Cis-regulatory programs in the development and evolution of vertebrate paired appendages

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Abstract

Differential gene expression is the core of development, mediating the genetic changes necessary for determining cell identity. The regulation of gene activity by *cis*-acting elements (e.g., enhancers) is a crucial mechanism for determining differential gene activity by precise control of gene expression in embryonic space and time. Modifications to regulatory regions can have profound impacts on phenotype, and therefore developmental and evolutionary biologists have increasingly focused on elucidating the transcriptional control of genes that build and pattern body plans. Here, we trace the evolutionary history of transcriptional control of three loci key to vertebrate appendage development (*Fgf8*, *Shh*, and *HoxD/A*). Within and across these regulatory modules, we find both complex and flexible regulation in contrast with more fixed enhancers that appear unchanged over vast timescales of vertebrate evolution. The transcriptional control of vertebrate appendage development was likely already incredibly complex in the common ancestor of fish, implying that subtle changes to regulatory networks were more likely responsible for alterations in phenotype rather than the *de novo* addition of whole regulatory domains. Finally, we discuss the dangers of relying on inter-species transgenesis when testing enhancer function, and call for more controlled regulatory swap experiments when inferring the evolutionary history of enhancer elements.

Keywords: gene regulation, evo devo, fin-to-limb, transgenesis, developmental systems drift, topological domain

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1. Introduction

The vast majority (> 95%) of mammalian genomes consist of sequence that does not code for proteins, and much of this non-genic sequence harbors regulatory elements that control the activity of surrounding target genes. Genomic regions that drive specific patterns of target gene expression in time and space are deemed “enhancers” or “Cis-Regulatory Elements” (CREs), and consist of a series of short (~10 bp) transcription factor (TF) binding sites that can vary in both length and number for any given target gene [1-3]. As vertebrate genes involved in development are largely conserved (e.g., Carroll, 2008), there has been increasing interest in CREs as the potential substrate for the evolution of morphological features [4]. Evolutionary developmental biology (“evo-devo”) has seen an increasing number of examples revealing how even slight changes to a CRE can have profound effects on phenotypes [5-7].

Recent advances in functional genomics have allowed biologists to move beyond a snapshot of activity for a single CRE to identification and functional analyses of entire suites of CREs. Chromosome conformation capture techniques (e.g. 4C, 5C, HiC) provide the interaction profiles of specific loci or even entire genomes, without prior knowledge of enhancer-promoter interaction [8]. ChIP-seq can identify genome-wide binding profiles for a specific protein or provide patterns of histone modifications, and ATAC-seq can rapidly identify specific areas of open chromatin at the level of transcription factor binding sites [9, 10]. Thus, a more holistic picture of gene regulation is beginning to arise, revealing a dynamic and complex landscape of enhancer activity spread over hundreds of thousands or even millions of base pairs amidst changing landscapes of chromatin in different tissues types and species [11]. To complement the array of functional genomic techniques, we are experiencing an influx of whole genome sequence from across the tree of life. Thus, evolutionary and developmental biologists are beginning to identify, characterize, and manipulate entire complements of regulatory elements that are active in an embryonic structure of interest, and compare them among taxa in a phylogenetically-informed way. Armed with these data, we can ask: how are *cis*-regulatory programs orchestrated both between loci and across taxa?

The fins of bony fish and the limbs of tetrapods are composed of fundamentally distinct anatomies (Fig. 1B). How the pectoral fins of fish evolved into the limbs of tetrapods is a fundamental issue in evolutionary biology. Recent work in this field has increasingly turned attention to how modifications to regulatory regions have influenced the fin to limb transition. In this review, we summarize the regulatory architecture of three loci crucial to building the vertebrate appendage to understand how these programs have been assembled and deployed to build and pattern fins and limbs. These loci were chosen due to their established roles in shaping limb morphology (see mutants in Fig. 1C), and their regulatory control has been studied at some level. For each gene (*Fgf8* and *Shh*) and locus (*HoxD* and *HoxA* gene clusters), we begin by summarizing its role in tetrapod limb development and what is known about its transcriptional control in mouse. We then compare these regulatory programs to those enacting upon the orthologous locus in a variety of fish species. We discuss how regulatory regions might have been assembled over time and the potential implications of changes to these regions for the fin to limb transition. Overall, comparisons of regulatory programs between fish and tetrapods reveal complex and dynamic regulatory landscapes that have been deeply

conserved in throughout vertebrate evolution. While large domains of regulatory regions appear conserved, some loci appear to tolerate a high degree of enhancer position shuffling, while enhancers at other loci seem “locked” in their position. We end by discussing challenges in understanding the evolution of enhancer function across large phylogenetic distances, and call for increased attention to potential *trans*-regulatory changes when interpreting cross-species transgenics.

2. *Fgf8* enhancers in phylogeny: conservation, flexibility, and redundancy

In tetrapods, limb outgrowth is driven by a distal structure of ectodermal cells called the “apical ectodermal ridge” (AER), the removal of which results in the elimination of distal structures in a stage-specific manner (Fig. 1). Studies in chick identified members of the Fibroblast Growth Factor (*Fgf*) family as being important for AER function, as adding beads soaked in *Fgf* proteins could rescue removal of the AER in chick limbs [12, 13]. While multiple *Fgf* genes (*Fgf4*, *Fgf9*, *Fgf17*) are active in the AER, *Fgf8* is the only member expressed at the onset of the AER and throughout all cells of the structure [14]. In addition, *Fgf8* seems to be the only member whose activity is essential for limb development (Fig. 1)[14].

As the AER (of which *Fgf8* is a crucial component) is necessary for proper limb development, considerable attention has been taken to understanding how changes to AER dynamics may have influenced the evolution of vertebrate appendages [15, 16]. An AER with active *fgf8* has been revealed in a variety of fish species, where it shares a similar role with its murine ortholog to drive appendage bud outgrowth [17-19]. One noticeable difference when compared to tetrapod limbs is the transition from an AER to an apical fold (AF) in fish, where the AER transforms into a layer of cylindrical cells that will eventually build the actinotrichia (supporting structures) and lepidotrichia (rays) of fins [19]. This transition is important because it marks the addition of dermal skeleton to the endochondral elements of the proximal fin (Fig. 1). As the tetrapod limb consists only of endochondral bone, one potential hypothesis for the fin to limb transition is the postponing (or elimination) of the AF by extension of AER activity, possibly mediated by prolonged or bolstered *fgf8* [15]. Thus, understanding how *fgf8* is regulated in appendages across vertebrates may shed light on how changes in gene expression in the AER have influenced the fin to limb transition.

In mouse limbs, *Fgf8* expression in the AER is controlled by a series of enhancers that lie centromeric to the gene, spanning a region of ~170 kb. Marinic et al. identified 5 enhancers (CE58, 59, 61, 66, 80) using phylogenetic footprinting that drove distinct patterns of AER expression in transgenic mice (Fig. 2) [20]. These are an addition to two previously described elements (CR3 and CR4) by Beerman and colleagues that also drove transgene AER expression [21]. These studies reveal that *Fgf8* expression in the mouse limb is controlled by at least 7 distinct enhancers that drive nearly identical expression in a narrow strip of cells that make up the AER (Fig. 2). Moreover, the enhancers lie in a relatively gene-dense region, where the nearby “bystander” genes *Poll*, *Dpcd*, and *Fbxw4* somehow escape the activity of these AER enhancers and are instead expressed at ubiquitous low levels [20]. These studies present a complex, layered regulation of *Fgf8* in the AER of mouse limb buds.

To understand how *fgf8a* is regulated in developing pectoral fins, Komisarczuk et al. used phylogenetic footprinting to identify a series of candidate enhancers in zebrafish [22]. The authors found 11 conserved sequences that, when introduced into as reporter constructs into zebrafish, drove expression in a distinct AER pattern (Fig. 2). Of these regions, 4 of their murine orthologs were tested in mouse in other studies (dr11/CE62, dr10/CE64, dr1/CR3, dr17/CE80), yet only two of these regions (dr17/CE80 and dr1/CR3) were active in both mouse limbs and fish fins [20-22]. The zebrafish elements dr11 and dr10 drove expression in fins, but their counterparts in mouse did not drive limb expression, which could be due to either a loss of activity of these elements in the tetrapod lineage, or simply local position effects of the reporter cassette in the transgenic animal [20, 22]. Komisarczuk and colleagues identified an additional 7 zebrafish CREs that drove AER expression that did not overlap with those tested in previous mouse studies [22]. The majority of these elements have orthologs in mouse, raising the potential number of shared *Fgf8* enhancers between tetrapods and bony fish to 13 (Fig. 2).

Recently, genome sequences of a variety of non-model vertebrates have become available, allowing a more refined understanding of how the *Fgf8* locus and its regulatory elements have evolved. Teleosts (bony fish, including zebrafish and pufferfish) have an inversion of the *fgf8* and *fbxw4* genes when compared to tetrapods, and are missing the *btrc*, *poll*, and *dpcd* genes (Fig. 2) [22]. The genome of the spotted gar, *Lepisosteus oculatus*, a bony fish whose lineage split before the teleost radiation, contains the same inversion of the *fgf8* and *fbxw4* genes (Fig. 2) [23]. Furthermore, the gar genome has retained the *btrc*, *dpcd* and *poll* genes, showing that the ancestral inversion involved a series of genes (*fgf8*, *fbxw4*, *dpcd*, and *poll*), some of which were lost in the teleost lineage (Fig. 2). This large inversion coincides with the boundaries of a topological associated domain (TAD), which are partitioned areas of the genome that define the level of interaction between genomic loci therein (Fig. 2) [24, 25]. Further insight has come from the genome sequence of the coelacanth, one of the two living groups of aquatic lobe-finned fish more closely related to tetrapods than fish [26]. The coelacanth exhibits a tetrapod-like pattern of synteny surrounding *fgf8*, showing that the gene order present in mouse was established at least in the common ancestor of lobe-finned fishes (Fig. 2).

The common ancestor of ray-finned (actinopterygian) and lobe-finned (sarcopterygian, including tetrapods) may have had as many as 13 enhancers that drove AER activity (Fig. 2). The gar genome reveals that an inversion involving four genes occurred during the evolution of vertebrates that was not specific to teleosts, and likely involved an entire topological domain (Fig. 2). However, as the coelacanth exhibits a tetrapod-like arrangement, it is difficult to ascertain which lineage experienced the inversion event. Genome sequence from cartilaginous fishes may provide insight into how this genomic region evolved (although the synteny of the *fgf8* locus is elephant shark is very different in comparison to zebrafish, gar, and mouse). Regardless of when the inversion transpired, the retention of nearly all enhancers despite rearrangement shows remarkable conservation of what seem to be redundant activities (Fig. 2). The combination of functional studies of *Fgf8* limb regulatory elements and comparisons of non-model genomic sequence reveals a complex and dynamic system of regulation for the seemingly simple structure of the AER.

3. *Shh* regulation in appendages: conservation of landscape and function

While *Fgf8* is crucial for outgrowth of the limb, patterning the distal skeleton is in part controlled by the genes of the *Shh* pathway [27]. During tetrapod limb development, *Shh* is expressed in a posterior patch of cells called the “Zone of Polarizing Activity” (ZPA), where it acts as a morphogen to determine both digit identity and number in a concentration-dependent manner (Fig. 1)[27]. *Shh* is essential for the development of distal structures of the limb, as mutants show a nearly complete and specific loss of the autopod (Fig. 1)[28]. Gain of function experiments in chicks show that when *Shh* is ectopically expressed in the anterior side of the developing forelimb, a mirror image pattern of digits develops [29].

Shh activity in the mouse limb is driven by a regulatory element lying nearly 1 megabase (MB) distant from the coding region, named the “ZPA Regulatory Element” (ZRS). The interval between *Shh* and the ZRS contains a number of CREs that regulate *Shh* in other tissues such as the brain, notochord, lungs, gut and many other organs [30]. Surprisingly, the ZRS is the only known CRE that drives *Shh* activity in the appendage. Deletion of the ZRS results in the elimination of *Shh* signal in the limb, making it likely that a single enhancer in the intron of a gene 1 MB away from is responsible for *Shh* expression in vertebrate appendages [31]. Functional studies of the ZPA have narrowed the enhancer activity to a segment of ~780 bps (which can be further broken down to “activator” and “tethering” sequences), bound in part by ETS transcription factors [32].

The ZPA has been identified in a number of fish species, including zebrafish and skate, revealing an ancient role for *Shh* in the patterning of vertebrate appendages [33, 34]. Both the sequence and the position of the ZRS have been highly conserved throughout vertebrate evolution (Fig. 3). In all fish that have paired appendages, the ZRS is present in the fifth intron of the gene *Lmbr1*, and is separated from the *Shh* target gene by a large gene desert in all species (~900 kb in mouse and ~215 kb in elephant shark)(Fig. 3). As this configuration is conserved in elephant shark (a member of an ancient group of cartilaginous fish) it is likely that *Shh* regulation by the distant action of the ZRS is a conserved feature of all jawed fishes (gnathostomes), pushing the origin of this *cis* regulatory program to at least ~450 mya (Fig. 3). The functional activity of the ZRS also appears conserved across vertebrates, as the ZRS from pufferfish is able to drive an identical pattern of expression in transgenic mice as the endogenous murine ZRS (Fig. 3)[35]. While this experiment suggests conservation of function, a wider taxonomic survey of ZRS elements in a transgenic context is needed to assess the level of functional conservation.

Taken together, the regulation of *Shh* in vertebrate limbs is an extreme example of conservation on topological, *cis*, and *trans* levels (see section 5). The position of the ZRS in the fifth intron of *Lmbr1* is conserved in all vertebrates tested; yet the constraint acting upon its location is unclear, as the ZRS is able to act upon reporter genes that lie in different configurations across the locus [30]. Both the CREs and the TFs that bind them also appear to be conserved despite nearly 1 billion years divergence among the two lineages, as the *Fugu* ZRS is able to active expression identical to the native mouse

enhancer. Do any other crucial loci in appendage development show this great level of conservation?

4. The regulatory landscape of *Hox* genes in appendage evolution

Tetrapods contain 4 clusters of *Hox* genes (A-D), which are responsible for patterning the primary axis in bilaterians [36]. Two of these clusters, *A* and *D*, have crucial roles in building the secondary axis (limbs) in tetrapods [37]. The limbs of mice are built via two successive “phases” of *HoxD* and *HoxA* expression: an “early” phase that is associated with the arm and forearm, and a “late” phase that orchestrates the construction of the wrist and digits (autopod) (Fig. 1)[37]. A loss of early phase expression via gene knockout results in the absence of proximal structures, such as the forearm, while loss of the late phase results in the complete absence of the autopod (Fig. 1)[37]. The CRE’s that control the early and late phases of the *HoxD* cluster have been elucidated and examined in detail in mouse [38-40]. Two enhancers residing 3’ to the cluster control the early phase of *HoxD* expression, which is followed by a topological shift of chromatin allowing portions of the cluster to contact 5’ enhancers that control late phase expression and autopod development [38, 39]. The digit “regulatory archipelago” contains as many as seven CREs, though not all may be involved in driving late phase *HoxD* expression (Fig. 4) [40, 41]. *HoxA* activity is controlled by a similar regulatory topology, where a series of enhancers on one side of the cluster contact 5’ *HoxA* genes to drive expression in developing digits (Fig. 4) [42, 43]. Due to their crucial role in limb development, the evolution of *Hox* gene expression has become a central point of study in deciphering molecular mechanisms behind the fin to limb transition [15, 44].

Patterns of gene expression resembling early and late phase *hox* activity have been described in a number of fish species, including zebrafish, paddlefish, and catshark [45-47]. Expression pattern alone cannot be taken as evidence for conservation of underlying gene regulatory mechanisms, which has shifted recent focus to the identification of early and late phase enhancers in fish genomes [44]. Expression patterns are likely homologous in two species if the genes utilize the same regulatory architecture (CREs, TFs), which are unlikely to have evolved by convergence. At the epigenomic level, the zebrafish genome contains the two topological domains at *hox* loci that are seen in mouse, strongly suggesting a regulatory system common in fish and tetrapods (Fig. 4)[48]. Furthermore, studies of non-model fish species have revealed the presence of mouse “digit” enhancer orthologs in the genomes of a variety of fish [41, 49]. Taken together, these studies reveal conservation at both the epigenomic and the sequence level of the regulatory network that drives *Hox* expression in both limbs and fins (Fig. 4).

Despite the aforementioned conservation, functional validation of fish enhancers via transgenesis have yielded conflicting results, which in turn have important implications for how we define homology and how we view the evolution of the autopod. When tested in transgenic mice, late phase enhancers from teleosts (zebrafish, *Fugu*, *Tetraodon*) drive expression only in proximal structures of the limb (or not at all), and are notably absent from the autopod (Fig. 4)[41, 48-50]. A late phase enhancer (CsB) from the little skate drives expression in the autopod of transgenic mice, but not robustly in the developing digits [49]. These observations have led to the hypothesis that although late

phase enhancers are present in fish genomes, they are not functionally capable of activating a digit-producing program, and thus a type of “retrofitting” of fish late phase CREs was necessary to produce digits during the fin to limb transition [48]. However, CREs from the spotted gar, an actinopterygian fish that is not a teleost, are able to drive patterns of late phase expression in developing digits that are nearly identical to those of the endogenous enhancers in mouse (Fig. 4)[41]. Thus, the activities of fish enhancers, when introduced into transgenic mice, are variable. What does the diversity of patterns revealed by using different species in transgenic experiments imply for the evolution of *Hox* gene regulation in appendages, and for our understanding of the homology of skeletal elements in fins and limbs? The next section will discuss caveats to interpreting transgenic experiments in an evolutionary context.

5. Inter-species transgenesis and developmental systems drift

The proper activation of an enhancer relies on the interaction between *cis* (the DNA binding site), and *trans* (the TFs that bind it). Decades of study in the evolution of gene regulation have shown that both the sequence of the enhancer and the properties of the TFs evolve, though recent studies show greater instances of changes in *cis* [51, 52]. The standard and most widely used assay to determine the function of an enhancer is transgenesis, where the regulatory element of interest is placed upstream of minimal promoter and a reporter gene and injected to make a transgenic host organism. Interpreting transgenics in a native organism is more straightforward (i.e. an element from the mouse genome tested in a transgenic mouse), as the *cis* element is tested in its endogenous *trans* environment. However, evolutionary developmental biologists, who are by definition interested in a comparative approach, are faced with a more challenging interpretation of inter-species transgenesis. Biologists are limited to model systems that are amenable to the assay of interest, and reliable transgenesis is a technique that often takes years to perfect in emerging systems. The result is a nearly limitless amount of donor sequences with only a few host species with which to test them. Thus, it is often impossible to test regulatory elements in their natural host, and the necessary evil of inter-species transgenesis must be relied upon. When testing a regulatory element in any organism other than the original host, careful consideration must be taken in what can be interpreted from the results.

Transgenic enhancer experiments involve two variables: donor (the *cis* DNA element), and host (the organism that will receive the *cis* element). As mentioned previously, when donor DNA and the host organism match (i.e. mouse in mouse), reproducible patterns of activity can be attributed solely to the regulatory element tested. When the donor and host species do not match (a “swap”), interpretations must take into account potential evolution in *cis*, *trans*, or both [52]. The number and type of conclusions that can be made from a “swap” experiment have been reviewed in detail elsewhere [52]. The most relevant type of swap for this discussion is one where the activity of the donor *cis* element in its native *trans* environment is either unknown or impossible to ascertain (often the case for non-model systems). Without knowledge of the enhancer capability in its native *trans* environment, no negative conclusion can be made concerning the activity of that element in another host organism. Foreign CREs may fail to drive expression in a transgenic host of a different species for a number of

reasons that are irrelevant to the evolution of phenotype. In such “non-controlled” swap experiments, evolution in *trans* cannot be ruled out, and thus causality or direction of evolution cannot be inferred [52]. The ability of underlying genetic systems of homologous characters to change in function is called “Developmental Systems Drift” (DSD), and is a phenomenon involving *cis* and *trans* coevolution that always must be considered when interpreting inter-species transgenic experiments [52-55].

When we consider the *Hox* appendage enhancer discussion in section 4 in this context, the manner in which inter-species transgenic experiments are interpreted becomes crucial for making statements about the evolutionary history of the limb. When assayed in transgenic mice, *hox* enhancers show a variety of different expression patterns depending on the phylogenetic position of the fish (Fig. 4 and Fig. 5)[41, 48]. While these regulatory elements have differing activities in the context of the developing mouse, all enhancers tested from fish are active in the pectoral fin of transgenic zebrafish (Fig. 5)[41, 49]. Thus, phylogenetic distance between the donor and host organism is a factor that cannot be ignored, as only when *hox* enhancers from some fish are placed in the context of a different *trans* environment (mouse) does the function drift (Fig. 5). The inability of some fish enhancers to drive expression in the autopods of transgenic mice is not due an intrinsic lack of function in these CREs, but developmental systems drift between donor and host species. Fish and tetrapods contain orthologous enhancers that may underlie homologous structures, yet the *trans* system of the host cannot always properly decode donor *cis* in a “swap” due to local evolution in each species.

6. Conclusions

This review has attempted to survey the evolution of regulatory control of three loci that are crucial to the development of paired appendages. This type of comparative analysis has been aided by the rise of functional genomics, allowing researchers to define entire landscapes of regulatory activity rather than a single enhancer-by-enhancer search. Comparisons of these loci and their regulatory control reveal a number of common themes.

First, and arguably most importantly, there appears to be a deep conservation of general regulatory architecture in fins and limbs. While there are degrees of flexibility in enhancer number and genomic position, in general we see the same regulatory logic in place in both fins and limbs. These commonalities are likely due to TADs, which are were likely defined early in vertebrate evolution and constraint contact between genes and their surrounding regulatory environment [56].

Second, there is a remarkably diverse level of enhancer flexibility within the general constraints of specific TADs. For example, the *Fgf8* locus contains ~12 enhancers that are shared between mouse and fish, a seemingly large number of regulatory elements to drive a small distal strip of gene expression in the limb and fin. Within this regulatory region, enhancers have persisted despite large local rearrangement via genomic inversion. One could argue that the plethora of *Fgf8* enhancers allows flexibility or redundancy to buffer the activity of a gene that is required for limb development. However, the opposite situation exists in the *Shh* locus, a gene equally important for appendage development. *Shh* expression in paired appendages is regulated by a single enhancer that lies close to 1 MB away from its promoter, in the same intron of

the same gene in every vertebrate taxon studied. The ZRS appears “locked” into position, whereas other enhancers (such as those regulating *Fgf8*) appear free to diverge in relative position after inversion of the TAD. Interestingly, transposon-induced rearrangement of reporter gene position within the *Shh* TAD has shown that the ZPA pattern of limb expression persists despite vastly different positions of the target gene relative to the ZRS [30]. This implies that the *Shh* TAD does not contain strict subunits, and the TAD is a generally open domain free to receive input from individual enhancers without affecting bystander genes [30]. It remains unclear why, despite the evidence provided by these experimental manipulations, certain loci appear to be more “flexible” in their regulatory control, while others appear “locked”.

Finally, while we see overall conservation of regulatory landscapes in *Fgf8*, *Shh*, and *Hox* loci, it is important to note the potential effects of enhancer number and/or activity potential within these domains. The *HoxD* and *HoxA* clusters of mouse appear to have a number of tetrapod-specific enhancers in the “digit archipelago”, implying that gain of additional late phase enhancers (such as CsC) may have had a role in the fin to limb transition and the evolution of the autopod (Fig. 4)[57]. However, as the presence/absence of these enhancers is based only on sequence conservation, an exhaustive definition of the complete regulatory landscape of *hox* genes in multiple fish species is necessary to ascertain whether there was a gain in enhancer number throughout evolution of the autopod. In addition, evolutionarily conserved enhancers have been found to evolve new intrinsic functions, again making it clear that functional assays must be performed when defining enhancers [58]. As functional assays are critical to elucidating enhancer landscapes, and often take the form of transgenesis between species, we have argued here for the careful analysis of the results of such experiments. DSD may make enhancers between species incompatible when swapped, even though they pattern homologous structures with conserved regulatory inputs in their native environments. Transgenesis in the native organism (or one as phylogenetically close as possible) must always be performed to make a controlled swap [52].

7. Future directions: a phylogenetic perspective on functional genomics

The development of high-throughput, whole-genome technologies that require a relatively small (or even single) input of cells has a potentially transformative impact on evolutionary studies [59-63]. Developmental biologists interested in the evolution of phenotype have the potential to complement the work done in model species, such as chicks and mice, with work on an ever-increasing diversity of species that was previously impossible due to technical limitations of bulk tissue needed. Armed with these tools, virtually any species has the potential to become a “model” of sorts. By being able to select taxa with relevant phenotypes, phylogenetic histories, and genomic structures, biologists can bring increasing granularity to comparisons, thereby allowing ever more refined tests of hypotheses of the genetic basis of morphological transformations. Finally, a greater understanding of regulatory landscapes across taxa will identify meaningful targets for direct genomic manipulation in order to test hypotheses of evolutionary scenarios [64]. Functional genomics, performed in the context of as wide a phylogenetic breadth as possible, will provide valuable insight for the future of evo devo.

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Figure legends

Fig. 1. Summary of vertebrate appendage development and mouse mutants highlighting the role of key genes. A) The forelimbs of mice and the pectoral fins of zebrafish utilize similar gene networks during development; *Fgfs* in the AER, *Shh* expression in the posterior mesenchyme, and both early and late phases of *Hox* gene expression. Late phase *Hox* expression (both *hoxa* and *hoxd*) is shown based on the activity pattern of late phase enhancers in zebrafish described in [41]. The blue and red colors are meant to denote the area in which early and late phases are active, and not the specific nested patterns of individual *Hox* genes. Note the early fin fold in the 48 hpf zebrafish fin (shown in yellow to denote *fgf8a* expression), a structure that does not develop in tetrapods. B) Despite these conserved expression patterns, tetrapod limbs and zebrafish fins are morphologically disparate. The mouse limb is composed entirely of endochondral bone, while the zebrafish fin consists of a base of endochondral bone followed by a long series of fin rays consisting of dermal bone. C) Mutant mice underscore the key nature of the genes discussed. Conditional knockouts of *Fgf8* in mouse results in either a lack of the stylopod and parts of the zeugopod [65], or are smaller and missing distal structures [66]. Removal of the AER (and thus *Fgf* activity) in chicken results in the arrest of limb development, in a time dependent context [67]. Mice lacking *Shh* expression the limb fail to develop an autopod, leaving only a single ambiguous digit [28]. Loss of early phase *HoxD* and *HoxA* expression leads to a loss of proximal structures of the limb, while loss of late phase expression results in complete deletion of the autopod [37].

Fig. 2. The evolution of the *Fgf8* regulatory landscape. Blue boxes denote enhancers that were identified and tested in zebrafish, red boxes indicate those that were identified and tested in mouse. Multicolored boxes indicate the same enhancers that were found independently in mouse and fish. In some instances, the murine ortholog of a validated

zebrafish enhancer did not drive expression in mouse (e.g. CE62 from mouse). The enhancer that did not show activity in transgenic mice is denoted by a lighter color. An inversion of the *Poll*, *Dpcd*, *Fbxw4*, and *Fgf8* genes took place either in the ancestor of bony fish (Actinopterygii) or in the ancestor of lobe-finned fish (Sarcopterygii) (denoted by grey segment). This was likely possible due to the inversion of an entire topological domain encompassing these genes, which can be seen in mouse Hi-C data (top). This topological domain is flanked by CTCF sites, a protein with a known role in chromatin organization that often marks the boundaries of topological domains [68]. Mouse genes are drawn according to scale to match the mouse Hi-C data, while the size of fish genes are stylized in order to best emphasize enhancer location and gene loss. Orthologs of previously reported enhancers were identified using the mVista LAGAN program, calc window: 100 bps; min cons width: 100 bps; cons identity: 70% [69]. Mouse Hi-C data and CTCF ChIP-seq images are from the 3D genome browser (<http://www.3dgenome.org>) according to mm9 region chr19:45,430,000-45,830,000.

Fig. 3. *Shh* expression in the paired appendages of vertebrates is controlled by a single regulatory element with deeply conserved topology and function. A) The *Shh* regulatory landscape showing the position of the ZRS in the fifth intron of *Lmbr1*. This enhancer traverses a topological domain (denoted by red triangle according to [30]) of nearly 1 MB in mouse to contact its target gene, a long distance regulation that is conserved in all vertebrates with paired appendages. B) The ZRS is functionally conserved, as the ZRS from *Fugu* is able to drive an identical pattern of expression to the endogenous enhancer in mouse [35].

Fig. 4. The evolutionary history of *Hox* gene regulation in distal appendages. A) Digit expression of *Hoxd13* and *Hoxa13* in mouse is driven by a series of enhancers that lie specifically on one side of the *Hox* cluster, spread over a distance of ~500 kb. These regulatory elements fall with topological associated domains (shown as red triangles) that define the regulatory landscape of *Hox* expression [40, 42]. Studies in zebrafish have shown that the same topological domains are present in fish [41, 48], along with orthologs of specific mouse digit enhancers [41, 49]. Potential mouse-specific enhancers are shown in blue, those that are shared with fish are colored purple. For the zebrafish/gar *HoxA* cluster, the topological domain was found in zebrafish, while the orthologs of the enhancers represented are from the gar genome. B) Summary of transgenic experiments with *Hox* digit enhancers. *Cis*-elements from mouse are denoted in blue, those from zebrafish.

Fig. 5. Interpreting inter-species transgenics in a phylogenetic context. A potential evolutionary scenario for the *cis/trans* interaction of the Island I *Hox* enhancer based on reciprocal transgenics [41]. Here, zebrafish likely underwent evolution in both *cis* and *trans* (developmental systems drift), making the host *trans* environment of mouse incapable of interpreting the *cis* element of zebrafish.

Figure_1

Figure_2

Figure_3

Figure_4

Figure_5