HHS Public Access

Author manuscript

Curr Opin Genet Dev. Author manuscript; available in PMC 2018 August 01.

Published in final edited form as:

Curr Opin Genet Dev. 2017 August ; 45: 115–123. doi:10.1016/j.gde.2017.04.006.

Enhancer evolution and the origins of morphological novelty

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Abstract

A central goal of evolutionary biology is to understand the genetic origin of morphological novelties – i.e. anatomical structures unique to a taxonomic group. Elaboration of morphology during development depends on networks of regulatory genes that activate patterned gene expression through transcriptional enhancer regions. We summarize recent case studies and genome-wide investigations that have uncovered diverse mechanisms though which new enhancers arise. We also discuss how these enhancer-originating mechanisms have clarified the history of genetic networks underlying diversification of genital structures in flies, limbs and neural crest in chordates, and plant leaves. These studies have identified enhancers that were pivotal for morphological divergence and highlighted how novel genetic networks shaping form emerged from pre-existing ones.

Keywords

enhancer origination; morphological novelty; gene regulatory network co-option

Introduction

A key problem in biology is to discern how the distinct features of different organisms arose at the genetic level. Of particular importance to morphological traits are the networks of transcription factors that control the expression of hundreds to thousands of downstream genes that confer upon each cell its distinctive physical properties [1]. Transcriptional control is mediated by *cis*-regulatory sequences, often called enhancers in cases of transcriptional activation and silencers in cases of repression, that recruit combinations of transcription factors to short binding sites that collectively determine when, where, and how much each gene is transcribed during development [2]. Thus, determining the evolutionary history of a morphological feature's regulatory network at the level of its participating enhancers provides key information on the origins of morphological novelty. We will review

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progress in studying the origins of enhancers and morphological novelties in the last two years.

How to build a new enhancer

New gene expression patterns may evolve through spatial or temporal changes in transcription (Figure 1A). Recent studies have highlighted a surprisingly wide range of molecular mechanisms that modify regulatory DNA (Figure 1B). Early papers by Britten and Davidson proposed that repetitive sequences such as those provided by transposons could contribute to gene regulation [3,4]. Many reports have since implicated transposable elements in the evolution of gene regulation [5–7]. Recently, genomic studies have investigated the significance of transposon-related enhancer birth genome wide. For example, transcriptome comparisons of uterine cells showed that thousands of genes gained expression during the evolution of mammalian pregnancy [8]. Transposons are enriched in the presumed regulatory regions of these genes, suggesting that they may have contributed to this major evolutionary transition. On the other hand, a comparative survey of enhancer-associated histone marks in the mammalian neocortex revealed that transposons were underrepresented in newly evolved neocortex enhancers [9]. These studies highlight that lineage specific trends may constrain the contribution of transposons to novel expression patterns, and raise the question of what direct impact they have on expression.

One recent study presented evidence that multiple families of transposons in mammalian genomes carry an interferon response element [10], and used CRISPR/Cas9 genome editing to demonstrate that these sequences are necessary for immune responses in cell culture. A striking example of a transposon insertion causing altered enhancer activity and concomitant morphological evolution was found in stickleback fish. Specifically, a change in body armor size that accompanied the transition from marine to freshwater environments was caused by a transposon insertion in the BMP-like *GDF6* gene which was associated with its increased expression [11]. While the insertion was necessary for this increase, it was itself insufficient to recapitulate the novel expression of GDF6 in a transgenic assay, indicating that other changes were involved in this morphological shift. The above findings underline how genome-wide and single gene approaches can provide complimentary insights into how molecular patterns shape differences in gene expression.

While transposition has made considerable contributions to the evolution of novel gene expression features, many additional mechanisms have been identified in mammals and other systems (Figure 1B). Although the evolution of enhancers *de novo* from mutations in non-functional sequences represents an obvious null hypothesis (Figure 1B), this mechanism has been difficult to study, and it more often turns out that new regulatory sequences have evolved from pre-existing ancestral ones. Studies of the domesticated chicken have shown how large-scale chromosome rearrangements and structural variations have contributed to diverse phenotypes by rearranging or duplicating regulatory elements and placing them in association with new genes (“promoter switching” in Figure 1B) [12–14]. Multiple studies have shown how novel expression domains can evolve from pre-existing enhancers that derive additional tissue specificities (“co-option” in Figure 1B) [15–18]. For example, novel domains of *Wingless* expression associated with unique spots of pigmentation in the

Drosophila guttifera wing evolved by modifying a pre-existing enhancer that was ancestrally active in a distinct wing location [17]. A study of the fly *Zaprionus capensis* uncovered an expression pattern in the larval wing disc that evolved through an extreme heterochronic shift (Figure 1A) that drove a pupal pattern much earlier into the larval life stage [18]. There is a growing appreciation that the introduction of mutations to an enhancer can often elicit its ectopic activation in additional locations [19,20]. Two recent papers by Farley and Levine illustrate how enhancers may either employ suboptimal binding affinity or suboptimal spacing between binding sites, which prevents ectopic activity [21,22].

Considering how frequently enhancers evolve by co-opting pre-existing activities, the role of pleiotropy in constraining their subsequent evolution has become increasingly appreciated. The loss of trichomes in *Drosophila sechellia* compared to other fly species, including *Drosophila melanogaster,* is one of the most well understood examples of morphological differences that have been dissected to the level of participating enhancers, mutations, and the binding sites they affect [23–25]. In an elegant set of experiments, Preger-Ben Noon *et al.* [26] performed a high-resolution identification of the binding sites that were gained and lost as an enhancer of the *shavenbaby* (*svb*) gene lost its trichome-patterning activity in the dorsal surface of *D. sechellia* larvae. Combining transcriptomic data on sorted epithelial cells with a computational analyses, they found that binding sites for the transcriptional activator Arrowhead were lost in *D. sechellia* [26]. Additionally, through a functional assay involving RNA interference for all detected transcription factors, they also found that the complete inactivation of this enhancer required evolution of a binding site for the spatially restricted repressor Abrupt. This transition was speculated to involve the gain of repressive inputs that allow maintenance of this enhancer's pleiotropic function in other tissues. In a similar case, the inactivation of a limb enhancer of the Tbx4 transcription factor in snakes occurred while preserving its function in the genitalia, likely contributing to evolution of the characteristic limbless body plan of these animals [27]. These findings illustrate how enhancers can disable linkages in gene regulatory networks, while maintaining pleiotropic functions in other tissues.

Network origins as a window into morphological novelties

To understand how new structures (i.e. "morphological novelties" [28–30]) evolve, one promising avenue of investigation is to trace the evolutionary history of their developmental networks. Several recent examples have leveraged the principles of enhancer evolution discussed above to study how novelties arose at a network level (Figure 2).

The posterior lobe of *Drosophila* male genitalia

Genital traits represent some of the most rapidly evolving morphologies in the animal kingdom, and among insects, these characters are key to species identification [31,32]. A recent study investigated the origins of a genital appendage, the posterior lobe (Figure 2A), present in the model organism *D. melanogaster* [33]. Development of this structure requires the transcription factor *Pox neuro* (*Poxn*) [34], and the authors used this pivotal gene to trace the network's evolutionary history. By examining an enhancer of *Poxn* that drives expression in the posterior lobe during pupal development, they found that its function had been co-

opted from another network deployed in the posterior spiracle, a structure that forms during embryonic development (Figure 3A). Interestingly, both the spiracle and lobe form in posterior regions of the *Drosophila* body plan, in a zone specified by the Hox gene *Abdominal-B* (*Abd-B*), a known regulator of several genes in the spiracle network [35]. The authors found that several genes of this ancestral network are active in the posterior lobe, and showed that at least seven enhancers active in this structure can be traced to activities in the posterior spiracle. In two enhancers, individual transcription factor binding sites were required for activity in both the spiracle and lobe contexts (Figure 3A). This demonstrates how tracing the origin of a network's enhancers can illuminate ancestral functions that would have been impossible to predict *a priori*.

Rewiring networks through cis and trans co-evolution during leaf shape evolution

Land plants show striking morphological variation, presenting attractive opportunities to study the contribution of regulatory evolution to morphological diversity in parallel to animals. One trait that has been extensively studied is leaf shape, particularly complex leaves with multiple leaflets, which have evolved repeatedly in seed plant lineages (Figure 2B). In the Brassicaceae family, complex leaves evolved from simpler forms, and work on two families of homeodomain transcription factors, KNOX (KNOTTED1-like homeobox) and REDUCED COMPLEXITY/LATE-MERISTEM IDENTITY1 (RCO/LMI1), has illuminated the molecular basis of this transition. Through a genetic screen in *C. hirsuta,* a complex-leaved *A. thaliana* relative [36] (Figure 2B), Vlad *et al*. identified the *RCO* gene [37]. *RCO* encodes an HD-ZIP class I transcription factor that promotes leaflet formation by repressing growth at focal points along leaf margins (Figure 3B). *RCO* arose in Brassicaceae through gene duplication of the floral regulator *LMI1,* and was secondarily lost in *A. thaliana*. Transgenic re-introduction of *RCO* from *C. hirsuta* into the genome of *A. thaliana* increased leaf complexity, indicating that its loss was a critical change for causing the simple leaf phenotype of this species. Diversification of *RCO* from *LMI1* arose through cis-regulatory evolution, which generated a novel and specific *RCO* expression domain at the base of developing leaflets in a region pivotal for shape determination. To investigate how this occurred, Vuolo *et al.* [38] discovered that a leaf-margin enhancer of *LMI1* which drives gene expression distally in leaf primordia was repurposed in the *RCO* paralog to drive expression proximally, flanking the emerging leaflets (Figure 3B). They also showed that a single amino acid substitution reduced RCO protein stability, which suppressed the potential pleiotropic effects of its altered expression. Both the regulatory and coding sequence changes in *RCO* show hallmarks of positive selection. Thus in this case, a potentially adaptive path for morphological evolution involved the neo-functionalization of an enhancer coupled with changes to its associated coding sequence, steps that limited pleiotropy while exploiting a novel expression domain. Interestingly, an *RCO*-like gene was also shown to underlie variation in leaf complexity between sister species in the related *Capsella* genus [39]. Hence, the *LMI1/RCO* genes likely define key nodes in an often-utilized network to modulate leaf shape.

While RCO restricts growth locally along the leaf margin, KNOX proteins actively promote outgrowth and patterning of leaflets. The expression of *KNOX* genes is associated with complex leaf forms, while simple leaves lack expression [40]. They are also active in the pluripotent Shoot Apical Meristem (SAM) from which leaves initiate. Their role in leaf complexity involves the partial redeployment of their SAM functions (for a review see [41]) of repressing differentiation and influencing cell polarity [42]. Previous work showed that *cis*-regulatory divergence at two *KNOX* genes, *SHOOTMERISTMLESS (STM)* and *BREVIPEDICELLUS* (*BP*), correlated with differences in leaf shape between *A. thaliana* and *C. hirsuta* [43]. However the functional significance of these regulatory differences were unknown. Rast-Somsich *et al*. showed that regulatory changes in the *C. hirsuta BP* gene were more potent than those at *STM* in terms of restoring complexity to *A. thaliana* leaves [44]. This result contrasted with the relative pleiotropy of these two genes, as mutations in *BP* had less widespread effects than *STM* on plant development in both *C. hirsuta* and *A. thaliana.* The resulting changes in *BP* expression introduced a new node in a small GRN that shapes leaf growth and promotes activity maxima of the indolic hormone auxin, which supports both leaf and leaflet initiation [44–46]. These findings indicate that regulatory divergence of weakly pleiotropic regulators like *BP* might offer favorable paths for morphological divergence to occur. Ichihashi *et al.* took a complementary genomics approach to study evolution of leaf complexity in the tomato lineage where this trait arose independently [47]. By conducting comparative transcriptome analyses between three species differing in leaflet number, they detected evolutionary changes in *KNOX*-related gene co-expression networks and identified a BOP transcription factor as an upstream modulator of KNOX activity and leaf shape [47].

The neural crest

The cartilage and skeletal elements of the vertebrate head embody an exceptionally complex novelty that allowed this group to transition to a predatory life style (Figure 2C). Of the cell types that contribute extensively to these structures, the neural crest stands out as a new tissue type whose origination was crucial to the evolution of this novelty [48]. Neural crest cells comprise a multipotent migratory population that invades multiple tissues along the anterior-posterior axis of the embryo, and subsequently differentiate into several different cell types. The neural domain that produces neural crest cells, the neural border, implements a highly conserved network that appears to predate the neural crest's emergence [49]. In contrast, the gene regulatory network underlying neural crest formation and migration (the NC-GRN) seems to be unique to vertebrates. Based upon comparative analyses of gene expression, it has been argued that many of the cell types derived from the neural crest (such as melanocytes, cartilage, connective tissues, and sensory neurons) already existed in basal lineages, and were redeployed in descendants of this new cell type [50]. This suggests many of the gene regulatory sub-circuits conferring neural crest-like behaviors and potencies predate final assembly of the full NC-GRN in the vertebrate lineage. One gene, *SoxE* plays an important role in the specification of neural crest fate. A recent study introduced a 186kb fragment encompassing the *SoxE* gene from amphioxus into zebrafish [51]. While this genomic fragment recapitulated the amphioxus pattern of *SoxE* expression, it failed to drive neural crest expression, suggesting that novel neural crest enhancers arose at this key gene.

However, a large number of cell types descend from the neural crest, and recent studies have made arguments for migratory populations in outgroup species that may share ancestry with the neural crest [52,53]. Detailed studies of enhancers within these networks may unveil their underlying homology relationships.

Novelties among vertebrate appendages

Some of the most striking morphological novelties reside in the appendages of vertebrates. Specifically, the tetrapod limb has novel elements in the wrist, ankle, and digits. As such, it represents a remarkably complex elaboration of the fin from an aquatic ancestor that had fewer skeletal and muscular elements (Figure 2C). The role of *Hox* genes in the evolution of the tetrapod limb has long been thought to correlate with a late phase of Hox expression in distal portions that form digits [54,55]. Two recent studies elegantly demonstrated that fish indeed have a late phase of *Hox* gene activity that is controlled by elements conserved with tetrapods [56,57]. Previous studies reported that the zebrafish versions of these elements fail to drive gene expression in the mouse limb bud, suggesting that these regions were novel to tetrapods [58]. However, this interpretation may have been complicated by derived features of zebrafish. Using the genome of the spotted gar [59], Gehrke *et al.* identified late Hox enhancers that drive distal fin expression [57]. While the zebrafish version of this enhancer lacks activity in a mouse reporter assay, the gar version is able to produce a pattern similar to that driven by the endogenous mouse enhancer. This result suggests that the lack of zebrafish activity likely reflects derived differences in zebrafish that cause its enhancer to no longer function in the mouse limb bud [57]. Such findings highlight the problem that tests for novelty in gene regulatory elements implicitly depend upon negative results (lack of activity), which may result from drift in the lineage displaying the ancestral trait rather than active changes in the lineage developing the novelty. Lineage tracing of cell populations marked by *Hoxd13a* enhancers, coupled with CRISPR/Cas9 knockout of *Hox13* paralogs in fish confirmed how this late phase of expression is required to pattern distal fin elements [56]. Collectively, these new findings suggest that known networks regulating limb development are ancient and that the changes underlying the evolution of the tetrapod limb lie in genes outside the *Hox* loci in this network. This work also illustrates how tracing a network's enhancers can clarify homology relationships among highly divergent traits. Given the age of the tetrapod limb (~370 MYA) it is likely that its evolution required multiple changes of small effect scattered throughout the genome.

Concluding remarks

The above studies show how the examination of enhancer history provides an important perspective into network origins and diversification. They underscore how enhancers lie at the heart of pleiotropic connections between different networks and also direct our attention towards their most evolutionarily relevant feature: the nodes that underlie trait diversity. The morphological features discussed above arose millions, or hundreds of millions of years ago, and their evolution probably involved coordinated changes in dozens to hundreds of genes. Thus, an important future challenge is to understand and quantify how the integration of multiple genetic changes produced such complex traits. In parallel, studies of the effectors of these GRNs that mediate morphogenesis will be a critical area of research. What are the key

genes causing cells to grow, collapse, mineralize or move to produce phenotypic diversity and how do they exert their effects? Answering these questions will require the combination of classical genetic approaches and genomics coupled with recently developed methods for quantitative and computational studies of development [60–62], cell biology, and precision genome engineering [63,64].

Acknowledgments

We thank members of the Tsiantis and Rebeiz lab groups for helpful discussions, and Sheila McCormick, Thomas M. Williams, and Daniel M. Medeiros for comments on the manuscript. Work on *Drosophila* genital evolution in the Rebeiz lab is supported by grants from the National Institutes of Health (GM107387 and GM112758). The Tsiantis lab is supported by Deutsche Forschungsgemeinschaft grants "Adaptomics" TS 229/1-1, SFB (Sonderforschungbereit) 680, and a core grant by the Max Planck Society. We thank Nicolas Gompel and Benjamin Prud'homme for organizing the 2014 Fondation de Treilles meeting on "The mechanisms of evolutionary changes and adaptation", where discussions relating to this review started.

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Highlights

- Genome-wide and single gene studies have revealed a variety of mechanisms by which new expression patterns arise
- Studying newly evolved morphologies (novelties) at the level of their regulatory sequences has provided key insights into the history of their genetic networks
- Pleiotropic connections between networks have resulted both from wholesale network co-options and expansion of regulatory sequence activity to new developmental contexts
- Targeted developmental changes in regulatory sequences have been shown to underlie morphological novelty in both animals and plants

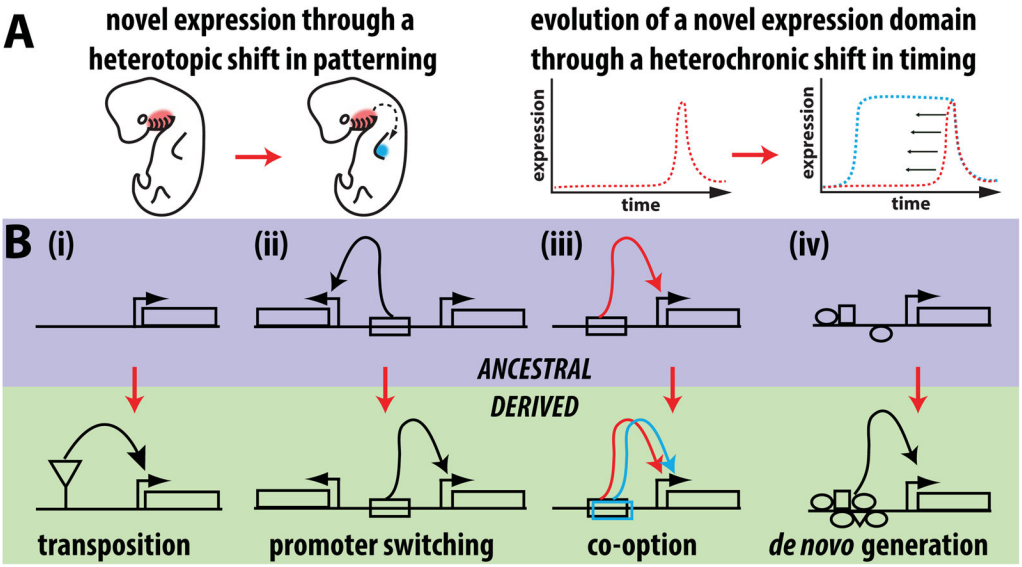

Figure 1. The genetic underpinnings of novel gene expression patterns and enhancer activities

(A) Novel expression patterns can arise through heterotopic shifts that cause a gene to be expressed in a spatially distinct pattern (left) or by temporal shifts that cause a gene to be expressed much earlier or later during development (right). (B) Genetic models for the origins of new enhancer activities. The ancestral state for each model depicts the status of a locus before a new expression domain evolved by changes in its cis-regulatory sequences. (i) A gene may gain a novel expression pattern through the introduction of a transposon that can carry regulatory information resulting in a new enhancer activity. (ii) Changes in mechanisms targeting enhancers to specific promoters (e.g. point mutations or large-scale chromosomal rearrangements) can cause a pre-existing enhancer to target a different promoter. (iii) A pre-existing enhancer active in an ancestral tissue may gain or lose inputs that allow it to be expressed in a novel domain. (iv) A stretch of DNA that ancestrally lacked regulatory function may evolve a *de novo* enhancer activity.

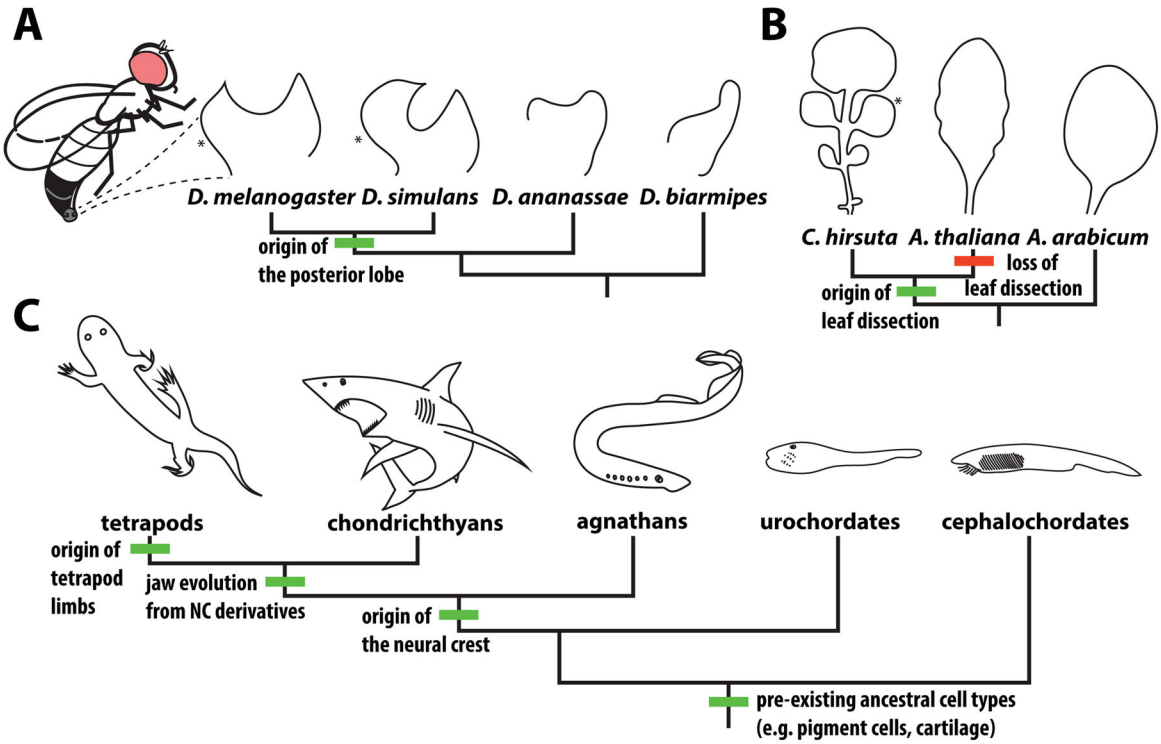

Figure 2. Morphological structures whose origins have been illuminated through the study of gene regulatory networks and their constituent enhancers

(A) The posterior lobe (*) is a genital outgrowth unique to males of *Drosophila* (*D.*) *melanogaster* and its close relatives that is required for mating. (B) Leaf dissection and the consequent presence of distinct leaflets (*) has arisen multiple times in seed plants. A convenient model for studying this trait is the complex leaf of the *Cardamine* (*C.*) *hirsuta*, which likely evolved from an ancestral simple leaf exemplified by *Aethionema arabicum,* a basally branching species in the Brassicaceae family. The well-studied model organism *Arabidopsis thaliana* has lost leaf dissection and has simple leaves with only slight serrations at their margins. (C) Chordate novelties. The neural crest (NC) is a novel migratory cell population that invades multiple tissues along the body axis, differentiating into several different cell types. NC derivatives play key roles in the development of the vertebrate jaw. Several of the cell types that neural crest cells ultimately adopt pre-date the origins of this dynamic cell population, which arose in the ancestor of vertebrates. The tetrapod limb evolved from fins that first appeared in jawless fish.

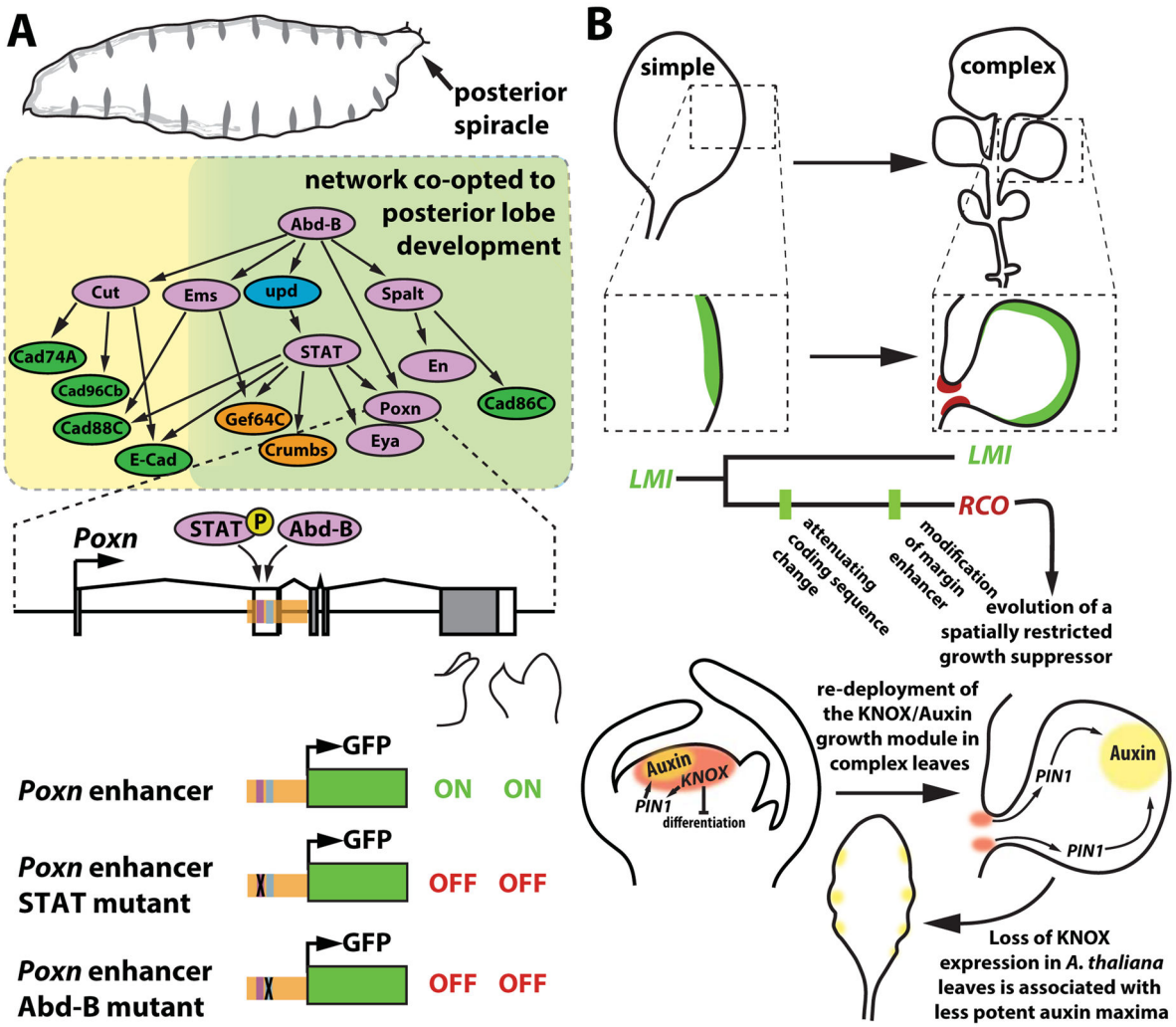

Figure 3. Tracing the origins of networks that participate in novelties

(A) Co-option of genes to the posterior lobe network. (top) Multiple genes of the posterior lobe network participate in the development of a larval breathing structure termed the posterior spiracle. Green shading delineates genes shared between the two networks. (bottom) The posterior lobe enhancer of the *Pox neuro* (*Poxn*) gene (orange shading) contains binding sites for STAT and Abdominal-B that are required for gene activity in both the novel posterior lobe and the ancestral posterior spiracle contexts. (B) The genetic basis for diversification of leaf shape in the Brassicaceae family. (top) In simple leaves, *LMI* is expressed at the leaf margin (green shading) and in floral tissues (not shown). The duplication of *LMI* resulted in a second copy of this transcription factor, named *RCO*, which evolved an enhancer specific to the base of leaflets (red shading), as well as an amino-acid substitution that reduced its pleiotropic effects on growth. The relative order of these regulatory and coding changes is unknown. (bottom) KNOX transcription factors have an ancestral role in the apical meristem, which involves suppressing differentiation and enabling organization of activity maxima of auxin that supports leaf initiation through the PIN1 transporter. This module was re-deployed in complex leaves to generate distal foci of

auxin that promote growth. The secondary loss of complex leaves in *A. thaliana* is partly due to loss of KNOX expression in developing leaf primordia. This loss likely reduced the morphogenetic potency of marginal auxin foci, contributing to shallower outgrowths. Note that leaves likely first evolved from ancestral branched shoots expressing meristem genes [65] which may account for the predisposition to reactivate meristem genes that contributes to repeated independent origins of leaf dissection in seed plants.