation and modulation of developmental GRN sub-circuits. This is for three reasons: first, all other levels of control follow after the primary recognition of genomic regulatory sequence by transcription factors; second, there is a wealth of experimental evidence for the evolutionary significance of morphological evolution of changes in genomic regulatory sequence that alter GRN linkages, as reviewed below and elsewhere[11,12]; and third, as GRNs control the developmental process they can be related to their developmental effects only by considering how *cis*-regulatory changes affect GRN structure.

We review recent studies that illustrate the diverse effects of regulatory changes in GRNs at different levels. Mutations that affect the *cis*-regulatory nodes of a GRN might cause the gain, loss or redeployment (that is, co-option) of regulatory genes; as a result, the architecture of a GRN might be altered and affect the developmental outcome[1,13–15]. We end with the message that soon the mechanisms of morphological evolution will become generally accessible to laboratory tests, an approach we term 'synthetic experimental evolution'.

Classes of regulatory change

Many DNA-level mutational mechanisms can cause gain, loss, or redeployment of *cis*-regulatory modules, as summarized in BOX 2. These include: point mutations; insertion and deletion of sequence tracts of various size; import and transposition of *cis*-regulatory modules by transposable elements; and conversion, translocation and replication slippage. These processes occur at different rates and by distinct molecular mechanisms, and they are repaired, or not repaired, by different systems. Some, such as point mutations, can be reversible, but others, such as translocations or deletions or many kinds of insertion or duplication, are essentially irreversible. Although changes to GRN architecture that are due

to *cis*-regulatory change have occurred throughout animal evolution, we argue that their effects are highly dependent on where in the GRN architecture they occur.

On the basis of the empirical structures of developmental GRNs and the theory summarized above, we suggest that the potential evolutionary effects of *cis*-regulatory change depend on both the nature of the change and the region of the hierarchical GRN affected (FIG. 2).

In this section we review some examples of different types of evolutionary change that have affected different aspects of developmental GRN architecture and that have therefore affected the function of the GRN. These examples range from pigmentation patterns reflecting changes in differentiation gene batteries, to the switch-like functions of some Hox genes, to the redeployment of GRN sub-circuits to form butterfly eyespot patterns. We have chosen cases in which the specific regulatory element that has been altered during evolution is known at some level of detail, so that we can identify the position of that element in the GRN hierarchy; we also describe the mutational mechanism when it is known.

The simplest evolutionary changes in developmental GRN architecture. At the periphery of the developmental GRN are differentiation gene batteries and their immediate controllers. They can be regarded as peripheral because their operation is the terminal activity of the developmental process; furthermore, they exercise little feedback into the internal regions of the GRN compared with the dense feedback linkages that are characteristic of regulatory state specification networks[1] (FIG. 1a).

An excellent example is the *yellow* gene in *Drosophila*. This gene encodes an enzyme that is required for the synthesis of melanin pigment[16], and thus is a differentiation gene. The different pigment patterns of *Drosophila* species partly depend on sequence differences in the *yellow cis*-regulatory modules that control wing pigment pattern[17,18] and, in the male, posterior abdominal colouring[19]. In each case the polarity of the change is known from studies on close outgroups. For example, an anterior–distal wing pigment spot in *Drosophila biarmipes* is lacking in *Drosophila melanogaster*. Underlying the evolution of the *D. biarmipes* pattern was the acquisition of new transcription factor target sites in a pleisiomorphic wing *cis*-regulatory module of the *yellow* gene, specifically including sites for the posterior wing repressor Engrailed. Regulatory changes have also altered the expression of other pigmentation genes that are active in the wing, and *cis*-regulatory sequence change is also responsible for loss of male abdominal pigmentation in another *Drosophila* species[16,17,19].

Box 1 | **Gene regulatory networks (GRNs)**

What is a GRN?

Development is controlled directly by progressive changes in the regulatory state in the spatial domains of the developing organism. As regulatory genes regulate one another as well as other genes, and because every regulatory gene responds to multiple inputs while regulating multiple other genes, the total map of their interactions has the form of a network. These GRNs consist of regulatory genes, which encode transcription factors, and signalling genes, which encode ligands and receptors for intercellular communication, plus the sequences that control the expression of each of these genes. Thus their components are elements of coding and non-coding DNA sequence, and together they constitute the 'regulatory genome'.

Importantly, these interactions depend causally on the DNA sequences that determine which transcription factors control each gene, and so the architecture of the GRN also depends on the ensemble of genomic regulatory DNA sequences. When these change in evolution, GRN structure changes and the developmental process changes, resulting in great or small changes in the outcome of development (that is, morphology).

Key features of GRNs

GRNs are hierarchical, so that the portions controlling the initial stages of development are at the top of the hierarchy, the portions controlling intermediate processes of spatial subdivision or the formation of future morphological pattern are in the middle, and the portions controlling the detailed functions of cell differentiation and morphogenesis are at the periphery.

The modular sub-circuits of developmental GRNs differ in evolutionary lability[10]. The most slowly changing components — called kernels — consist of highly conserved regulatory interactions that establish the progenitor field of a developing structure[1,10]. Kernels are sub-circuits composed of recursively wired regulatory genes (that is, they share inputs through multiple *cis*-regulatory interactions), which operate during the initial phase of regional pattern formation for a particular body part. If any of the genes in the sub-circuit are prevented from functioning, the body part fails to develop[30]. A kernel interacts with regional regulatory state sub-circuits, which in turn activate or repress the activity of differentiation gene batteries at the periphery of the GRN (FIG. 1c). The conserved structure of developmental GRN kernels might be responsible for the phenotypic stability of animal body plans that has persisted at least since the Early Cambrian period, 520 million years ago[63,64].

The evolutionary stability of kernels contrasts with the lability of other GRN sub-circuits. Minor changes, such as those that affect patterns of pigmentation[16–18], occur at high rates. Other changes have a greater impact on phenotype, particularly those that alter the conserved aspects of morphology in major clades[1,10].

GRNs in evolution

There are a number of attributes that can be used to describe GRNs and their role in evolution; some of these are summarized below.

- Developmental specification GRN. A gene regulatory network that establishes the developmental identity of a previously undefined cellular domain. Specification GRNs therefore determine which genes will be active or inactive in each spatial domain of the developing animal.
- *Cis*-regulatory module. DNA sequence, usually a few hundred base pairs long, that includes clusters of transcription factor target sites and that is functionally responsible for an aspect of expression of the gene it controls.
- Differentiation gene battery. A set of genes that encode proteins required for cell type-specific function, under common regulatory control by a small set of transcriptional drivers.
- GRN sub-circuit. A modular component of a GRN consisting of ~3–8 regulatory genes plus their regulatory interactions; the output of the sub-circuit executes a given developmental function.
- GRN plug-in. A GRN sub-circuit that is frequently re-deployed in a flexible, non-conserved way in evolution, even though its internal structure remains the same.
- GRN architecture. The topology of the map of functional linkages among the genes of a sub-circuit or of a whole GRN.
- Switch functions. Control circuitry that permits or forbids activity of a whole sub-circuit that has some pattern formation function.
- Signal deployment. *Cis*-regulatory features causing utilization of one of the canonical intercellular signalling devices in a given developmental context, for example, expression of the signal ligand in certain cells at certain times.

In studies of the evolutionary loss of function of particular differentiation genes the question often arises of whether, in a lineage with a derived gene expression pattern, the change occurred in the protein-coding sequence or in a *cis*-regulatory sequence. Single mutational events can easily destroy either kind of function. But lesions in the coding sequence of a pleiotropic gene are more likely to eliminate function in all phases of development[20,21]. Conversely, as individual *cis*-regulatory modules mediate distinct phases of regulatory activity[1], lesions in just one of these modules will only affect its particular phase of activity. For this reason, changes in particular *cis*-regulatory modules are commonly observed in pleiotropic genes; *yellow* is one such example.

The differentiation gene batteries at the periphery of developmental GRNs consist of effector or structural genes, plus the small set of regulatory genes that determine their developmental deployment. Thus, a potent mechanism of change in regional details of anatomy and function is an alteration of the *cis*-regulatory modules that determine the developmental expression of differentiation gene battery controllers as well as the structural genes themselves. A canonical example is the regulatory gene *ovo* (also known as *shavenbaby*), which encodes a zinc-finger transcription factor that drives the effector genes that build the trichomes on *Drosophila* larvae. These downstream genes constitute a 'trichome morphological module'[22]: they control actin filament reorganization, modification of the cuticle–cell interaction and pigmentation through direct *cis*-regulatory control of *yellow* expression. Indeed, species-specific patterns of trichome deposition depend on *cis*-regulatory differences in the *ovo* genes of different *Drosophila* species[23,24]. In *Drosophila sechellia*, for example, alterations in three separate *cis*-regulatory modules account for the differences between its pattern of trichome deposition and that of *D. melanogaster*.

Switch-like functions in developmental GRNs. Some *cis*-regulatory genes function as Input/Output (I/O) switches (FIG. 1c), enhancing or forbidding the operation of developmental GRN sub-circuits that initiate the development of morphological features. I/O switches appear at every level of GRN architecture — from high in the hierarchy, where the progenitor fields of body parts or embryonic territories are specified, to downstream peripheral functions.

Homeobox (Hox) genes have many functions, but a number of positive and negative functions are apparently of the switch type[1,10]. One elegant example is provided by the *Drosophila* Hox gene *Ultrabithorax* (*Ubx*). *Ubx* prevents wing development in the haltere imaginal disc of the third thoracic segment by repressing *cis*-regulatory modules at multiple nodes of the wing patterning GRN[25–28]. *Ubx* also provides positive inputs to genes of the

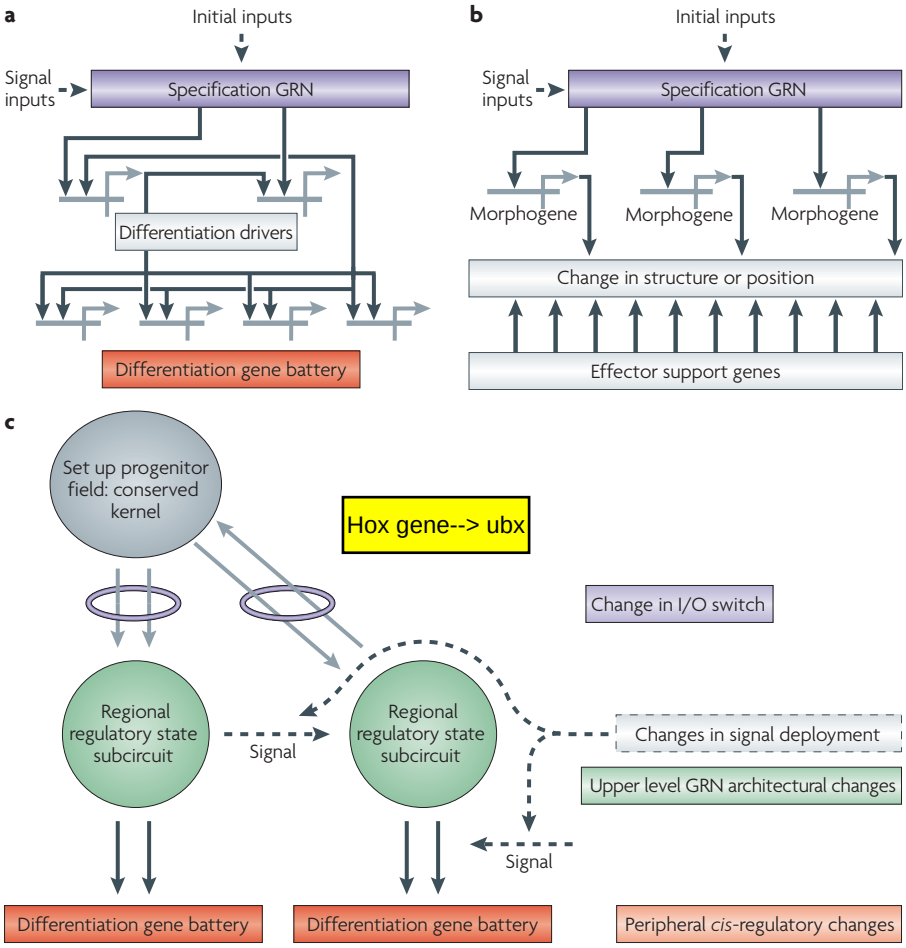


Figure 1 | **Key features of gene regulatory networks (GRNs), and consequences of changes in GRN components. a** | The control system of a typical differentiation gene battery. The output of the specification GRN is expression in given cells of a small set of 'differentiation driver' transcription factors. In combination, these drivers transcriptionally activate the protein-coding genes of the 'differentiation gene battery', because these genes are all controlled by *cis*-regulatory modules that include target sites for subsets of the drivers. The driver regulatory genes are often cross-regulated, as in the feedback circuit shown, and they provide the multiple inputs into the downstream differentiation genes. These are typically 'wired' in parallel, often in feed-forward circuitry[1,2,65]. **b** | The control system for a morphogenetic function, such as invagination or migration. Based on the few available examples (including REFS 22,66), the output of the specification GRN is expression of transcription factors that activate (or repress) a few key checkpoint genes (so-called morphogenes), which are required to trigger the process. Most of the genes encoding proteins that contribute to the process (the effector support genes) are broadly expressed rather than directly controlled by the specification GRN (in contrast to the way differentiation gene batteries are controlled). **c** | The components and topology of a specification GRN (BOX 1) and diverse types of evolutionary change that might occur therein[1,2]. The GRN includes a kernel of highly conserved regulatory interactions that establish the progenitor field for regional specification of a developing structure (shown in grey). This kernel in turn provides key inputs into regionally active sub-circuits, the role of which is to establish regional regulatory states (green). The outputs of these activate or repress the activity of differentiation gene batteries at the periphery of the GRN (red). GRNs also encompass Input/Output (I/O) switches that permit or prohibit the operation of the regulatory sub-circuits (purple), and signals between the regulatory sub-circuits (dashed line). At the right of the figure, using the same colour code, are listed the different types of change that might occur as *cis*-regulatory modules evolve, according to where in the hierarchical GRN the affected *cis*-regulatory modules operate.

haltere patterning network[29]. In many developmental GRN contexts Hox genes are separate from the patterning sub-circuits, as they do not receive inputs from other genes of the sub-circuit; like *Ubx* in the haltere, they can sit on the outside of the patterning GRNs, where they act as I/O switches. The deployment of these I/O switches is extremely plastic in evolution, as it depends on individual *cis*-regulatory modules of the Hox genes.

The most common developmental GRN switches affecting the spatial location of GRN sub-circuit activity during development are those that are regulated by intercellular signals. Specifically because of this role, *cis*-regulatory change in the expression of signal ligand genes and receptors must *a priori* be regarded as a potent source of evolutionary variation. We have referred to these signal-driven regulatory switches as the 'plug-ins' of developmental GRNs (BOX 1), by analogy to canonical electrical switches that can be attached onto any kind of circuitry[10]. The implication is that evolutionary redeployment of these signal systems might cause either large or small alterations in the developmental process, depending on where in the GRN they occur.

Experimental identification of an evolutionary signalling change high up in the hierarchy was discovered by comparing the embryonic developmental GRNs of the sea star and sea urchin[30,31]. Embryonic Notch signalling has been deployed in strikingly different ways in the two organisms: in both the sea urchin and sea star, mesoderm to endoderm Notch signalling is required for endoderm specification but, in the sea urchin only, specification of the mesoderm also requires Notch signalling. If Notch signalling is interrupted in the sea star, the result is lack of endoderm and default conversion of what should become endoderm into excess mesoderm; by contrast, in the sea urchin the primary result is the opposite — that is, loss of mesoderm.

At an intermediate level of the developmental GRN, comparative studies in insect orders[32] revealed tremendous variation in the deployment of identical signals during development of the divergent appendage forms, although the full *cis*-regulatory network remains to be elucidated. The same is true of appendage divergence in tetrapod orders and classes[33]. In both cases these changes are correlated with changes in the transcription of regulatory genes.

Examples of evolutionary signal redeployment and of modulation of signal strength at the developmental GRN periphery that have species-specific morphological effects are becoming more common. Among species of Darwin's Galapagos finches[34,35], the between-species variation in expression of the transforming growth factor-β ligand bone morphogenetic protein 4 is correlated with the breadth and depth of the beak, whereas calcium signalling mediated by calmodulin (CaM) expression determines beak length. Similarly, the evolutionary divergence of integumental appendages such as hair, feathers and scales in tetrapods can be interpreted as alterations in the expression of signal systems, many of which are utilized in the development of the follicles from which these appendages emerge[36,37].

Unfortunately, there is no direct *cis*-regulatory evidence for the particular cases cited here, except for Notch signalling in the sea urchin[38,39]. Evolutionary analyses of *cis*-regulatory modules that control the developmental expression of relevant signalling functions will be a fertile area for evolutionary research.

Cis-regulatory alteration of interior GRN architecture. The internal linkages in a developmental GRN functionally connect genes encoding transcription factors, which regulate one another and generate a progression of spatially defined regulatory states. The highly conserved sub-circuits of the GRN — its kernels — cannot be changed without disastrous effects[10]. In other sub-circuits we might expect that changes in the genomic sequences that execute the control functions at the nodes of the GRN would be a source of pleiotropic evolutionary alterations. Such changes alter developmental GRN architecture and might rearrange the spatial distribution of given regulatory states in the developing organism, affecting the expression of multiple genes downstream. Fixation of such an altered architecture in the population might result in significant morphological change.

The *pitx1* homeodomain transcription factor gene is an essential component of the developmental GRN controlling specification and morphogenesis of the vertebrate pelvis and associated structures[20], but the actual architecture of this GRN has not been fully elucidated. Genetic analysis of the recent evolutionary loss of pelvic spines in isolated populations of the stickleback fish identified mutations in *pitx1* (REFS 20,40). Mouse knockouts of the gene also affect morphogenesis, which further demonstrates that *pitx1* lies in the interior of the GRN architecture. Populations of sticklebacks lacking pelvic spines have reduced pelvic development and the *pitx1* gene fails to be expressed in the pelvic region of the larvae. However, other essential aspects of *pitx1* expression are normal, and the sequence of the coding region of the gene is identical irrespective of this phenotype. It follows that loss-of-function mutations specifically in a pelvic *cis*-regulatory module of the *pitx1* gene account for this evolutionary change. Furthermore, similar mutations in the same gene have occurred repeatedly in different stickleback populations.

The proneural genes of the *achaete–scute* (*ac–sc*) complex in *Drosophila* species are expressed in the developing peripheral nervous system bristles, which appear on the notum and wing in species-specific,

Box 2 | **Modes of *cis*-regulatory change**

There are a number of changes that can have an effect on *cis*-regulatory modules (CRMs). Three of these changes are summarized below.

Internal changes that could affect the function of a pre-existing CRM

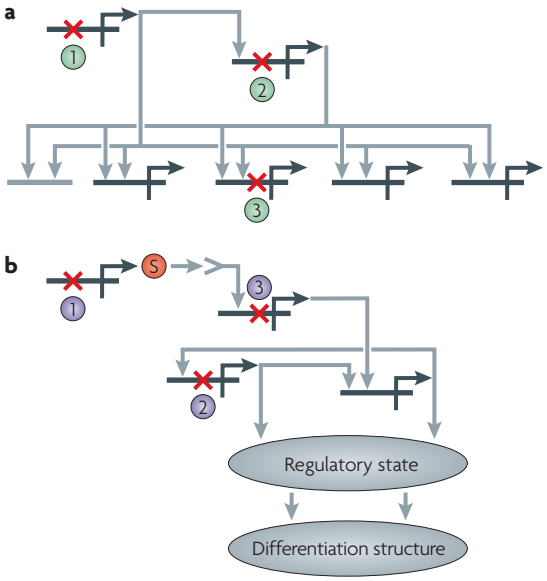
- SNPs that are due to single base-pair mutation can cause gain of new binding sites, loss of sites, or strengthening or weakening of binding to sites.
- Insertions and deletions can change the distance between interacting sites, cause gain or loss of sites, or an increase in the copy number of given sites.
- Insertion of mobile element carrying regulatory sequences can cause gain or potential loss of site, change in the distance between interacting sites and increase in copy number, as well as alter the strength of binding at the site.

Changes that alter CRM repertoire of pre-existing genes

- Insertion of CRMs from elsewhere, carried by mobile elements, by inversions, by translocations, or by intronic retrotranspositions can cause gain of developmental functions without loss of the gene.
- Loss of a CRM by translocation, large deletion, inversion breakage or insertion of mobile element can cause loss of specific developmental function without loss of the gene.

Large-scale rearrangements that produce novel gene–CRM complexes

- Regional duplications can result in subfunctionalization and neofunctionalization.
- Translocations can bring new genes into large regulatory domains.



	Cis-regulatory module loss	*Cis*-regulatory module redeployment
a		
1	Lose expression of whole battery	Battery expressed in a new developmental domain
2	Lose expression of whole battery	Lose expression of whole battery
3	Lose expression of differentiation gene	Redeploy gene
b		
1	Lose development of structure	Can lose sub-circuit that is expressed adjacent to new domain, and signal expression
2	Lose stabilization of state; development starts but fades out	Lose stabilization of state: development starts but fades out
3	Lose structure	Produce structure in another location

Figure 2 | **Developmental effect of gene regulatory network change depends on location.** The effect of loss or redeployment of a *cis*-regulatory module depends on the position of the module in the regulatory hierarchy. **a** | The effect of loss or redeployment of *cis*-regulatory modules in a terminal gene-differentiation battery (FIG. 1a), depending on whether the change occurs in a sequence upstream of the battery (1), in a sequence directly controlling the battery (2), or in a differentiation gene (3). **b** | The effect of loss or redeployment of a *cis*-regulatory module controlling a sub-circuit (S) in a gene regulatory network, depending on whether the change occurs upstream of the sub-circuit (1), producing a loss or shift of the structure; in a *cis*-regulatory module that is part of a feedback structure (2), leading to the loss of lockdown of the feedback loop and fading out of development; or in a *cis*-regulatory module directly controlling the sub-circuit (3), leading to a loss or redeployment of the structure.

stereotypical positions. Each bristle arises from a proneural cluster of cells marked in the imaginal disc by *ac–sc* expression, which is controlled by a large number of *cis*-regulatory modules that interpret the complex spatial regulatory landscape in the disc[41]. The patterning GRN contains many regulatory genes, expressed in specific overlapping domains[42], so that activators and repressors produce diverse combinatorial regulatory states in the various local regions of the disc. Downstream of *ac–sc* are other transcriptional regulatory genes, in particular the *Enhancer of split* genes[43,44]. Thus the position of *ac–sc* is clearly internal with respect to GRN structure. But both the wing regulatory landscape and the *ac–sc cis*-regulatory systems of *Drosophila* are greatly different from their respective pleisiomorphic states, as revealed by several studies of basal dipterans[45–47]. These dipterans lack bristle-specific *ac–sc* control modules; instead, broad areas are defined, each of which contains many bristles. The evolution of the *Drosophila* pattern has required the gain of the multiple unique *cis*-regulatory modules of the *ac–sc* complex, as well as the currently undefined *cis*-regulatory changes required to generate the multiple localized regulatory states upstream of *ac–sc*. This means the *de novo* generation of regional patterning GRN sub-circuits occurred since the divergence of the Diptera.

Redeployment of whole developmental GRN sub-circuits. Just as whole differentiation gene batteries can be redeployed in evolution, so can the internal specification sub-circuits of GRNs, the role of which is to set up new regulatory states in given places in the developing organism. A demonstration of this is the redeployment of the *patched–hedgehog–engrailed–cubitus interruptus* repression sub-circuit to establish the foci of the pigmented eyespots in the wings of some butterflies[48]. This same signalling sub-circuit is probably a pleisiomorphic feature of transcriptional patterning at the anterior–posterior boundary of the insect wing, where its purpose is to cause *engrailed* expression in intervein cells. In such cases, genes that are already linked in regulatory circuits retain their functional connections, and thus the *cis*-regulatory alterations produce a large effect for a small change, as they result in the transfer of a whole GRN sub-circuit to a new embryological or larval address.

A prominent derived feature of sea urchin embryos and larvae is their biomineral skeleton, which arose 250 million years ago. Since then the developmental GRN responsible for producing the embryonic skeleton was loaded into the regulatory apparatus of the micromere cell lineage, which builds the skeleton in modern sea urchins. This almost 30-gene network is now well characterized[2]; this made it possible to test the idea that the embryonic skeleton is a heterochronic redeployment of the adult skeletogenic GRN module that is common to all echinoderms. A study[49] of almost every gene of the embryonic skeletogenic lineage shows that the differentiation gene batteries, their regulatory drivers and the skeletogenic regulatory state sub-circuits of the adult skeletogenesis centres were redeployed *en masse* by hooking the whole of this circuitry into the embryonic micromere lineage-specification system. The micromere lineage GRN turns on its functions by use of a double negative gate (that is, a repressor repressing a repressor); to hijack the whole adult skeletogenic circuitry the *cis*-regulatory modules operating its upstream genes merely had to be brought under the control of this gate.

Protein-coding changes. Although we are concerned here with the evolution of animal morphology through *cis*-regulatory alteration, the same arguments pertain to changes in the protein-coding regions of genes. Most of these are changes in differentiation proteins. However, alterations also occur in the coding regions of transcription factors. Their evolutionary effects, in the same way we have discussed, depend on where in the developmental GRN these regulatory proteins operate. A good example is the

acquisition of a repressive regulatory domain in the insect UBX protein, which affects the I/O function of UBX and prevents the development of abdominal legs[50]. Similarly, variation in the number of tandemly repeated coding-sequence elements in regulatory genes of skeletal patterning systems in dogs and other mammals is a rapidly occurring mechanism of change in morphology[51].

In summary, although we can describe all of the DNA-level evolutionary changes discussed in this article as '*cis*-regulatory alteration', this term conflates many mechanisms that produce many effects. There is no equivalence between the processes of single base-pair mutation, the one-time insertion of a transposon-borne *cis*-regulatory module in the vicinity of a regulatory gene, the translocation of a gene to the regulatory environment of another gene, or the common and usually irreversible processes of sequence deletion and insertion. When there is sufficient experimental detail on the structure of the developmental GRN, we have described the effects of these *cis*-regulatory alterations in the context of the hierarchical organization of GRNs and their diverse developmental effects. As developmental GRNs continue to be elucidated, our ability to understand the context of these changes will correspondingly increase.

Evolutionary implications

This emerging view of the evolution of developmental GRNs presents a challenge to our understanding of the evolutionary process. Over evolutionary time, GRNs seem to have modified the range of accessible variation by changing the probability of evolution in different parts of the network. The formation and subsequent conservation of developmental kernels provide a paradigmatic example. After regional patterning interactions form a kernel it becomes resistant to subsequent modification, shifting the locus of selection to the kernel, rather than its component genes. The extreme conservation of network structure in kernels and their resistance to perturbation experiments indicates that they are subject to purifying internal selection[52], and present almost no additive genetic variation to external selection. Thus, the hierarchical structure of developmental GRN controls the nature of the variation available, in effect 'packaging' entire GRN sub-circuits for selection. Some of these sub-circuits have remained static for hundreds of millions of years[11], and this has forced evolutionary changes upstream and downstream in the GRN. As this space of 'evolutionarily relevant mutations'[53] has been modified there has been a temporal vector to the likelihood of evolutionary success of different types of GRN modification, particularly those responsible for body plan formation: this is a non-uniformitarian view of evolution. Although the mechanistic explanation is new, Riedl referred to the concept as 'burden'[54] and Wimsatt as 'genetic entrenchment'[55,56].

Uniformitarianism is the concept that the processes of geology have not changed over time, and thus 'the present is the key to the past'; this is also an implicit assumption of most evolutionary theory. Strict microevolutionary theory assumes that changes in gene frequencies are sufficient to explain all evolutionary patterns. In terms of developmental GRN structure, this concerns mainly changes at the periphery of GRNs. The macroevolutionary theories favoured by many palaeontologists are similarly ahistorical, focusing on selection operating at the level of species and clades[57–60], but with no historical vector. Neither microevolution nor macroevolution takes into consideration the impact of past changes in developmental GRNs on the future course of evolution.

Synthetic experimental evolution

Understanding that the mechanistic foundation of major morphological changes lies in alterations in GRN architecture is opening the way to a new kind of evolutionary biology: this could be termed 'synthetic experimental evolution' (Supplementary information S1 (figure)). As we come to understand the structure and function of developmental GRNs, and as our technological abilities to re-engineer them in living developing systems improves, it will become possible to experimentally reproduce evolutionary pathways (see also REF. 61).

Glossary

Additive genetic variation
The portion of genetic variance that is attributable to the average effect of substitution of one allele for another at a locus; it is used to predict the rate of response to selection for quantitative traits.

Body plan
The conserved aspects of morphology that define major clades, recognized in a Linnean hierarchy as phyla, classes and perhaps orders.

Fixation
Describes the situation in which a mutation has achieved a frequency of 100% in a natural population.

Haltere
Club-shaped modified hind wings on *Drosophila* species that serve as gyroscopic sense organs.

Heterochronic
An evolutionary change in the timing of a developmental process, so that a character or process occurs earlier or later in ontogeny, or grows at a different rate.

Imaginal disc
Epidermal thickenings in the larvae of holometabolous insects. The discs contain mesodermal cells that give rise to adult organs.

Integumental
Relating to the skin; appendages that grow out of the skin.

Lockdown
Establishment of a stable regulatory state by instituting positive feedback between regulatory genes that ensures continued transcription of the participating genes and those operating downstream of them in the GRN.

Macroevolution
Evolution above the species level, including species-level trends, and putatively encompassing selection at the level of species and clades.

Microevolution
Evolution within species and often the formation of new species; this is experimentally accessible through studies of shifting gene frequencies in populations.

Neofunctionalization
Acquisition by a duplicated gene of a new function compared with the original common ancestral function.

Notum
The dorsal portion of an insect's thoracic segment.

Outgroup
The most distantly related group in a phylogenetic analysis; it is used to establish the sequence and polarity of evolutionary changes.

Pleisiomorphic
Character states of an organism that were present before the last common ancestor of a clade.

Purifying internal selection
The elimination of genetic variation except around a single mode, by selection against non-viable developmental mutants.

Regulatory state
The set of active transcription factors in every cell at any time point.

Subfunctionalization
Retention by duplicated genes of different components of the original common ancestral function, which allows both gene copies to be preserved.

Trichome
Small hairs, specifically on the epidermis of *Drosophila* species.

Uniformitarianism
The assumption that studies of present processes are sufficient to understand past events, because there is a single common underlying evolutionary process that accounts for changes at every level.

This is already happening at the level of single-gene interventions. For example, as noted above, variation in beak length in Darwin's finches is driven by regulatory variation in the levels of CaM-dependent signalling in the developing beak mesenchyme[34]. This was confirmed by forcing expression of CaM in the developing chicken beak, and showing that this caused beak elongation. In another example the difference in forelimb length between bats and mice was addressed by inserting into the mouse genome a bat *cis*-regulatory module of the *Prx1* homeodomain gene[62]. In mammals, *Prx1* functions as an essential limb patterning regulatory gene. Introduction of the bat *cis*-regulatory module caused the lengthening of the embryonic mouse forelimb, thus demonstrating experimentally the evolutionary mechanism.

We believe, however, that the possibilities for synthetic experimental evolution are more far reaching, provided that three requirements are met: as thorough knowledge as possible of the developmental mechanism for formation of a derived character; the availability of a related experimental organism that manifests the pleisiomorphic state and into which gene transfer is possible; and the necessary technology for genomic transfer of regulatory circuitry. Such requirements are easily met for recent divergences but also apply to much deeper divergences, for example, between orders of echinoids. In other words, when we can re-engineer development experimentally, we should be able to reproduce much more fundamental evolutionary changes in morphology.

Conclusions

These arguments point to three complementary avenues of research into the basic problems of understanding mechanisms of animal body plan evolution. First, we need vastly augmented sets of comparative GRN analyses, which in turn require vastly expanded knowledge of developmental GRNs, obtained in reference to the cladistic relationships of organisms. Second, we need a deeper and far more extensive understanding of the mechanisms and functional consequences of *cis*-regulatory sequence change, and of constraints and flexibility in *cis*-regulatory sequence organization. Finally, we need the ability to carry out synthetic experimental evolution, which will provide experimental tests of the results gleaned from comparative GRN studies. Evolution of the animal body plan can be considered the most sophisticated, interdisciplinary and demanding branch of gene regulation bioscience, but viewed in this way it is clear that its mechanisms will be solved, as the basic features of gene regulation bioscience are solved.

Douglas H. Erwin is at the Department of Paleobiology, MRC-121, National Museum of Natural History, PO BOX 37012, Washington, Washington DC 20013-7012, USA; and the Santa Fe Institute, 1399 Hyde Park Road, Santa Fe, New Mexico 87501, USA.

Eric H. Davidson is at the Division of Biology 156-29, California Institute of Technology, Pasadena, California 91125, USA.

Correspondence to D.H.E.
e-mail: erwind@si.edu

doi:10.1038/nrg2499
Published online 13 January 2009

1. Davidson, E. H. *The Regulatory Genome* (Academic, San Diego, 2006).
2. Oliveri, P., Tu, Q. & Davidson, E. H. Global regulatory logic for specification of an embryonic cell lineage. *Proc. Natl Acad. Sci. USA* **105**, 5955–5962 (2008).
3. Smith, J. & Davidson, E. H. Gene regulatory network subcircuit controlling a dynamic spatial pattern of signaling in the sea urchin embryo. *Proc. Natl Acad. Sci. USA* **105**, 20089–20094 (2008).
4. Stathopoulos, A. & Levine, M. Genomic regulatory networks and animal development. *Dev. Cell* **9**, 449–462 (2005).
5. Nikitina, N., Sauka-Spengler, T. & Bronner-Fraser, M. Dissecting early regulatory relationships in the lamprey neural crest gene regulatory network. *Proc. Natl Acad. Sci. USA* **105**, 20083–20088 (2008).
6. Georgescu, C. *et al.* A gene regulatory network armature for T-lymphocyte specification. *Proc. Natl Acad. Sci. USA* **105**, 200100–200105 (2008).
7. Ben-Tabou de-Leon, S. & Davidson, E. H. Experimentally based sea urchin gene regulatory network and the causal explanation of developmental phenomenology. *Wiley Interdiscip. Rev. Syst. Biol. Med.* (in the press).
8. Gene networks in animal development and evolution special feature Sackler Colloquium. *Proc. Natl Acad. Sci. USA* (in the press).
9. Davidson, E. H. Developmental biology at the systems level. *Biochim. Biophys. Acta Gene Reg. Mech.* (in the press).
10. Davidson, E. H. & Erwin, D. H. Gene regulatory networks and the evolution of animal body plans. *Science* **311**, 796–800 (2006).
11. Wray, G. A. The evolutionary significance of *cis*-regulatory mutations. *Nature Rev. Genet.* **8**, 206–216 (2007).
12. Carroll, S. B. Evo–devo and an expanding evolutionary synthesis: a genetic theory of morphological evolution. *Cell* **134**, 25–36 (2008).
13. Prud'homme, B. *et al.* Repeated morphological evolution through *cis*-regulatory changes in a pleiotropic gene. *Nature* **440**, 1060–1053 (2006).
14. Wray, G. A. *et al.* The evolution of transcriptional regulation in eukaryotes. *Mol. Biol. Evol.* **20**, 1377–1419 (2003).
15. Davidson, E. H. A view from the genome: spatial control of transcription in sea urchin development. *Curr. Opin. Genet. Dev.* **9**, 530–541 (1999).
16. Wittkopp, P. J., True, J. R. & Carroll, S. B. Reciprocal functions of the *Drosophila* Yellow and Ebony proteins in the development and evolution of pigment patterns. *Development* **129**, 1849–1858 (2002).
17. Gompel, N., Prud'homme, B., Wittkopp, P. J., Kassner, V. A. & Carroll, S. B. Chance caught on the wing: *cis*-regulatory evolution and the origin of pigment patterns in *Drosophila*. *Nature* **433**, 481–487 (2005).
18. Wittkopp, P. J., Vaccaro, K. & Carroll, S. B. Evolution of *yellow* gene regulation and pigmentation in *Drosophila*. *Curr. Biol.* **12**, 1547–1556 (2002).
19. Jeong, S., Rokas, A. & Carroll, S. B. Regulation of body pigmentation by the Abdominal-B Hox protein and its gain and loss in *Drosophila* evolution. *Cell* **125**, 1387–1399 (2006).
20. Shapiro, M. D. *et al.* Genetic and developmental basis of evolutionary pelvic reduction in threespine sticklebacks. *Nature* **428**, 717–723 (2004).
21. Jeong, S. *et al.* The evolution of gene regulation underlies a morphological difference between two *Drosophila* sister species. *Cell* **132**, 783–793 (2008).
22. Chanut-Delalande, H., Fernandes, I., Roch, F., Payre, F. & Plaza, S. *Shavenbaby* couples patterning to epidermal cell shape control. *PLoS Biol.* **4**, e290 (2006).
23. McGregor, A. P. *et al.* Morphological evolution through multiple *cis*-regulatory mutations at a single gene. *Nature* **448**, 587–590 (2007).
24. Sucena, E., Delon, I., Jones, I., Payre, F. & Stern, D. L. Regulatory evolution of *shavenbaby*/*ovo* underlies multiple cases of morphological parallelism. *Nature* **424**, 935–938 (2003).
25. Hersh, B. M. & Carroll, S. B. Direct regulation of *knot* gene expression by Ultrabithorax and the evolution of *cis*-regulatory elements in *Drosophila*. *Development* **132**, 1567–1577 (2005).
26. Walsh, C. M. & Carroll, S. B. Collaboration between Smads and a Hox protein in target gene repression. *Development* **134**, 3585–3592 (2007).
27. Weatherbee, S. D., Halder, G., Kim, J., Hudson, A. & Carroll, S. Ultrabithorax regulates genes at several levels of the wing-patterning hierarchy to shape the development of the *Drosophila* haltere. *Genes Dev.* **12**, 1474–1482 (1998).
28. Galant, R. & Carroll, S. B. Evolution of a transcriptional repression domain in an insect Hox protein. *Nature* **415**, 910–913 (2002).
29. Hersh, B. M. *et al.* The UBX-regulated network in the haltere imaginal disc of *D. melanogaster*. *Dev. Biol.* **302**, 717–727 (2007).
30. Hinman, V. F., Nguyen, A. T., Cameron, R. A. & Davidson, E. H. Developmental gene regulatory network architecture across 500 million years of echinoderm evolution. *Proc. Natl Acad. Sci. USA* **100**, 13356–13361 (2003).
31. Hinman, V. F., Nguyen, A. & Davidson, E. H. 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 (2007).
32. Angelini, D. R. & Kaufman, T. C. Insect appendages and comparative ontogenetics. *Dev. Biol.* **286**, 57–77 (2005).
33. Stopper, G. F. & Wagner, G. P. Of chicken wings and frog legs: a smorgasbord of evolutionary variation in mechanisms of tetrapod limb development. *Dev. Biol.* **288**, 21–39 (2005).
34. Abzhanov, A. *et al.* The calmodulin pathway and evolution of elongated beak morphology in Darwin's finches. *Nature* **442**, 563–567 (2006).
35. Abzhanov, A., Protas, M., Grant, B. R., Grant, P. R. & Tabin, C. J. *Bmp4* and morphological variation of beaks in Darwin's finches. *Science* **305**, 1462–1465 (2004).
36. Lin, C. M., Jiang, T. X., Widelitz, R. B. & Chuong, C. M. Molecular signaling in feather morphogenesis. *Curr. Opin. Cell Biol.* **18**, 730–741 (2006).
37. Wu, P. *et al.* Evo–devo of amniote integuments and appendages. *Int. J. Dev. Biol.* **48**, 249–270 (2004).
38. Revilla-i-Domingo, R., Minokawa, T. & Davidson, E. H. R11: a *cis*-regulatory node of the sea urchin embryo gene network that controls early expression of *SpDelta* in micromeres. *Dev. Biol.* **274**, 438–451 (2004).
39. Ransick, A. & Davidson, E. H. *cis*-regulatory processing of Notch signaling input to the sea urchin *glial cells missing* gene during mesoderm specification. *Dev. Biol.* **297**, 587–602 (2006).
40. Shapiro, M. D., Bell, M. A. & Kingsley, D. M. Parallel genetic origins of pelvic reduction in vertebrates. *Proc. Natl Acad. Sci. USA* **103**, 13753–13758 (2006).
41. Gomez-Skarmeta, J. L. *et al.* *Cis*-regulation of achaete and scute: shared enhancer-like elements drive their coexpression in proneural clusters of the imaginal discs. *Genes Dev.* **9**, 1869–1882 (1995).
42. Calleja, M. *et al.* How to pattern an epithelium: lessons from *achaete–scute* regulation on the notum of *Drosophila*. *Gene* **292**, 1–12 (2002).
43. Maeder, M. L., Polansky, B. J., Robson, B. E. & Eastman, D. A. Phylogenetic footprinting analysis in the upstream regulatory regions of the *Drosophila Enhancer of split* genes. *Genetics* **177**, 1377–1394 (2007).

44. Singson, A., Leviten, M. W., Bang, A. G., Hua, X. H. & Posakony, J. W. Direct downstream targets of proneural activators in the imaginal disc include genes involved in lateral inhibitory signaling. *Genes Dev.* **8**, 2058–2071 (1994).
45. Wulbeck, C. & Simpson, P. Expression of *achaete–scute* homologues in discrete proneural clusters on the developing notum of the medfly *Ceratitis capitata*, suggests a common origin for the stereotyped bristle patterns of higher Diptera. *Development* **127**, 1411–1420 (2000).
46. Wulbeck, C. & Simpson, P. The expression of *pannier* and *achaete–scute* homologues in a mosquito suggests an ancient role of *pannier* as a selector gene in the regulation of the dorsal body pattern. *Development* **129**, 3861–3871 (2002).
47. Pistillo, D., Skaer, N. & Simpson, P. *scute* expression in *Calliphora vicina* reveals an ancestral pattern of longitudinal stripes on the thorax of higher Diptera. *Development* **129**, 563–572 (2002).
48. Keys, D. N. *et al.* Recruitment of a hedgehog regulatory circuit in butterfly eyespot evolution. *Science* **283**, 532–534 (1999).
49. Gao, F. & Davidson, E. H. Transfer of a large regulatory apparatus to a new developmental address in echinoid evolution. *Proc. Natl Acad. Sci. USA* **105**, 6091–6096 (2008).
50. Ronshaugen, M., McGinnis, N. & McGinnis, W. Hox protein mutation and macroevolution of the insect body plan. *Nature* **415**, 914–917 (2002).
51. Fondon, J. W. III & Garner, H. R. Molecular origins of rapid and continuous morphological evolution. *Proc. Natl Acad. Sci. USA* **101**, 18058–18063 (2004).
52. Schwenk, K. & Wagner, G. P. Function and the evolution of phenotypic stability: connecting pattern to process. *Am. Zool.* **41**, 552–563 (2001).
53. Stern, D. L. Evolutionary developmental biology and the problem of variation. *Evolution* **54**, 1079–1091 (2000).
54. Riedl, R. *Order in Living Organisms* (John Wiley & Sons, Chichester, 1978).
55. Wimsatt, W. C. in *From Embryology to Evo–Devo* (eds Laubichler, M. D. & Mainenschein, J.) 310–355 (MIT Press, Cambridge, Massachusetts, 2007).
56. Wimsatt, W. C. in *Cycles of Contingency* (eds Oyama, S., Griffiths, P. E. & Gray, R. D.) 219–237 (MIT Press, Cambridge, Massachusetts, 2001).
57. Jablonski, D. Scale and hierarchy in macroevolution. *Palaeontology* **50**, 87–109 (2007).
58. Gould, S. J. *The Structure of Evolutionary Theory* (Harvard Univ. Press, Cambridge, Massachusetts, 2002).
59. Stanley, S. M. *Macroevolution* (W. H. Freeman, San Francisco, 1979).
60. Jablonski, D. Micro- and macroevolution: scale and hierarchy in evolutionary biology and paleobiology. *Paleobiology* **26**, 15–52 (2000).
61. Dean, A. M. & Thornton, J. W. Mechanistic approaches to the study of evolution: the functional synthesis. *Nature Rev. Genet.* **8**, 675–688 (2007).
62. Cretekos, C. J. *et al.* Regulatory divergence modifies limb length between mammals. *Genes Dev.* **22**, 141–151 (2008).
63. Valentine, J. W. *On the Origin of Phyla* (Univ. Chicago Press, Chicago, 2004).
64. Erwin, D. H. Disparity: morphologic pattern and developmental context. *Palaeontology* **50**, 57–73 (2007).
65. Gilchrist, M. *et al.* Systems biology approaches identify ATF3as a negative regulator of Toll-like receptor 4. *Nature* **441**, 173–178 (2006).
66. Christiaen, L. *et al.* The transcription/migration interface in heart precursors of *Ciona intestinalis*. *Science* **320**, 1349–1352 (2008).

Acknowledgements

We appreciate the helpful comments from several reviewers. D.H.E. acknowledges support from the NASA Astrobiology Program, and E.H.D. from the National Science Foundation (IOS 0641398).

FURTHER INFORMATION

Douglas H. Erwin's homepage: http://paleobiology.si.edu/staff/individuals/erwin.html
Eric H. Davidson's homepage: http://www.its.caltech.edu/~mirsky/ehdavidson.htm
Biotapestry: http://www.biotapestry.org

SUPPLEMENTARY INFORMATION

See online article: S1 (figure)

ALL LINKS ARE ACTIVE IN THE ONLINE PDF