Contributions of 5’HoxA/D regulation to actinodin evolution and the fin-to-limb transition

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ABSTRACT The evolution of tetrapod limbs from paired fish fins comprised major changes to the appendicular dermal and endochondral skeleton. Fish fin rays were lost, and the endochondral bone was modified and elaborated to form three distinct segments common to all tetrapod limbs: the stylopod, the zeugopod and the autopod. Identifying the molecular mechanisms that contributed to these morphological changes presents a unique insight into our own evolutionary history. This review first summarizes previously identified cis-acting regulatory elements for the 5’HoxA/D genes and actinodin1 that were tested using transgenic swap experiments between fish and tetrapods. Conserved regulatory networks provide evidence for a deep homology between distal fin structures and the autopod, while diverging regulatory strategies highlight potential molecular mechanisms that contributed to the fin-to-limb transition. Next, we summarize studies that performed functional analysis to recapitulate fish-tetrapod diverging regulatory strategies and then discuss their potential morphological consequences during limb evolution. Finally, we also discuss here some of the advantages and disadvantages of using zebrafish to study molecular and morphological changes during the fin-to-limb transition.

KEY WORDS: actinodin, 5’HoxA/D, fin-to-limb transition, regulatory conservation, regulatory divergence

Introduction

The evolution of the tetrapod limbs from paired fish fins involved drastic changes to the appendicular dermal and endochondral skeleton (Ahlberg and Clack 2006; Long et al., 2006; Shubin et al., 1997; Shubin et al., 2006; Schneider and Shubin 2013). The fin dermal skeleton of extant teleosts, consisting of the calcified lepidodotrichia fin rays and collagenous actinotrichia fibrils, is completely absent in tetrapod limbs and the limb endochondral skeleton has been modified to form three distinct segments: the stylopod, the zeugopod, and the autopod. (Ahlberg and Clack 2006; Grandel and Schulte-Merker 1998; Mari-Beffa and Murciano 2010; Schneider and Shubin 2013; Shubin et al., 2006; Tamura et al., 2008; Yano and Tamura 2013). Using genomic, molecular, and developmental data from a phylogenetically-broad range of fish species, in comparison with existing tetrapod models, researchers are now focused on identifying the molecular mechanisms that contributed to the evolution of limbs from paired fish fins (Fromental-Ramain et al., 1996; Nakamura et al., 2016; Scotti et al., 2015; Shubin et al., 1997; Standen et al., 2014; Tulenko et al., 2017; Zakany and Duboule 2007).

Understanding mechanisms of developmental divergence between fins and limbs provides insight into molecular and morphological changes during the fin-to-limb transition. Early fin and limb development are remarkably similar, with both structures relying on signalling from the apical ectodermal ridge (AER) and the zone of polarizing activity (ZPA) to establish axial patterning (Harfe et al., 2004; Harfe 2011; Heikinheimo et al., 1994; Heude et al., 2014; Mercader 2007; Ohuchi et al., 1997; Saunders 1948; Summerbell 1974; Suzuki 2013; Tickle and Eichele 1994; Yano et al., 2012) (Fig. 1A, D). One of the earliest morphological differences between fin and limb development is the transition of the AER into the apical fin fold in fish and the formation of rigid fibrils (actinotrichia) supporting the fold (Bouvet 1974; Géraudie et al., 1977, 1985; Wood and Thorogood 1984; Zhang et al., 2010) (Fig. 1B). During tetrapod limb development, no apical fin fold or actinotrichia form, and the AER is maintained until later stages (E14 in the mouse

Abbreviations used in this paper: actinodin; 5’HoxA/D, cis-acting regulatory element; Hox, Homeobox-containing gene; NTR/MTZ, Nitroreductase/Metronidazole.
forelimb) (Lu et al., 2008; Martin 1990; Wanek et al., 1989) (Fig. 1G). Following the formation of the fin fold, mesenchymal cells will invade the fold distally using the actinotrichia as a scaffold and the proximal fin mesenchyme will condense and differentiate into chondrocytes to form the endoskeletal disc (Dewitt et al., 2011; Grandel and Schulte-Merker 1998; Lalonde et al., 2016; Wood and Thorogood 1984) (Fig. 1B). The adult zebrafish pectoral fin skeleton consists of both intramembranous (lepidotrichia) and endochondral bone (proximal and distal radials) (Dewitt et al., 2011; Konig et al., 2017; Grandel and Schulte-Merker 1998) (Fig. 1C). In contrast, the tetrapod limb skeleton is composed entirely of endochondral bone (Fig. 1E-G) (Kronenberg 2006; Mackie et al., 2008; Martin 1990; Patton and Kaufman 1995; Wanek et al., 1989).

A powerful molecular tool for uncovering evidence of regulatory conservation or divergence during the fin-to-limb transition is to perform fish-tetrapod transgenic swap experiments, where cis-acting regulatory elements (CREs) are tested for activity between species (Gehrke and Shubin 2016; Gordon and Ruvinsky 2012). The existence and activity of appendicular-specific cis-acting regulatory elements for the Homeobox-containing A (HoxA) and Homeobox-containing D (HoxD) clusters, and actinodin (and) genes has been previously studied (Amemiya et al., 2013; Berlivet et al., 2013; Kherdjemil et al., 2016; Lalonde et al., 2016; Schneider et al., 2011; Schneider and Shubin 2013) (Table 1). This review first summarizes 5’HoxA/D and actinodin enhancer transgenic swap experiments that provide evidence for regulatory conservation and divergence between fish and tetrapods, and is followed by a review of experimental data testing the morphological implications of 5’HoxA/D and actinodin regulatory changes on fin and limb evolution.

Homeobox-containing (Hox) genes code for transcription factors that contribute to axial patterning of many structures during development, including fins and limbs (Burke et al., 1995; Kessel and Gruss 1991; Krumlauf 1994). Amniotes possess four Hox clusters (HoxA, HoxB, HoxC, HoxD), while teleosts, including zebrafish, have had implications for the loss of the dermal skeleton (Lalonde et al., 2010). The loss of the actinodin gene family during tetrapod evolution is proposed to have contributed to the loss of fin dermal bone (Lalonde et al., 2016; Zhang et al., 2010). We uncovered evidence that modulation of 5’HoxA/D activity may have had consequences for actinodin regulatory evolution as well. Transgenic swap experiments provide insight into differential activity of actinodin enhancers between fish and tetrapod species, including the potential contributions to the loss of this gene family in tetrapods, and functional analysis can test the effects of modulated actinodin activity on fin dermal bone formation.

### Regulatory conservation and the autopod debate

It has long been debated whether the autopod represents a distinct do novo structure that evolved during the fin-to-limb transition or whether it is a modification on pre-existing distal fish fin structures (Shubin et al., 2006; Shubin and Alberch 1986; Sordino et al., 1995; Woltering et al., 2010; 2014). Due to major morphological differences between distal fish fin elements and the tetrapod autopod, these structures tend to not be considered homologous in a classical sense (Grandel and Schulte-Merker 1998; Mari-Belfa and Murciano 2010; Tamura et al., 2008; Yano and Tamura 2013). Nevertheless, advances in the fields of molecular and developmental biology have revealed a deep homology between distal fin structures and the autopod (Davis 2013; Fromental-Ramain et al.,

### TABLE 1

<table>
<thead>
<tr>
<th>Element (Donor Species)</th>
<th>Host Mouse</th>
<th>Host Zebrafish</th>
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<tr>
<td>actinodin regulatory elements</td>
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<tr>
<td>Epi (Zebrafish)</td>
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<td>2PAEpi (Zebrafish)</td>
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<td>m-inta11 (Mouse)</td>
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<td>HoxD/hoxD regulatory elements</td>
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<tr>
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<td>Island I (Coelacanth)</td>
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<td>Island I (Zebrafish)</td>
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<td>Island II (Mouse)</td>
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<td>CsB (Spotted gar)</td>
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<td>CsB (Skate)</td>
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<td>CsC (Mouse)</td>
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<td>HoxA/hoxA regulatory elements</td>
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<td>e13 (Mouse)</td>
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<td>e16 (Mouse)</td>
<td>Functional</td>
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Enhancer name and donor species are indicated in the first column. Functionality in mice and zebrafish is presented in the second and third column respectively. If the elements were not tested in a host species, this is indicated (Amemiya et al., 2013; Berlivet et al., 2013; Gehrke et al., 2015; Kherdjemil et al., 2016; Lalonde et al., 2016; Schneider et al., 2011; Schneider and Shubin 2013).
1996; Nakamura et al., 2016; Schneider and Shubin 2013; Scotti et al., 2015; Shubin et al., 1997; Zakany and Duboule, 2007). Deep homology describes the evolutionary relationship of two structures that develop through shared genetic regulatory networks, even when they are morphologically and phylogenetically distinct (Shubin et al., 2009). While conserved regulatory activity during transgenic swap experiments may support homology between two tissues, it must also be noted alternatively that distinct groups of cells may have independently coopted regulatory strategies during the course of fin and limb evolution. This section will summarize 5’HoxA/D and actinodin1 fin/limb enhancers that show conserved regulatory activity when tested using transgenic swap experiments between different fish species and mice (Gehrke and Shubin 2016; Gordon and Ruvinisky 2012).

**Fish (donor) to mouse (host)**

5’HoxA/D genes contribute to the appendicular skeletal patterning in fish and tetrapods (Ahn and Ho 2008; Fromental-Ramain et al., 1996; Nakamura et al., 2016; Zakany and duboule 2007). During both fin and limb development 5’HoxD genes have two distinct waves, or phases, of expression that are controlled by limb enhancers are active in the zebrafish distal fin fold mesenchyme, while “CsB” drives reporter activity more proximally, aligning with their activity in the mouse.

Actinotrichia in fish fins are composed of Collagen type I and II, and Actinodins, a structural protein that is secreted from both the fin fold ectoderm and fin fold mesenchyme (Duran et al., 2011; Zhang et al., 2010). We have previously identified and described cis-acting regulatory elements that drive actinodin1 (and 1 and 2) expression in the fin fold ectoderm and mesenchyme, known respectively as “Epi” and “2PAEpi” (Lalonde et al., 2016) (Fig. 1B). Despite the disappearance of the actinodin (and) genes from the tetrapod genome, the and1 ectodermal fin fold enhancer “Epi” shows conserved regulatory activity in the presumptive autopod ectoderm of reporter transgenic mouse, supporting a homology between the fin fold and autopod ectoderm (Lalonde et al., 2016). Using *in silico* analysis, a putative binding domain for Tcf proteins has been identified within the “Epi” enhancer. This protein family represents a good candidate for “Epi” activation as multiple tcf/Tcf genes are

Multiple fish late-phase 5’hoxA/D enhancers that show activity in the zebrafish fin fold mesenchyme also show regulatory conservation in the presumptive autopod mesenchyme. These results have been extensively covered in other reviews (Gehrke and Shubin 2016; Paço and Freitas 2017; Pieretti et al., 2015), and support homology between fish larval fin fold mesenchyme and the autopod mesenchyme of tetrapods. Briefly, the orthologous late-phase hoxD enhancer “Island I” from the spotted gar and coelacanth, the orthologous late-phase hoxD enhancer “CsB” from the spotted gar and the skate, and the orthologous late-phase hoxA enhancer “e16” from the spotted gar all show conserved functionality when tested in the mouse (Amemiya et al., 2013; Gehrke et al., 2015; Schneider et al., 2011; 2013). The spotted gar “Island I”, and “e16” enhancers are active in the zebrafish distal fin fold mesenchyme, while “CsB” drives reporter activity more proximally, aligning with their activity in the mouse.

![Fig. 1. Overview of zebrafish pectoral fin and mouse limb skeletal development.](image)

(A-C) Zebrafish pectoral fin development at 2 days post fertilization (dpf), 3dpf and 25-30dpf. (D-G) Mouse forelimb development at E11.5, E12.5 and E14.5. At 2dpf, the zebrafish pectoral fin consists of a bud possessing an apical ectodermal ridge (A). At 3dpf, the fin fold is supported by actinotrichia (purple lines) and distal fin mesenchyme migrates though the fin fold using the actinotrichia as a scaffold (green cells) (B). The proximal mesenchyme condenses and chondrifies to form the endoskeletal disc (blue cells) (B). At 25-30dpf, the proximal radials are still composed of cartilage (numbered 1-4), and the lepidotrichia have started calcifying. Actinotrichia are restricted to the distal tip of each fin ray (C). The mouse forelimb starts with the formation of a bud very similar to the pectoral fin bud (D). From E11.5 to E14.5 cartilaginous templates will form for the three limb segments: stylopod, zeugopod and autopod (E-G). The AER regresses after E12.5 in the mouse forelimb (F,G). AC, actinotrichia; AER, apical ectodermal ridge; ED, endoskeletal disc; LP, lepidotrichia; PR, proximal radials.
expressed in the fin fold ectoderm of zebrafish and the autopod ectoderm of mice, and tcf7 GFP enhancer trap zebrafish mutants show median and pectoral fin defects, with GFP localizing in the fin fold ectoderm (Gray et al., 2004; Nagayoshi et al., 2008).

Mouse (donor) to fish (host)

A tetrapod-specific cis-acting regulatory element has been previously identified and described located within the intron of the mouse Hoxa11, titled “m-Inta11”, that shows activity in the presumptive autopod during mouse limb development (Kherdjemil et al., 2016; Kherdjemil and Kmita 2017). ChIP experiments have shown both HOXA13, and HOXD13 preferentially bind to this regulatory element during mouse limb development, and Hoxa13 (-/-)/Hoxd13 (-/-) double knockout mice show loss of enhancer activity (Kherdjemil et al., 2016). When tested in zebrafish, this enhancer is able to drive reporter expression in the distal fin fold mesenchyme, specifically within a subpopulation of hoxa13b- and hoxd13a-expressing cells (Fig. 2), supporting a homology between distal fin mesenchyme and presumptive autopod mesenchyme (Kherdjemil et al., 2016; Lalonde and Akimenko 2018). Although we recognize the enhancer itself is an example of regulatory divergence, the results reveal the signalling pathways required for enhancer activation are conserved between ray-finned fish and tetrapods.

Interestingly, “m-Inta11” is only activated in the region where the three hox13 genes (hoxa13a, hoxa13b, hoxd13a) are expressed (Fig. 2C). It is not activated in the proximal-posterior region endoskeletal disc where only hoxa13b and hoxd13a are expressed (Fig. 2C), nor the anterior fin fold mesenchyme where hoxa13a and hoxa13b are expressed (Fig. 2). This observation provides evidence that either all three Hox13 proteins are required to activate this enhancer in the pectoral fin, or simply Hoxa13a and Hoxd13a together. Other possibilities are that the activation of this enhancer is “Hox” dose-dependent or relies on transcription factor heterodimer formation (Funnell and Crossley 2012). Several developmental processes, including digit patterning, are regulated in a dose-dependent manner by multiple 5’HoxA/D proteins (Zakany et al., 1997). Furthermore, the heterodimeric complexes of Hox:Meis or Hox:Pbx with various other protein partners have been shown to yield different functional outcomes depending on the proteins involved (Amin et al., 2015; Gordon et al., 2010). One final alternative is that differential enhancer activating capacities may exist between Hoxa13 paralogous proteins in zebrafish (Fig. 2C).

Mouse late-phase HoxD enhancers “Island I, II, IV” also show conserved activity in the limb presumptive autopod and the zebrafish distal fin. All three regulatory elements drive reporter expression in the distal fin fold mesenchyme, in addition to more proximal fin regions (Gehrke et al., 2015).

Regulatory divergence: zebrafish to tetrapods

Although an initial conserved genetic regulatory system underlies both fin and limb development, it is also known that major differences in fin/limb morphology, including novel autopodial identity, are supported by diverging regulatory strategies (Freitas et al., 2012; Leite-Castro et al., 2016; Paço and Freitas 2017; Yano and Tamura 2013). This section will first discuss instances of tetrapod-specific cis- and trans-regulatory evolution that have been linked to changes in S’HoxA/D and actinodin expression.
during limb evolution. *Cis*-regulatory evolution refers to changes in enhancer elements/transcription factor binding domains, while *trans*-regulatory evolution refers to changes to the transcription factors (Gehrke and Shubin 2016; Gordon and Ruvinsky 2012). Absence of conserved enhancer elements in fish provides additional evidence for regulatory novelty during autopod evolution. We will also summarize evidence that the expression pattern of Hoxa11 in the pectoral fin of zebrafish may represent a derived state in teleost fish.

**Novel regulatory elements: tetrapod-specific Innovations**

The “m-Inta11” element represents a 5’HoxA/D regulatory change that can be directly attributed to endochondral bone changes during the fin-to-limb transition (Kherdjemil et al., 2016; Kherdjemil and Kmita 2017). This regulatory element is activated by HOXA13 and HOXD13 in the distal limb mesenchyme of mice, and shows conserved functionality in distal fin fold mesenchyme of zebrafish (as discussed in section I) (Fig. 2, 3A-B, Fig. 5). In tetrapods, the “m-Inta11” enhancer drives the expression of long non-coding RNAs starting from the Hoxa11 exon 1 locus, resulting in the repression of Hoxa11 from the distal limb bud domain where Hoxa13 and Hoxd13 are expressed (Fig. 3D). Prior to Hoxa13 and Hoxd13 activation, conserved functionality in distal fin fold mesenchyme of zebrafish (as discussed in section I) (Fig. 2, 3A-B, Fig. 5). In tetrapods, the “m-Inta11” enhancer drives the expression of long non-coding RNAs starting from the Hoxa11 exon 1 locus, resulting in the repression of Hoxa11 from the distal limb bud domain where Hoxa13 and Hoxd13 are expressed (Fig. 3D). Prior to Hoxa13 and Hoxd13 activation,
hoxa13a, hoxa13b, and partially with hoxd13a (Fig. 3C) and no enhancers were identified in the hoxa11a, and hoxa11b intronic regions when these regions were tested in transgenic reporter constructs in zebrafish (Ahn and Ho 2008; Kherdjemil et al., 2016). The “m-Inta11” regulatory element therefore represents a novel tetrapod-specific enhancer that can be directly linked to changes in regulation of Hoxa11 during limb evolution.

Mouse late-phase HoxD enhancers “Island II, III, IV”, and “CsC” may also represent novel tetrapod enhancers that contributed to changes in regulation of 5′HoxD genes. Using sequence alignment, only “Island III” was found to be conserved in the spotted gar genome (Gehrke et al., 2015; Gonzalez et al., 2007; Montavon et al., 2011). When the putative “Island III” enhancer, and orthologous “Island II”, and “Island IV” regions from the spotted gar were tested in zebrafish, they were not able to drive reporter expression in the pectoral fin suggesting an absence of cis-regulatory elements at these loci in fish (Gehrke et al., 2015). However, it should be noted that the activity of these elements was only observed in primary injected fish with mosaic transgene integration. It may be beneficial to observe these elements in stable transgenic lines with the addition of eye/heart markers (cryaa, cmloc2) for screening purposes (for example, see methods in Kherdjemil et al., 2016). Furthermore, only 3 of 7 mouse autopodial HoxA enhancers (e10, e13, and e16) were identified by sequence analysis in spotaged gar, with only “e16” being tested for activity in zebrafish (Berlivet et al., 2013; Gehrke et al., 2015). Importantly, absence of sequence conservation is not sufficient enough evidence that these enhancers do not exist in fish. The zebrafish “Island I” enhancer was not identified from sequence alignments, yet this element was shown to drive reporter expression in the distal pectoral fin fold mesenchyme (Gehrke et al., 2015). The existence of 5′HoxA/D autopodial enhancers in mice that are not conserved in fish provide evidence that enhancer evolution may have contributed to novel autopodial identity in tetrapods (Berlivet et al., 2013; Gehrke et al., 2015; Montavon et al., 2011).

**actinodin regulatory evolution**

We have previously shown the mesenchymal and1 enhancer “2PΔEpi”, which drives expression in the zebrafish pectoral and median fin fold mesenchyme, is not functional in the mouse highlighting the possibility that changes in regulation occurred during the fin-to-limb transition (trans evolution in tetrapods) (Lalonde et al., 2016). Strong evidence suggests that mesenchymal actinodin1 activation may be dependent on one or more 5′HoxA/D proteins. Firstly, the “2PΔEpi” regulatory element contains five HoxA13 putative binding sites, as well as one site for Hoxd13 and Hoxa11 each (Lalonde et al., 2016). When testing this element for enhancer activity using transgenic reporter zebrafish, we see a drastic decrease in its ability to drive reporter expression when a putative binding site for Hox proteins, termed “Mes 1”, has been removed (Fig. 4). Secondly, “2PΔEpi” drives reporter expression in the pectoral fin in a manner very similarly to that of “m-Inta11”, which is activated by Hoxa13a and Hoxd13a (Fig. 3B, 4). We therefore propose that 5′HoxA/D regulatory changes during the fin-to-limb transition may have also had consequences on the expression of actinodin1 in the mesenchyme.

Due to the phylogenetic distance between zebrafish and mice, the trans machinery of the host (mouse) may not be able to decode the donor (zebrafish) cis sequence (Gehrke and Shubin 2016; Gordon and Ruvinsky 2012; True and Haag 2001). This process is called Developmental Systems Drift (DSD) and suggests that inability of “2PΔEpi” to drive reporter expression in the mouse limb autopod may not be due to the intrinsic lack of activity of the enhancer, but due to cis and trans coevolution in zebrafish (Gehrke and Shubin 2016; Gordon and Ruvinsky 2012; True and Haag 2001) (Fig. 6). Previous cases of mouse trans machinery being unable to decode zebrafish cis sequences has been described with teleost 5′hoxA/D Enhancers. The zebrafish and pufferfish orthologous “CsB” and the zebrafish orthologous “Island I” regulatory elements are not functional in transgenic reporter mice (Gehrke et al., 2015; Woltering et al., 2014). In contrast, when the spotted gar orthologous “CsB” and “Island I” were tested in transgenic reporter mice, they showed conserved regulatory activity with their mammalian counterparts, as summarized in Gehrke et al., 2015 (Gehrke et al., 2015). Indeed, the zebrafish “2PΔEpi” may have experienced sequence divergence (relative to more basal ray-finned fish) (cis evolution), paired with transcription factor modulation (trans evolution) that render it unrecognizable to the tetrapod orthologous transcription factors (cis trans coevolution) (Gehrke and Shubin 2016; Gordon and Ruvinsky 2012; True and Haag 2001). Therefore limited conclusions and interpretations can be drawn when testing the functionality of the zebrafish “2PΔEpi” in mice. This observation raises the questions about the choice of animal species when investigating evolutionary research questions. The spotted gar is a more basal ray-finned fish, whose lineage split off prior to the teleost-specific whole genome duplication. The spotted gar genome is believed to have experienced less sequence divergence compared to zebrafish due to its unduplicated state,
et al., et al., Nonfunc

hours post fertilization (hpf), the zebrafish “Island I” is restricted tested for reporter activity in zebrafish (Gehrke gar or mouse version (Fig. 7). All three “Island I” elements were “Island I” shows differential activity when compared to the spotted teleosts. This portion will summarize enhancer data that support expression in zebrafish may represent a derived modification in regulatory modulation, the absent or partial late-phase that the evolution of the autopod involved tetrapod-specific 5 HoxD expansion may be linked to autopod evolution (Ahn and Ho 2008; Woltering and Duboule 2010) (Fig. 3A-B). More recent expression analysis across a phylogenetically broad range of fish species, however, has concluded fish have a definite second phase of 5’HoxA/D regulation and actinodin evolution (Ahn and Ho 2008; Lalonde et al., 2016) (Fig. 3B, 4). The “m-Inta11” expression, supporting the notion that 5’HoxD expression during the fin-to-limb transition, the spotted gar orthologous “2PΔEpi” cis-acting regulatory element should exist (Tulenko et al., 2017).

“Posteriorization” of hoxd13a: a teleost-specific modification

It has been shown that zebrafish display absent or partial second-phase of hoxd13a expression, supporting the notion that 5’HoxD expansion may be linked to autopod evolution (Ahn and Ho 2008; Woltering and Duboule 2010) (Fig. 3A-B). More recent expression analysis across a phylogenetically broad range of fish species, however, has concluded fish have a definite second phase of 5’HoxD expression during fin development; however there appears to be species-specific variation in the degree of this second phase (Paço and Freitas 2017). Therefore, while it is generally accepted that the evolution of the autopod involved tetrapod-specific 5’HoxD regulatory modulation, the absent or partial late-phase hoxd13a expression in zebrafish may represent a derived modification in teleosts. This portion will summarize enhancer data that support this hypothesis.

Firstly, the zebrafish orthologous late-phase hoxD enhancer “Island I” shows differential activity when compared to the spotted gar or mouse version (Fig. 7). All three “Island I” elements were tested for reporter activity in zebrafish (Gehrke et al., 2015). At 55 hours post fertilization (hpf), the zebrafish “Island I” is restricted to the posterior half of the pectoral fin fold while the spotted gar “Island I” extends much more anteriorly (Fig. 7). Similarly, the mouse “Island I” shows more anterior activity in the pectoral fin at 48hpf compared to the zebrafish version (Gehrke et al., 2015) (Fig. 7). However, it should be noted that late phase 5’HoxA/D expression occurs uniquely in the distal fin fold mesenchyme starting around 60hpf and data regarding later transgene expression has not been reported (Ahn and Ho 2008). By 72hpf, the pectoral fin fold has significantly elongated compared to 55hpf, and much more mesenchyme has invaded the fin fold (Grandel and Schulte-Merker 1998; Mari-Beffa and Murciano 2010). Therefore, these elements should be observed at 72hpf to get a more accurate picture of their late-phase activity in the distal pectoral fin fold. In addition to these enhancer activity analyses in zebrafish, the zebrafish “Island I” enhancer is not functional in mice providing evidence of teleost-specific enhancer sequence divergence (Gehrke et al., 2015). It should also be noted that the zebrafish “Island I” enhancer was not detectable by sequence analysis and therefore the zebrafish amplified region might not contain all elements corresponding to the orthologous spotted gar and mouse “Island I”.

Next, both “m-Inta11” and “2PΔEpi” enhancers show stronger activity in the posterior pectoral fin fold mesenchyme (Kherdjemil et al., 2016; Lalonde et al., 2016) (Fig. 3B, 4). The “m-Inta11” enhancer is activated by Hoxa13a and Hoxd13a, and due to the similar domains of activity, we believe “2PΔEpi” element is regulated similarly in the zebrafish pectoral fin (Fig. 5). The regulatory consequence of “m-Inta11” activity in the tetrapod lineage is the complete repression of Hoxa11 from the distal limb domain (Kherdjemil et al., 2016) (Fig. 3C). Therefore we predict the activity of “m-Inta11” to correspond completely with the regions of distal limb Hoxa11 expression when this enhancer element would have evolved. In zebrafish, the expression of hoxa11b extends much more anteri-
and expression occurs in both the anterior and posterior fin fold ectoderm (Lalonde et al., 2016; Zhang et al., 2010).

**Morphological consequences during fin and limb evolution**

The past section summarized major advances in identifying potential regulatory changes during limb evolution; however the most exciting discoveries are able to link changes at a molecular level to functional consequences at a morphological level. This section will first highlight the evidence linking the “m-Inta11” element to the evolution of the “pentadactyl” or 5-digit state, in tetrapods, followed by a summary of experimental data predicting the potential implications of actinodin changes during the fin-to-limb transition, and finally will discuss the implications of 5’HoxA/D modulation during both fin and limb evolution.

**Hoxa11 distal repression and the pentadactyl state in tetrapods**

To determine the functional consequences of Hoxa11 distal repression on appendicular development following the evolution of the “m-Inta11” regulatory element, a Hoxa11 conditional gain-of-function allele (Rosa<sup>Hoxa11</sup>ΔEpi) was used to ectopically express Hoxa11 in the presumptive autopod mesenchyme. All homozygous gain-of-function mutants displayed polydactylous limbs, an ancestral state, providing evidence that the distal repression of Hoxa11 during evolution contributed to the “pentadactyl” or 5-digit state in tetrapods (Kherdjemil et al., 2016; Kherdjemil and Kmita 2017). In addition, “m-Inta11” deletion mutants and Hoxa11<sup>-/-</sup> Hoxd13<sup>Δ/-</sup> loss-of-function mutants express Hoxa11 in the distal limb bud, further confirming the regulatory consequences of “m-Inta11” activity during limb development (Fig. 3D). Finally, the functionality of “m-Inta11” in zebrafish (as discussed in section I) reveals the elements required for activation, including HOXA13/HOXD13 proteins, were conserved in the common ancestor of tetrapods and bony fish (Kherdjemil et al., 2016).

**Loss/downregulation of actinodin genes and appendicular dermal bone loss in tetrapods**

Morpholino-mediated knockdown of actinodin1 and actinodin2 leads to an absence of actinotrichia, impaired fin fold development and defects in fin fold mesenchyme migration (Zhang et al., 2010). Knockdown of either paralog individually yields no actinotrichia defects, highlighting their ability to compensate for one another. No functional analysis has been performed on the shorter actinodin paralogs and3/4; however their expression patterns are similar to and1/2 during larval fin development (Zhang et al., 2010). Fin fold mesenchyme has been shown to directly contribute to fin ray fibroblast and osteoblast populations (Lee et al., 2013; Nakamura et al., 2016). It is therefore proposed that actinotrichia defects, through changes in actinodin expression, may have contributed to the loss of fin rays during the fin-to-limb transition (Zhang et al., 2010; Lalonde et al., 2016). Due to the transitory limitations of morpholino oligonucleotides effects once injected in zebrafish embryos, the impact of actinotrichia defects on fin ray formation is unknown. Loss-of-function actinodin mutants are required to observe how fin ray development proceeds in the absence of actinotrichia.

To directly observe the effects of fin fold mesenchyme defects on fin ray formation, these cells were ablated during zebrafish
larval development using the nitroreductase/metrondazole (NTR/MTZ) system (Lalonde and Akimenko 2018; Mathias et al., 2014). Briefly, this system allows for the conditional ablation of NTR targeted cells of transgenic zebrafish upon addition of MTZ to the fish water. The MTZ prodrug is converted into a cytotoxic compound by the NTR, inducing cell death. The ablation of these cells resulted in impaired larval fin fold development, actinotrichia defects, and defects during fin ray formation. The presence of fin ray defects supports the hypothesis that these cells are crucial for proper fin ray formation, and that mis-migration of these cells during the fin-to-limb transition may have contributed to the loss of dermal rays in fish (Lalonde et al., 2016; Nakamura et al., 2016; Ahn and Ho 2008). These results also reveal fin fold mesenchyme defects may also have implications for actinodin expression and actinotrichia maintenance. As these cells migrate distally through the larval fin fold they secrete actinodin proteins (Duran et al., 2011; Lalonde et al., 2016). Following ablation of these cells, mesenchymal actinodin1 activity is decreased; in turn, actinotrichia defects occur, which may lead to a failure of surviving mesenchymal cells to migrate correctly (Lalonde and Akimenko 2018).

Based on the phenotypes observed following fin fold mesenchyme ablation, it is proposed that these cells contribute to their own successful migration through their production and secretion of actinodin proteins. This concept raises the intriguing possibility that even minor changes in actinodin regulation during the fin-to-limb transition may have yielded drastic fin ray defects (Ahn and Ho 2008; Lalonde and Akimenko 2018; Nakamura et al., 2016).

5’HoxA/D modulation during fin and limb evolution

During limb evolution the proximal anterior endoskeletal bone elements were lost (pro- and mesopterygium), and the distal endoskeletal bone elements became more expanded and elaborate to form the autopod (Fig. 8). The tetrapod stylopod is considered to be homologous to the posterior most proximal bone elements in chondrichthyan and basal actinopterygian fish, the metapterygium (Fig. 8A-C) (Onimaru et al., 2015; Tanaka 2016). In contrast, zebrafish possess highly reduced pectoral fin endochondral bone, having lost the metapterygium and the proximal-most pro- and mesopterygial bones (Mabee et al., 2004). The pro- and mesopterygial radials are retained (Fig. 8D). In this final section, we will summarize overexpression data, mutant models and cell ablation experiments that investigate the role of 5’HoxA/D regulatory changes in endochondral bone evolution during the fin-to-limb transition.

Although we have proposed that the absence or partial late phase hoxd13a expression in zebrafish may be a teleost-specific modification, it does not preclude the possibility that changes in 5’HoxA/D regulation during tetrapod evolution contributed to limb endochondral bone expansion (Paco and Freitas 2017). To assess the effects of hoxd13a modulation on endochondral bone formation, transient methods were used to overexpress hoxd13a in the zebrafish distal fin domain (Freitas et al., 2012). Briefly, hoxd13a was fused to a glucocorticoid receptor to create a hormone-inducible construct that allows for temporal control of overexpression. Freitas et al., 2012 found that 40% of fish display properties consistent with endochondral bone expansion and fin fold reduction (Freitas et al., 2012). Endochondral bone identity was confirmed using cartilage stais and chondrocyte markers and additional molecular markers were used to highlight a shift towards a “distal limb fate” (Freitas et al., 2012). Unfortunately due to the transient nature of these experiments, and high mortality rates among affected fish, analysis on radial and fin ray formation was not possible. It may be beneficial to revisit these experiments using stable transgenic zebrafish lines incorporating other methods of inducible transgene expression (Akerberg et al., 2014; Knopf et al., 2010; Mosimann et al., 2011). Consistently, the ablation of hoxa13a/hoxd13a-expressing mesenchyme during larval development, using the NTR/MTZ system described above, shows a decrease in expression of these genes and decreased endoskeletal disc size; further implicating 5’HoxA/D genes during endochondral bone formation and evolution (Lalonde and Akimenko 2018). Furthermore, it is predicted that modulation of 5’HoxA/D may be linked to shifts in anterior-posterior positional identity and the loss of the proximal anterior bone elements (pro- and mesopterygium) during limb evolution (Onimaru et al., 2015; Tanaka 2016). Early fin and limb anterior-posterior patterning is in part established by opposing gradients of SHH and GLI3R (Hill et al., 2009; Litingtung et al., 2002; Prykozhij and Neumann 2008). Increased transcript levels of 5’HoxA/D during limb evolution would have promoted SHH-signaling and the inhibition of GLI3R conversion, leading to an expansion of posterior positional identity (Onimaru et al., 2015; Tanaka 2016). This hypothesis awaits experimental investigation.

Functional analysis of hox13 genes (hoxa13a, hoxa13b and hoxd13a) in zebrafish has recently been performed using loss-of-function CRISPR mutants. Double knockout (hoxa13a-/-, hoxa13b-/-) and triple mosaic knockout (hoxa13a-/-, hoxa13b-/-, hoxd13a-/-) mutants formed shorter defective fin rays, and produced
an increased number of distal radial bones, a potential phenotype predicted during the fin-to-limb transition (Nakamura et al., 2016; Sordino et al., 1995; Tamura and Yano 2013; Tamura et al., 2008). It should be noted that while the resulting morphology at a tissue level may mimic processes that occurred during tetrapod evolution, the molecular mechanisms are evidently different as both Hoxa13 and Hoxd13 are retained in tetrapods (Nakamura et al., 2016, Yano and Tamura 2008). It is proposed that hox13 zebrafish mutants show defects in fin fold mesenchyme migration, resulting in a differential allocation of these cells to the proximal fin bud and a shift in fate from dermal to endochondral bone progenitors (Nakamura et al., 2016). Fin fold mesenchyme migration defects are not explored in hox13 mutant fish, and should be considered a priority to determine the mechanisms of fin ray loss/distal radial expansion. While multiple fluorescent reporter lines are available that label fin fold mesenchyme, we recognize the time constraints of recreating hoxa13a-/-, hox13b-/- homozygous mutants within transgenic reporter backgrounds (Kawakami 2007; Kherdjemil et al., 2016; Lalonde et al., 2016). As hox13 loss-of-function zebrafish mutants do not recreate the expression of Hoxa13 and Hoxd13 in tetrapods, additional information is required to integrate these results with proposed 5'HoxA/D regulatory changes during the fin-to-limb transition.

In the hoxd13a overexpression study and the hox13 loss-of-function zebrafish mutants, larval fin fold structure is observed by performing an in situ hybridization for and1 (Freitas et al., 2012; Nakamura et al., 2016). We have previously discussed the inclusion of Hoxa13a and Hoxd13 as positive regulators of and1 in the mesenchyme. While the results of hoxa13a overexpression (decreased and1) seem to dispute this conclusion, we would argue these results are not mutually exclusive and would like to highlight the importance of discussing tissue-specificity when investigating actinodin1 expression (Freitas et al., 2012; Zhang et al., 2010). Fin fold ectodermal and mesenchymal and1 expression begins at different stages of pectoral fin development, relying on distinct sets of transcription factors (Lalonde et al., 2016; Zhang et al., 2010). Conclusions using "global" and1 fin transcript levels on whole mount samples may therefore be difficult to interpret. While and1 expression is sufficient as a fin fold marker, examining tissue-specific changes using and1 reporter lines could shed light on actinodin regulation by 5'HoxA/D proteins and the implications during the fin-to-limb transition (Lalonde et al., 2016; Lalonde and Akimenko 2018).

Conclusions

Combining regulatory data and functional analysis provides crucial insight into some of the molecular mechanisms that contributed to morphological changes during the fin-to-limb transition. Conserved regulatory strategies between fish and tetrapods highlight a deep homology between distal fin and limb structures, while diverging strategies illuminate instances of evolutionary novelty and trait loss (autopod and appendicular dermal bone, respectively). We have highlighted how the zebrafish has remained a powerful model organism for performing transgenic swap experiments, however the derived nature of the genome and fin morphology limit interpretations regarding the fin-to-limb transition. Instead, it is becoming increasingly more common to study a phylogenetically broad range of fish species to draw conclusions regarding molecular and morphological change during limb evolution. The advances in fish genome availability and genome editing technology will serve as invaluable tools that will greatly facilitate future studies.

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