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

ROBERT L. LALONDE* and MARIE-ANDRÉE AKIMENKO*

Department of Biology, University of Ottawa, Ottawa, ON, Canada

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 lepidotrichia 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: and, actinodin; CRE, *cis*-acting regulatory element; *Hox, Homeobox-containing* gene; NTR/MTZ, Nitroreductase/Metronidazole.

***Address correspondence to:** Robert L. Lalonde. Department of Biology, University of Ottawa, 30, Marie Curie, Ottawa, ON, K1N 6N5, Canada. Fax: 613 562 5486. Tel: 613 562 5800 ext: 6329. E-mail: RobertL.Lalonde@gmail.com - web: https://akimenkolabuottawa.wordpress.com - https://orcid.org/0000-0002-1803-0983 or Marie-Andree Akimenko. Department of Biology, University of Ottawa, 30, Marie Curie, Ottawa, ON, K1N 6N5, Canada. Fax: 613 562 5486. Tel: 613 562 5800 ext: 7399. E-mail: Marie-Andree.Akimenko@uottawa.ca - web: https://akimenkolabuottawa.wordpress.com - https://orcid.org/0000-0002-1905-7731

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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; Kherdjmeil *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 7 clusters due to a whole genome duplication event followed by a loss of the entire paralogous second *HoxD* cluster (Ahn and Ho 2008; Burke *et al.,* 1995; Kessel and Gruss 1991; Krumlauf 1994).The spatial and temporal expression of these genes along the head-tail axis correlates with their position on the chromosome, with those located at the telomeric (3') side being expressed earlier and more anteriorly than those at the centromeric (5') side of the cluster (Zakany *et al.,* 2004; Zakany and Duboule 2007). Modulation of 5*'HoxA/D* activity has been consistently linked to endochondral skeletal changes during the fin-to-limb transition, including the evolution of the autopod (Freitas *et al.,* 2012; Leite-Castro *et al.,* 2016; Kherdjemil *et al.,* 2016; Kherdjemil and Kmita 2017; Paço and Freitas 2017; Tanaka 2016; Yano and Tamura 2013). We have also uncovered evidence that modulation in *5'HoxA/D* activity may have had implications for the loss of the dermal skeleton (Lalonde *et al.,* 2016). Transgenic swap experiments provide insight into the differential existence and activity of *5'HoxA/D* enhancers between fish and tetrapod species, and functional analysis can test the effects of modulated *5'HoxA/D* activity during fin and limb skeletal development.

The *actinodin* (*and*) genes code for structural proteins in the actinotrichia, the first exoskeletal elements formed during embryonic fin development (Fig. 1B). Zebrafish possess four *actinodin* genes (*actinodin1-4* (*and1-4*)), two long paralogs and two short paralogs, owing to the teleost-specific whole genome duplication event (Zhang *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.

TABLE 1

SUMMARY OF TRANSGENIC SWAP EXPERIMENTS BETWEEN FISH SPECIES AND MICE

Element (Donor Species)	Host Mouse	Host Zebrafish
***actinodin* regulatory elements**		
Epi (Zebrafish)	Functional	Functional
2PΔEpi (Zebrafish)	Not Functional	Functional
***Hoxa11* regulatory elements**		
m-Inta11 (Mouse)	Functional	Functional
***HoxD/hoxD* regulatory elements**		
Island I (Spotted gar)	Functional	Functional
Island I (Coelacanth)	Functional	Not Tested
Island I (Zebrafish)	Not Functional	Functional
Island II (Mouse)	Functional	Functional
Island II (Spotted gar)	Not Tested	Not Functional
Island III (Mouse)	Functional	Not Tested
Island III (Spotted gar)	Not Tested	Not Functional
Island IV (Mouse)	Functional	Functional
Island IV (Spotted gar)	Not Tested	Not Functional
CsB (Mouse)	Functional	Functional
CsB (Zebrafish)	Not Functional	Functional
CsB (Spotted gar)	Functional	Functional
CsB (Skate)	Functional	Not Tested
CsC (Mouse)	Functional	Not Tested
HoxA/hoxA regulatory elements		
e10 (Mouse)	Functional	Not Tested
e13 (Mouse)	Functional	Not Tested
e16 (Mouse)	Functional	Not Tested
e16 (Mouse)	Functional	Functional

Enhancer name and donor species are indicated in the first column, functionality in mice and zebrafish are 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).

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-Beffa 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.,*

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 Ruvinsky 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 regulatory landscapes on either side of the cluster (Ahn and Ho 2008; Freitas and Zhang 2007; Woltering *et al.,* 2014; Zakany *et al.,* 2004). The first phase (Fig. 3A) is controlled by enhancer elements located on the telomeric side of the cluster, known as the early limb control region (ELCR), and the second or "late" phase (Fig. 3B-C) is regulated by enhancer elements on the centromeric side of the cluster, called the global control region (GCR) (Tarchini and Duboule 2006; Zakany *et al.,* 2004). All *5'HoxA* limb enhancers are located on the telomeric side of the cluster interspersed between neighbouring genes (Berlivet *et al.,* 2013; Gehrke *et al.,* 2015). 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.

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* (*and1*) expression in the fin fold ectoderm and mesenchyme, known respectively as "Epi" and "2PΔEpi" (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

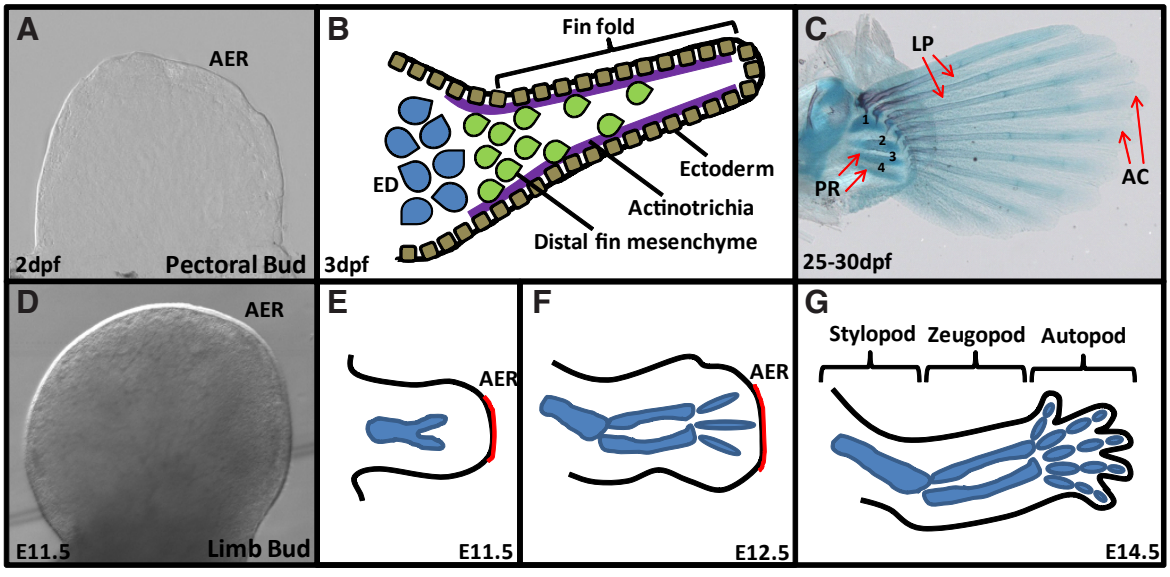

Fig. 1. Overview of zebrafish pectoral fin and mouse limb skeletal development. (A-C) *Zebrafish pectoral fin development at 2 days post fertilization (dpf), 3dpf and 25-30dpf.* **(D-G)** *Mouse forelimb development at E11.5, E12.5 and E14.5. At 2dpf, the zebrafish pectoral fin consists of a bud possessing 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 E.14.5 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 *hoxa13a/b-* 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 *5'HoxA/D* and *actinodin* expression

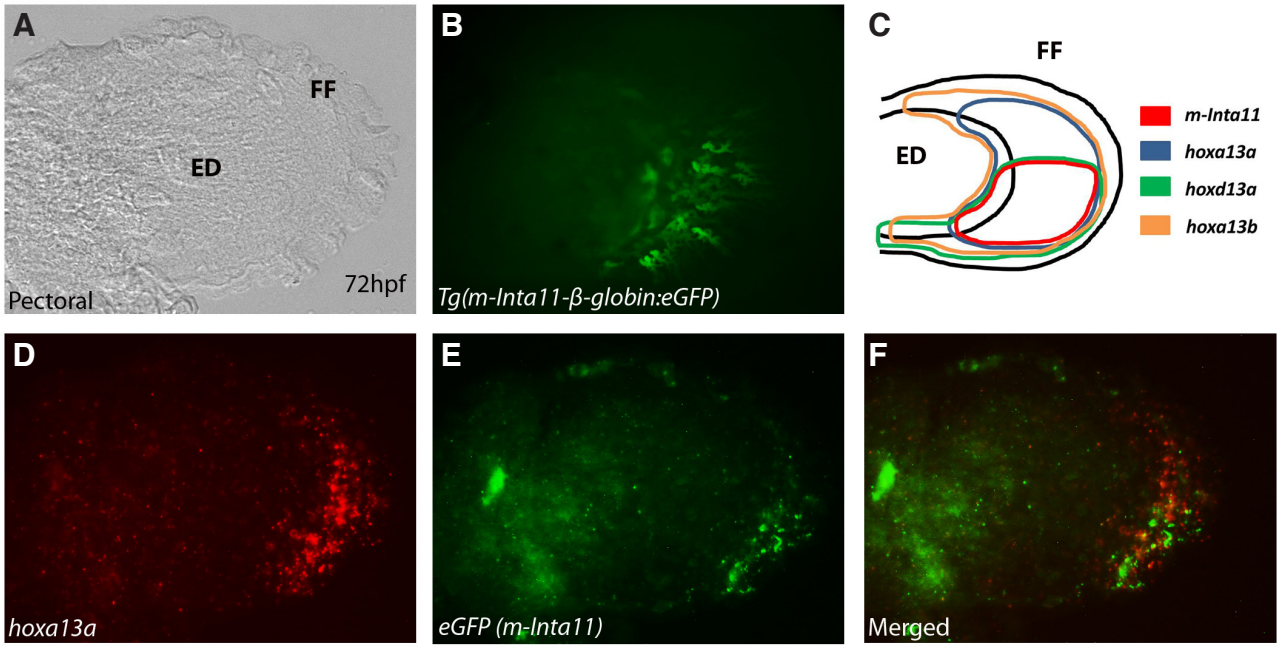

Fig. 2. The"m-Inta11" regulatory element is active in a subpopulation of *hoxd13a- and hoxa13a*-expressing cells in the zebrafish pectoral fin at 72hpf. (A, D-F) *Double fluorescent ISH for* hoxa13a, eGFP, *and* **(B)** Tg(m-Inta11:eGFP) *reporter activity in 72hpf pectoral fin.* **(C)** *Summary of expression patterns for* hoxa13a, hoxd13a,hoxa13b *and* m-Inta11 *activity in 72hpf pectoral fin. The "m-Inta11" regulatory element drives expression in the posterior fin fold mesenchyme (red arrow): eGFP fluoresence* **(B)** *and eGFP transcripts are presented* **(E)**. *The expression of* hoxa13a *extends to the anterior fin fold mesenchyme (white arrow)* **(D,F)**, *outside the region where "m-Inta11" is active (red arrow)* **(E,F)**. *The expression of* hoxd13a *is posteriorly restricted and partially mimics "m-Inta11" activity* **(C)**. *The "m-Inta11" element is not active in the proximal-posterior endoskeletal disc and fin fold regions where* hoxd13a, *and* hoxa13b *are co-expressed (purple arrow)* **(C)**. *Brightfield (A), fluorescent (B,D,E) and merged (F) images are present. ED, endoskeletal disc; FF, fin fold. Scale bar, 3* μm.

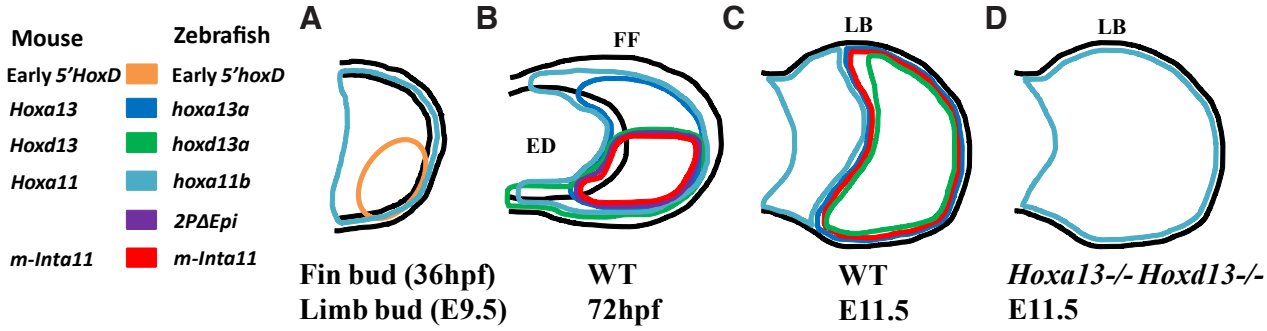

Fig. 3. Expression patterns (*Hoxa13/hoxa13a), Hoxd13/hoxd13a, Hoxa11/hoxa11b*) and regions of enhancer activity (m-Inta11, 2PΔEpi) in 36 and 72hpf zebrafish pectoral fin, and E9.5 and E11.5 WT and *Hoxa13 -/- Hoxd13a -/-* mutant mouse forelimb bud. (A) *Fin and limb bud at 36hpf, and E9.5 respectively,* **(B)** *72hpf zebrafish pectoral fin,* **(C)** *E11.5 WT mouse forelimb bud, and* **(D)** *E11.5* Hoxa13 -/- Hoxd13-/- *mutant mouse forelimb bud.* Hoxa11(hoxa11b) *is similarly expressed in the distal fin and limb bud at equivalent stages, before* Hoxa13(hoxa13a)*expression begins (A). Early 5'*HoxD *expression is similar in the fin and limb bud at respective stages (A). 2PΔEpi and m-Inta11 enhancers are not active in the fin and limb bud at 36hpf, and E9.5 respectively (A). Enhancers 2PΔEpi (purple) and m-Inta11 (red) show overlapping activity in a subpopulation of* hoxa13a/hoxd13a-*expressing cells (blue/green) in the zebrafish pectoral fin at 72hpf (B). Note* hoxa13a *expression expands more anteriorly (blue) and* hoxd13a *expression extends to the proximal-posterior disc and fin fold regions (green) (B). The expression of* hoxa11b *(turquoise) extends anteriorly outside the domain of activity of m-Inta11 (red) (B). In WT E11.5 limb buds,* Hoxa11 *is restricted to the proximal domain due to m-Inta11 activity in the distal regions (turquoise) (C). The activity of m-Inta11 (red) overlaps completely with* Hoxa13 *expression (blue) (C). Note* Hoxd13a *expression (green) does not completely overlap with* Hoxa13 *(blue) and m-Inta11 activity (red) (C). m-Inta11 activity in the WT E11.5 limb bud is inferred through absence of distal* Hoxa11 *transcripts (C). Enhancer activity was only observed at E12.5 in transient transgenic mice (*Tg(m-Inta11:LacZ)*). In* Hoxa13 -/- Hoxd13 -/- *E11.5 limb buds, m-Inta11 shows no activity and* Hoxa11 *is expressed in both the proximal and distal regions (turquoise) (D). Absence of m-Inta11 activity is inferred by the presence of* Hoxa11b *transcripts in the distal limb bud region (D). Note 2PΔEpi is not functional in WT or mutant limb buds (C,D). E, embryonic day; ED, endoskeletal disc; FF, fin fold; hpf, hours post fertilization; LB, limb bud; WT, wild type.*

during limb evolution. *Cis*-regulatory evolution refers to changes to 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 *hoxd13a* 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,

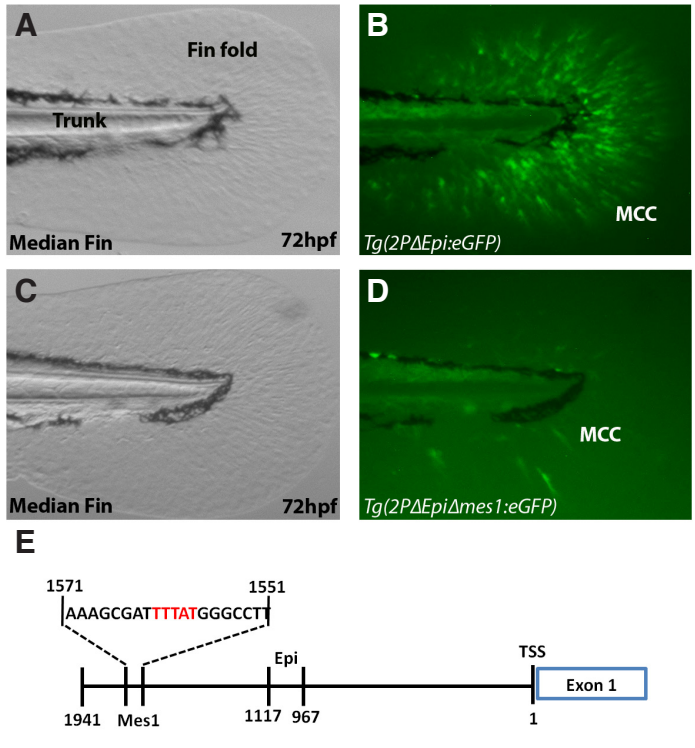

Fig. 4. Enhancer activity of 2PΔEpi drastically reduced when putative binding site for Hox (mes1 site) removed. (A-D) *Zebrafish median fins at 72hpf comparing eGFP reporter expression when driven by either 2PΔEpi or 2PΔEpiΔmes1 regulatory elements.* **(E)** *Schematic of* actinodin1 *1941bp region of the first non-coding exon. The 2PΔEpi regulatory element is able to drive reporter expression in the migrating mesenchymal cells of the median and pectoral fin folds (Median fin: A-B, Pectoral fin: Fig. 5B). Reporter expression is drastically reduced in the median fin when mes1 site is removed from the 2PΔEpi regulatory element (C-D). No eGFP-positive cells are visible in the pectoral fin (data not shown). Mes1 site consists of a 20bp region containing the consensus Hox binding domain TTTAT (Red text) (E). 2PΔEpi regulatory contains the entire 1941bp fragment with Epi region removed (E). Two independent lines were obtained to confirmed the expression pattern of Tg(2PΔEpiΔmes1:eGFP). Brightfield (A,C) and fluorescent (B,D) images are displayed. MMC, migrating mesenchymal cells; TSS, transcription start site.*

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, cmlc2*) 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 spotted 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).

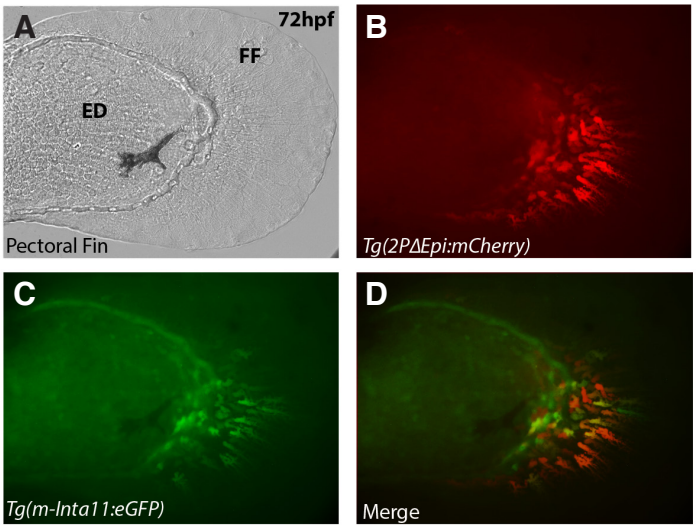

Fig. 5. The "m-Inta11" and "2PΔEpi" enhancer elements display overlapping domains of activity in the zebrafish pectoral fin fold mesenchyme at 72hpf. (A-D) *Zebrafish pectoral fin at 72hpf of* Tg(2PΔEpi:mCherry*) x* Tg(m-Inta11:eGFP) *double transgenic fish. Both regulatory elements drive overlapping reporter expression in posterior fin fold mesenchyme (yellow arrow) (B-D), providing evidence they may both be co-activated by Hoxa13a and Hoxd13a proteins. Note the absence of reporter activity in anterior fin fold mesenchyme (B-D). Due to inconsistencies within transgenic lines, individual cells contain variable amounts of eGFP and mCherry protein and accounts for the colour variation observed in different mesenchymal cells (D). Brightfield (A), Fluorescent (B, C), and merged (D) images are displayed. ED, endoskeletal disc; FF, fin fold. Scale bar, 3 μm.*

***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 the 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 have 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,

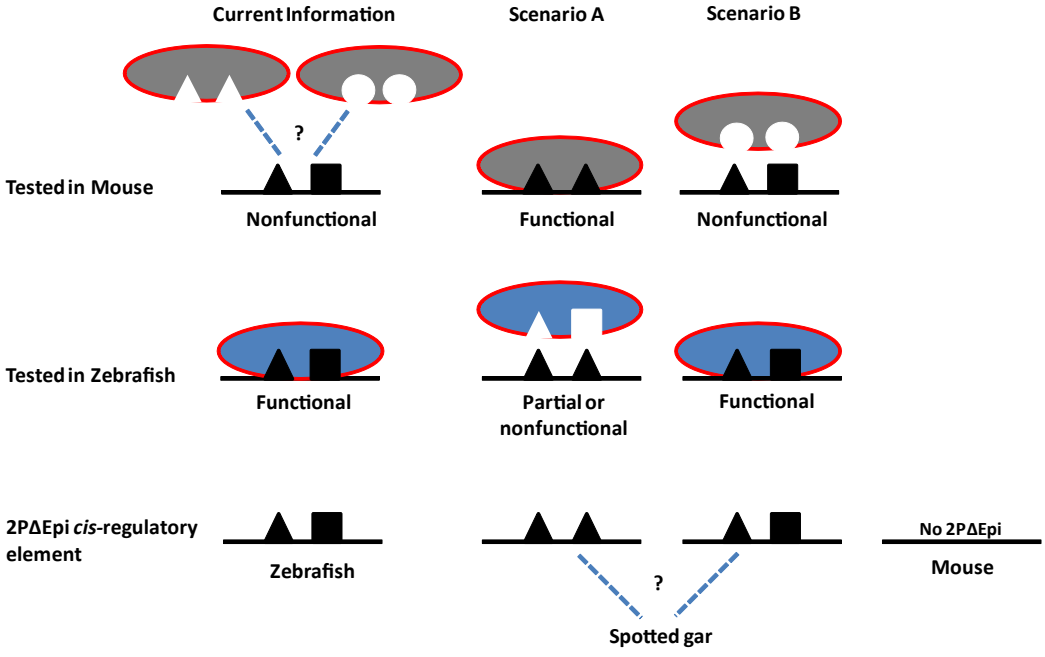

Fig. 6. Orthologous spotted gar "2PΔEpi" regulatory element is required to support or contradict possibility of *trans actinodin* evolution in tetrapods during the fin-to-limb transition. *Hypothetical scenarios if spotted gar orthologous "2PΔEpi" regulatory element is tested via transgenic swap experiments in the zebrafish and mouse. Current information represents experiments performed in Lalonde* et al., *2016, where the zebrafish "2PΔEpi" element is not functional in the mouse. It is impossible to determine from these results if lack of enhancer functionality is due to changes to the* trans *environment in tetrapods, or* cis trans *coevolution in the zebrafish. In scenario A, the spotted gar orthologous "2PΔEpi" is functional in mice. From this result, we could conclude there is no evidence for* trans *evolution in tetrapods, and that the zebrafish has undergone* cis *and* trans *coevolution which the* trans *environment of the mouse is not able to decode. In this scenario, the gar element may be partially functional or non-functional when tested in the zebrafish. In scenario B, the spotted gar "2PΔEpi" element shows conserved functionality in zebrafish however is unable to function in the mouse. This result supports the hypothesis that there was a change in regulation of* actinodin *due to* trans *evolution in tetrapods. If the spotted gar element is not functional in either zebrafish or mouse (not displayed), we could not conclude anything more than what is currently known.*

and therefore the *trans* machinery of the host (mouse) is able to decode the *cis* enhancer sequences of the donor (Braasch *et al.,* 2016). To support a change in *actinodin1* mesenchymal regulation during the fin-to-limb transition, the spotted gar orthologous "2PΔEpi" regulatory element should be identified and tested for functionality in the zebrafish and mouse (Fig. 6). To date, sequence analysis has not revealed any conserved putative enhancers upstream of *actinodin1* in the spotted gar (Lalonde *et al.,* 2016), however due to the expression of *actinodin* and the presence of actinotrichia in this species, a *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-

orly compared to the activity of "m-Inta11" (Lalonde and Akimenko 2018), and we predict this is due to the derived "posteriorization" of *hoxd13a* (Fig. 3B). Similarly, despite actinotrichia being present in the entire pectoral fin fold, "2PΔEpi" activity is posteriorly restricted and this may again be due to the derived expression pattern of *hoxd13a* (Lalonde *et al.,* 2016) (Fig. 3). Changes in mesenchymal *actinodin1* regulation in zebrafish may have yielded no functional consequences, as other *actinodin* paralogs can compensate (Zhang *et al.,* 2010). The expression of *actinodin* was observed in paddlefish fins, a basal actinopterygian, and results from *in situ* hybridization experiments on whole mount samples are suggestive of an anterior extension of the domain of expression (Tulenko *et al.,* 2017). However this approach does not allow distinction between ectodermal and mesodermal expression. It is important to remember that, in zebrafish, unlike *actinodin1* mesenchymal activation, ectodermal activation is not dependent on Hoxd13a and expression occurs in both the anterior and posterior fin fold ectoderm (Lalonde *et al.,* 2016; Zhang *et al.,* 2010).

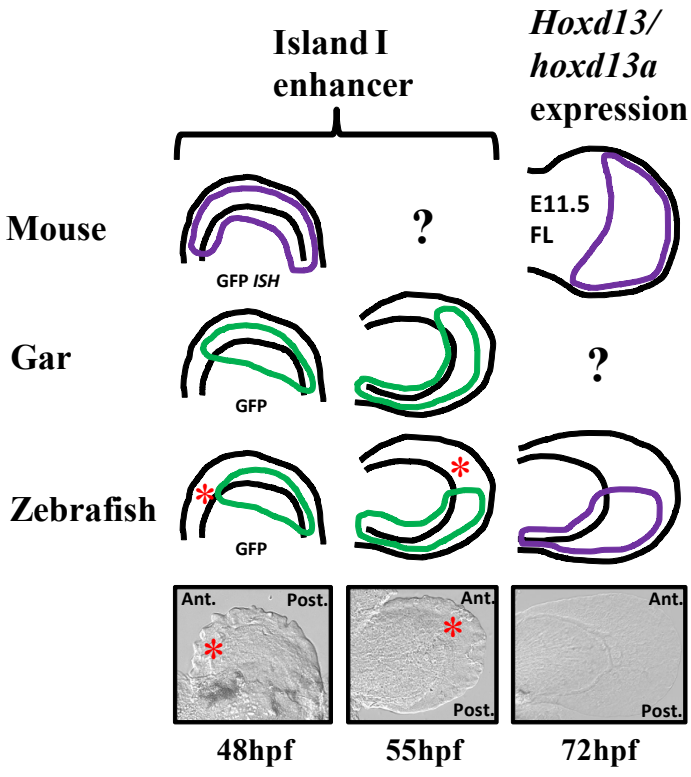

Fig. 7. Orthologous gar and mouse Island I enhancers drive reporter expression in the anterior zebrafish pectoral fin. *Mouse, gar, and zebrafish orthologous Island I enhancer activity at 48, and 55hpf in the zebrafish pectoral fin, and endogenous* Hoxd13/hoxd13a *expression in the mouse forelimb at E11.5 and the zebrafish pectoral fin at 72hpf. Mouse and gar Island I enhancer drive activity more anteriorly in the zebrafish pectoral fin compared to the orthologous zebrafish Island I (red asterisks). Endogenous* Hoxd13 *expression extends to the anterior presumptive autopod at E11.5 in the mouse forelimb. Endogenous* hoxd13a *expression is absent from the anterior pectoral fin fold mesenchyme at 72hpf in the zebrafish. Mouse Island I enhancer activity at 55hpf, and endogenous* hoxd13 *expression in the gar are unknown (Question mark). Donor organism indicated on the left. Brightfield representative pectoral fins shown below. Purple =* in situ *hybridization (ISH), green = GFP fluorescence. Schematic representation of Island I enhancer activity based on data from Gehrke* et al., *2015. Ant, anterior; FL, forelimb; Post, posterior.*

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^{Hoxa11}) 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 *Hoxa13 -/- Hoxd13 -/-* 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/metronidazole (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 (Paço 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 stains 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; Prykhozhij 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

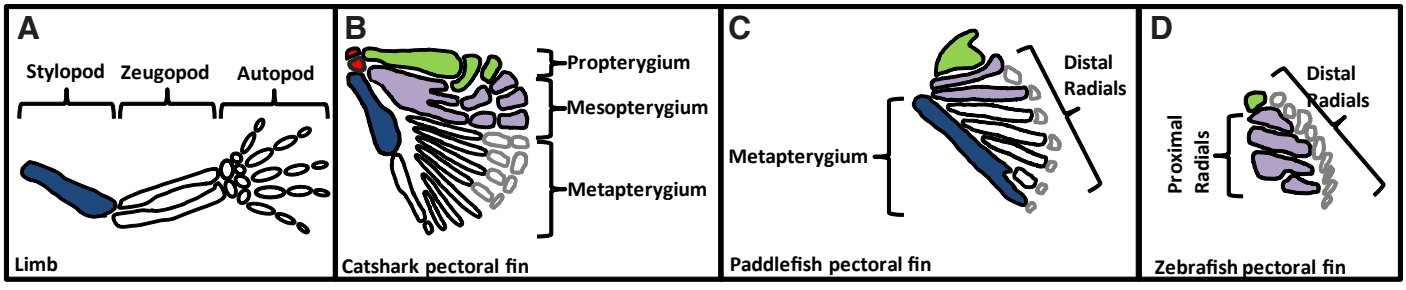

Fig. 8. Appendicular bone/cartilage structure in tetrapods, catshark, paddlefish, and zebrafish. *The autopod is unique to tetrapods, having evolved from the expansion and elaboration of distal endochondral bone elements of lobe-finned fish (sarcopterygian)* **(A)**. *The tetrapod stylopod is thought to be homologous to the metapterygium present in the catshark and paddlefish pectoral fin (blue)* **(A-C)**. *The tetrapod limb has lost the pro- and mesopterygium that is present in the catshark, paddlefish, and zebrafish pectoral fin (green, and purple)* **(A-C)**. *Zebrafish have lost the posterior-most proximal endochondral bone (metapterygium), and retain only the pro- and mesopterygial radials* **(D)**.

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 Hoxd13a as positive regulators of *and1* in the mesenchyme. While the results of *hoxd13a* 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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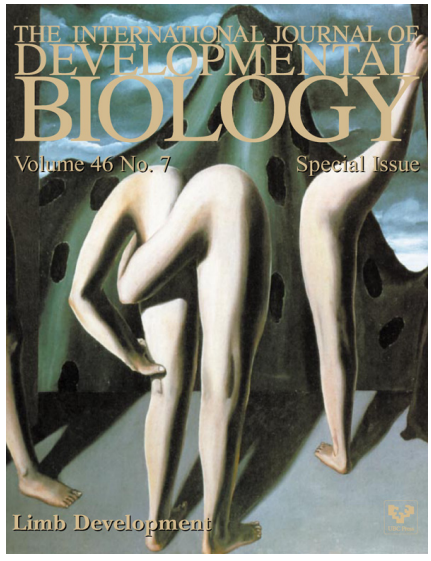

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