8-2018

Characterization of FGF-mediated Sonic Hedgehog Expression during Limb Development

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in conjunction with the
Faculty of Graduate Studies

Characterization of FGF-mediated Sonic Hedgehog Expression during Limb Development

by

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A Dissertation submitted in partial satisfaction of
the requirements for the degree
Doctor of Philosophy in Microbiology and Molecular Genetics

August 2018

Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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ACKNOWLEDGEMENTS

The process of attaining this degree has been filled with failures, successes, losses, victories, pain and joy. I, along with others, have made sacrifices along the way. I would therefore firstly like to thank God for this season of growth and change where I have gained not only knowledge but wisdom. May I be blessed with the opportunity to share with others. Next, I would like to thank my mentors, lecturers and professors for their time, energy and support throughout my training. I would particularly like to thank Dr. Kerby C. Oberg for taking me under his wings and providing support, understanding, insight and training for the next steps of my academic/professional journey. Thank you to Dr. H. M. Fletcher for providing me with opportunities to grow along the way. Thank you to Drs. Mackem and Mohan whose insights in limb development and gene regulation proved invaluable and to Dr. Wang for his insight in sequencing technologies. This milestone would not have been possible without any of you. Special thanks also to Ms. Charmaine Pira for her training and advice in planning and executing experiments.

To my family members and friends who have called, texted, prayed, cried and celebrated with me through this journey, I made it with your help. Words are not enough to express my gratitude to my immediate family, especially my parents Billy and Gloria Watson. To my wife, Nakia Watson, who I met and fell in love with along this journey, thank you for being the rock I need.

As I launch out into the world of work once more, I am eager to move forward with God's guidance and everyone's love and support. Thank you all.

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ABBREVIATIONS

FGF	Fibroblast growth factor
SHH	Sonic hedgehog
LHX2	Lim homeobox 2
HOX	Homeobox
ETS	E twenty six
SCUBE	Signal peptide, CUB domain, EGF-like
TFAP2C	Transcription factor AP2 gamma
AER	Apical ectodermal ridge
ZPA	Zone of polarizing activity
ZRS	Zone of polarizing activity resulatory sequence
WNT	Wingless-type MMTV
DLX	Distal-less homeobox
DUSP	Dual specificity phosphatase
BAMBI	BMP and Activin Membrane Bound Inhibitor
BMP	Bone morphogenic protein
SPRY	Sprouty
RSPO	R-spondin
GJA1	Gap junction A1
PCRM	Potential *cis*-regulatory module
MO	morpholino
PTCH	Patched
GLI	Gli-Kruppel family member

EMX	Empty spiracles homeobox
FGFR	Fibroblast growth factor receptor
SHOX	Short stature homeobox
TBX	T-box
DKK	Dickkopf WNT Signaling Pathway Inhibitor
EN1	Engrailed1
LMX1B	LIM Homeobox transcription factor 1 beta
FBXW	F-box/WD repeat-containing protein
TTC8	Tetratricopeptide repeat domain 8
WMISH	Whole mount *in situ* hybridization
GMPS	Guanine monophosphate synthase
SHFM	Split hand foot malformations
HSPG	Heparan sulfate proteoglycan
JAK/STAT	Janus kinase/signal transducer and activator of transcription
MAPK	Mitogen-activated protein kinase
ERK	Extracellular signal-regulated kinase
PI3K	Phosphatidylinositol 3-kinase
PLCγ	Phosphoinositide phospholipase C

ABSTRACT OF THE DISSERTATION

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by

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Doctor of Philosophy, Graduate Program in Microbiology and Molecular Genetics
Loma Linda University, August 2018
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Crosstalk between Fibroblast Growth Factors (FGFs) and Sonic hedgehog (SHH) is essential for proper limb development; however, the molecular mechanisms involved in this process remain unclear. Via comparative transcriptome analysis and gene knockdown and overexpression studies, we identified the transcription factor Lim homeobox 2 (LHX2) as an intermediate in FGF-directed *SHH* expression. We determined that FGF signaling was necessary for *LHX2* expression and demonstrated that LHX2 is a primary transcription target of FGF signaling through the RAS and AKT pathways. Additionally, loss of LHX2 in the distal limb results in decreased *SHH* expression and a truncated limb within 24 hours of treatment, while overexpression of LHX2 in the presence of ectopic FGF leads to increased *SHH* expression. While LHX2 is insufficient alone to induce SHH, we conclude that it likely serves as a competency factor for endogenous *SHH* expression, keeping it juxtaposed to the distal apical ectodermal ridge during limb outgrowth.

CHAPTER ONE

INTRODUCTION

Human limb development begins in the fourth week of gestation (Figure 1A) and coincides with the development of other organs such as the heart, kidney, liver and lungs. As a result, the molecular mechanisms governing the development of these organs are shared. Studying limb development therefore provides important insights into organogenesis. Vertebrate limb development is under tight molecular regulation directing the number, location and pattern of tetrapod limbs. Expression of Homeobox (Hox) genes along the cranial-caudal or anterior-posterior axis of the developing embryo provides segmental identity to the embryo and establishes presumptive limb fields within the lateral plate mesoderm from which the limb buds will emerge (Figure 1B) (Oliver et al., 1988; Burke et al., 1995; Wellik, 2009; Tanaka, 2016).

Limb Initiation

Limb bud initiation occurs as a complex interaction between wingless-type MMTV (WNT), fibroblast growth factor (FGF) signals and T-box (TBX) transcription factors in the mesenchyme of the presumptive limb field (Figure 1C). Murine FGF10 knockouts develop tetra-amelia or failure of limb bud initiation (Ng et al., 2002), while disruption of another member of the pathway, WNT3A, has been shown to cause tetra-amelia in humans (Niemann et al., 2004). TBX4 and TBX5 contribute to initiation and identity of the hindlimb and forelimb, respectively (Gibson-Brown et al., 1996). Holt-Oram syndrome, which is characterized by abnormalities of the heart and upper limb, occurs in humans with mutations in the *TBX5* gene (Basson et al., 1997; Q. Y. Li et al.,

1997; Mori & Bruneau, 2004). Through loss and gain of function experiments in chicken and mice, a role for *PITX1* in hindlimb specificity has also been identified (Lanctot, Moreau, Chamberland, Tremblay, & Drouin, 1999; Logan & Tabin, 1999; Szeto et al., 1999). Misexpression of *PITX1* in wing-field of chicks leads to ectopic *TBX4* expression and results in hindlimb-specific changes to the limb (Logan & Tabin, 1999). Pitx1 overexpression in mice and mis-regulation of PITX1 in humans (Liebenberg syndrome) lead to similar phenotypes with partial transformation of the upper limb into a hindlimb (Al-Qattan, Al-Thunayan, Alabdulkareem, & Al Balwi, 2013; Mennen, Mundlos, & Spielmann, 2014; Spielmann et al., 2012) .

Limb Patterning Along the Proximal-Distal Axis (Figure 1C)

Subsequent to limb bud initiation, limb development occurs along three axes: proximal-distal, anterior-posterior, and dorsal-ventral. Signaling centers are organized to govern growth and patterning along each axis. The apical ectodermal ridge (AER) forms as a thickened epithelium at the distal tip of the limb bud in response to mesodermal signals. FGFs, primarily FGF8 from the AER, direct growth and patterning along the proximal-distal axis (shoulder to fingers). A deficiency of Fgf8 and 4 (from the AER) (Boulet, Moon, Arenkiel, & Capecchi, 2004) or Fgf10 (from limb mesoderm) (Min et al., 1998) in mice results in the complete absence of fore- and hindlimbs. Interrupting AER-related FGF function either by AER removal in chick wings or conditional Fgf receptor knockouts at various developmental stages, truncates limbs along the proximal-distal axis corresponding to its progressive stage of distalization (P. Lu, Yu, Perdue, & Werb, 2008; Mahmood et al., 1995; J.W. Saunders, Jr., 1948; Yu & Ornitz, 2008). In addition,

maintenance of AER integrity and FGF secretion is important for proper digit formation. Several molecules involved in AER maintenance and FGF8 production have been described including: TP63, DLX5, WNT10B and FBXW4. Mutations in target genes or regulatory elements associated with this pathway disrupt AER structure and function, producing a phenotypic spectrum of abnormalities known as split hand foot malformations (SHFM) (Marinic, Aktas, Ruf, & Spitz, 2013; Naruse, Takahara, Takagi, Oberg, & Ogino, 2007; Restelli et al., 2014; Sowinska-Seidler, Socha, & Jamsheer, 2014).

Limb Patterning Along the Anterior-Posterior Axis (Figure 1D)

The zone of polarizing activity (ZPA) is a cluster of cells in the posterior distal mesoderm of the developing limb that is responsible for patterning along the anterior-posterior axis. These cells secrete sonic hedgehog (SHH), a potent morphogen that directs posterior expansion and patterning (Towers, Mahood, Yin, & Tickle, 2008; Zhu et al., 2008). In the absence of *SHH* expression, the posterior zeugopod bones (ulna and fibula) and posterior digits fail to develop (Chiang et al., 2001; Kraus, Fraidenraich, & Loomis, 2001). While ectopic anterior expression of *SHH* is associated with transformation of anterior zeugopod bone (radius) into a posterior bone (ulna) and mirror duplication of posterior digits (Riddle, Johnson, Laufer, & Tabin, 1993).

In the murine model, Gli3 transcription factor functions as a downstream effector of Shh signaling. In the absence of Shh signaling, Gli3 is processed into a truncated repressor variant (Gli3R) (Litingtung, Dahn, Li, Fallon, & Chiang, 2002). Gli3 is expressed in the limb bud prior to Shh expression and acts antagonistically to restrict

Hand2 expression to the posterior limb (te Welscher, Fernandez-Teran, Ros, & Zeller, 2002). Correspondingly, Hand2 restricts Gli3R to the anterior aspect of the limb. This reciprocal antagonism sets up the ZPA and *SHH* expression in the posterior distal mesenchyme. Shh subsequently counters Gli3R repression by blocking the local processing of Gli3, to keep it in its full-length activator form. In the developing autopod, Shh and Gli3 (Gli3/Gli3R ratio) regulate the number and identity of the digits. In the absence of Gli3, the digits increase in number, become syndactylous, and lose their identity (Litingtung et al., 2002). Mutations in Gli3 result in polydactyly in both mouse and humans (Figure 2) (Hinchcliffe, 1980 ; Vortkamp, Franz, Gessler, & Grzeschik, 1992; Vortkamp, Gessler, & Grzeschik, 1991).

A conserved *cis*-regulatory element, the ZPA regulatory sequence (ZRS), is found within intron 5 of the LMBR1 gene and is required for limb-specific *SHH* expression (Clark et al., 2001; L. A. Lettice et al., 2002). Mis-regulation of SHH by microduplication of the ZRS and presumed anterior ectopic expression causes Laurin-Sandrow Syndrome with tetramelic posterior zeugopod duplication (ulnar/fibular dimelia) and mirror-image polydactyly (Lohan et al., 2014). In a chicken model termed oligozeugodactyly (ozd), there is a deletion of the ZRS resulting in the absence of SHH expression and the absence of the posterior zeugopod elements and the entire autopod of the three-digit forelimb/wing (Figure 2). In humans, a deletion mapping upstream of this region results in an autosomal recessive disorder *Acheiropodia* (Ianakiev et al., 2001) that causes longitudinal postaxial deficiencies resembling the limb phenotype of ozd chicks and $Shh^{-/-}$ mice (Ros et al., 2003). Apart from Acheiropodia in humans, pre-axial polydactyly (L. A. Lettice et al., 2003) triphalangeal thumb (Heutink et al., 1994) and

acropectoral syndrome (Dundar et al., 2001), are other limb-specific disorders associated with mutations in this region.

Limb Patterning Along the Dorsal-Ventral Axis (Figure 1E)

Limb dorsalization is accomplished by the release of WNT7a from the dorsal ectoderm which induces the expression of LIM Homeobox transcription factor 1 beta (LMX1B) in the underlying mesoderm. Expression of Engrailed 1 (EN1) in the ventral ectoderm restricts WNT7a to the dorsal ectoderm thereby establishing the dorsal-ventral aspects of the limb. In mice lacking Wnt7a or Lmx1b function, limbs develop with near ventral-ventral symmetry. In contrast, over expression of Wnt7a or Lmx1b in the ventral limb generates a dorsal-dorsal limb. Mutations that disrupt LMX1B function and cause haploinsufficiency, impair limb dorsalization and cause Nail-Patella Syndrome in humans (H. Chen et al., 1998).

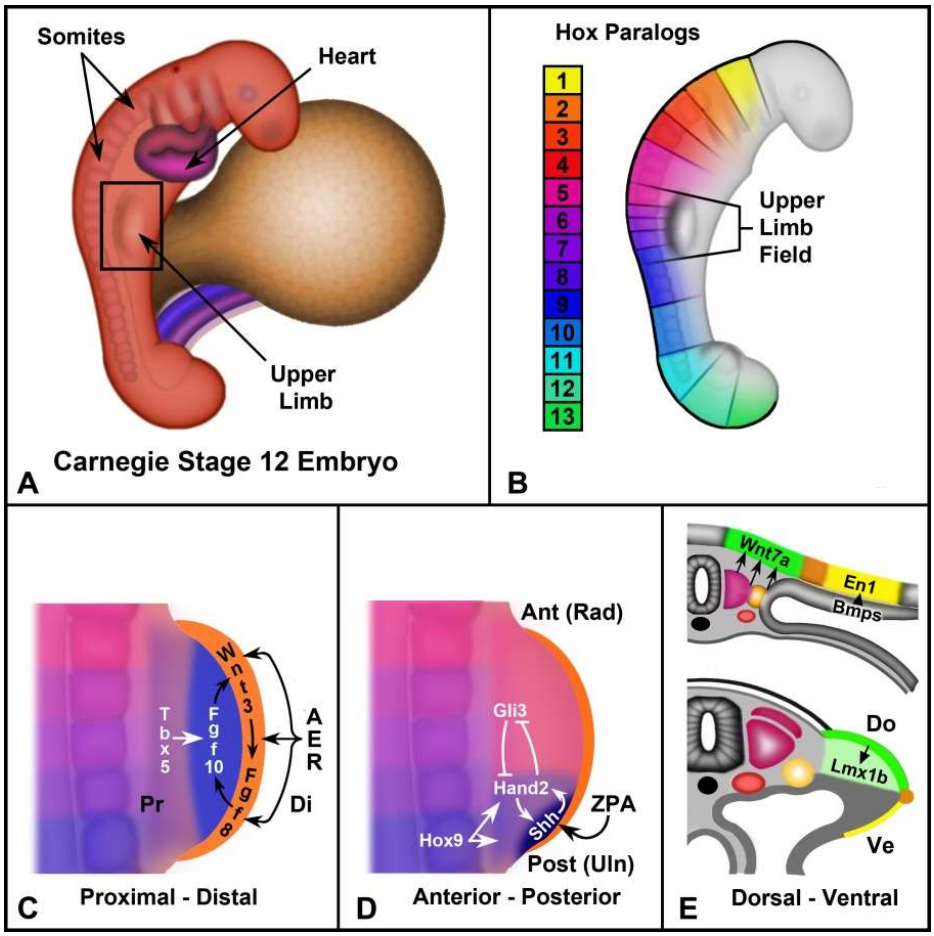

Figure 1. Early Limb Development and Axis Organization. A) Illustration of a human embryo at Carnegie stage 12 (~ 4 weeks gestation) showing an emerging upper limb bud. B) Evidence suggests that Hox genes establish upper limb position and polarity. C-E) Molecular pathways involved in forelimb initiation and patterning along the proximal-distal axis (C), anterior-posterior axis (D), and dorsal-ventral axis. Apical ectodermal ridge (AER) (orange), Pr – proximal, Di-distal, Ant(Rad)-anterior/radial, Post(Uln)-posterior/ulnar, zone of polarizing activity (ZPA) (dark purple), Do-dorsal, Ve-ventral, dorsal ectoderm and mesoderm (green). From Oberg et al., (Oberg et al., 2014) with permission.

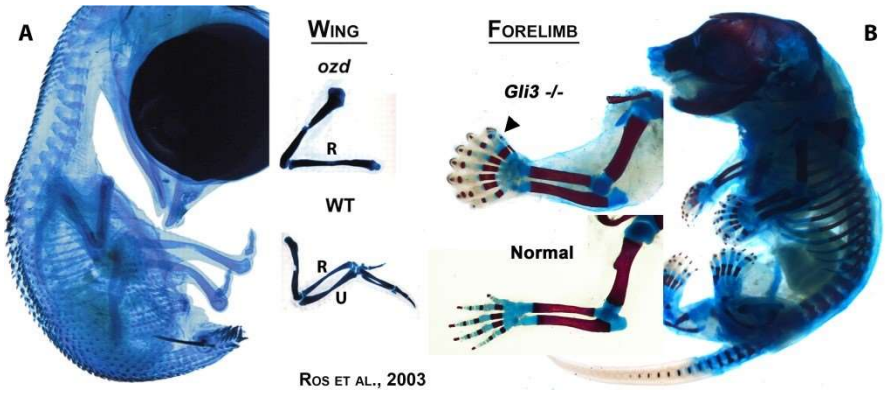

Figure 2. The Influence of SHH/GLI3 on Digit Formation. This figure illustrates the range of SHH/GLI3 signaling in two common models of limb development, chick and mouse. A) A spontaneous mutation in chickens deletes the limb-specific enhancer of SHH generating the Oligozeugodactyly (ozd) phenotype. Without the enhancer, no SHH is expressed in the limbs and Gli3R function is unopposed. The unopposed Gli3R inhibits the formation of the ulna and digits. B) In contrast, if GLI3 expression is absent as seen in the GLI3 knockout mouse, the number of digits is excessive, and the digits lack specific AP (radioulnar) identity, including a two phalangeal thumb. (Images courtesy of John F. Fallon, PhD, University of Wisconsin, and Maria A. Ros, MD, PhD, Instituto de Biomedicina y Biotechnologia de Cantabria; (A) adapted with permission from Ros et al. (Ros et al., 2003) and Oberg (Oberg, 2014).

Coordination of Inter-Axes Patterning

The signaling centers further coordinate patterning during limb outgrowth by inter-axes regulation. AER-related FGFs and SHH regulate each other in a positive feedback loop during limb development (Laufer, Nelson, Johnson, Morgan, & Tabin, 1994; Niswander, Jeffrey, Martin, & Tickle, 1994). Loss of FGF signaling from the AER diminishes SHH expression (Laufer et al., 1994) and removal of SHH downregulates FGF expression in the AER (Chiang et al., 2001).

SHH regulates FGFs in the AER via Formin, Gremlin, and bone morphogenic proteins (BMPs). A recessive mutation in mice termed *limb deformity* (*ld*), is characterized by mutations in the *Formin* locus. Within this locus a *cis*-regulatory region resides that negatively affects *Gremlin* expression (Zuniga et al., 2004). Gremlin is a BMP antagonist that aids in maintaining the structural/functional integrity of the AER. In Gremlin-deficient mouse embryos, a morphologically distinct and functional AER fails to develop which negatively affects FGF signaling and in turn leads to diminished *Shh* expression (Khokha, Hsu, Brunet, Dionne, & Harland, 2003; Michos et al., 2004). Homozygous mice for the *ld* mutation present with limb patterning defects characterized by synostosis of the zeugopod in combination with oligo- and syndactyly of metacarpal bones and digits (Zeller et al., 1999). Zuniga et al. in their 2004 report, hypothesize that homologous mutations in this *cis*-regulatory region, which maps to chromosome 15q13-14 in humans (Maas et al., 1991) may be linked to human congenital malformations such as Cenani-Lenz-like nonsyndromic oligosyndactyly which resembles the *ld* phenotype (Zuniga et al., 2004).

WNT7a links the dorsal-ventral axis to the anterior-posterior axis by promoting the expression of SHH (Yang & Niswander, 1995). Similarly, the loss of Lmx1b function in knockout mice causes a loss in distal ulna formation in addition to ventral-ventral limbs (H. Chen et al., 1998). In humans, homozygous missense mutations of WNT7A causes ulnar deficiency/absence with loss of one or more ulnar digits in a condition called Al-Awadi/Raas-Rothschild syndrome (AARRS) (Kantaputra, Mundlos, & Sripathomsawat, 2010).

Defining the Number and Identity of Digits

A Turing-type model is now being used to describe how the number of digits is determined in the autopod as opposed to a simpler diffusion-gradient type model of the polarizing agent, SHH (Sheth et al., 2012). Recent reports show this periodic patterning model integrates an activator (BMP) and an inhibitor (WNT) to specify a *SOX9* expression pattern which stimulates chondrogenesis and dictates digit number (Raspopovic, Marcon, Russo, & Sharpe, 2014). BMPs may play additional roles in defining digit identities by regulating SMADs and the signal transduction pathways of the phalanx forming regions (Suzuki, Hasso, & Fallon, 2008) .

FGF Signaling

FGFs are secreted morphogens with mainly paracrine activity; meaning they act on nearby cells. Reportedly, this is because they bind strongly to heparan sulfate proteoglycans (HSPGs) such as the syndecans, glypican, and perlecan on the cell surface and in the extracellular matrix (ECM) which restricts them from diffusing very far from

the cells that secreted them (Basilico and Moscatelli 1992). HSPGs are often considered co-receptors for FGFs and modulate ligand availability (Lanner 2010). FGFs binding to HSPGs facilitate signal transduction by presenting the ligands to high affinity transmembrane tyrosine kinase receptors also known as Fibroblast growth factor receptors (FGFRs) (Faham et al. 1996; for review, see Mason 1994). In vertebrate species, a family of four genes, FGFR1– FGFR4, encodes such high-affinity FGFRs, each of which can produce a variety of receptor RNA isoforms through alternative splicing. Abnormal limb development in humans as a consequence of deficiency/mutations in these receptors are well known and include Apert syndrome (FGFR2 mutation), Crouzon syndrome (FGFR2 mutation), Pfeiffer syndrome (FGFR1 and 2 mutations), achondroplasia (FGFR3 mutation), and thanatophoric dysplasia (FGFR3 mutation) (Toriello, Meck, Professional, & Guidelines, 2008).

The binding of FGFs activates the high-affinity receptor proteins by inducing the formation of receptor homo- or heterodimers, which results in receptor transphosphorylation and leads to the activation of one of the four main signaling pathways: the Janus kinase/signal transducer and activator of transcription (Jak/Stat), phosphoinositide phospholipase C (PLCγ), phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/Erk). These intracellular signaling pathways regulate cell proliferation, growth and differentiation (Martin, 1998).

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CHAPTER TWO

LHX2 MEDIATES THE FGF TO SHH REGULATORY LOOP DURING LIMB DEVELOPMENT

By

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The work presented in this chapter has been published.
J. Dev. Biol. **2018**, *6*(2), 13;

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Keywords: Limb Development, Fibroblast growth factor (FGF), Sonic hedgehog (SHH), Lim Homeobox2 (LHX2)

Abstract

During limb development, Fibroblast growth factors (Fgfs) govern proximal-distal outgrowth and patterning. FGFs also synchronize developmental patterning between the proximal-distal and anterior-posterior axes by maintaining Sonic hedgehog (Shh) expression in cells of the zone of polarizing activity (ZPA) in the distal posterior mesoderm. Shh, in turn, maintains Fgfs in the apical ectodermal ridge (AER) that caps the distal tip of the limb bud. Crosstalk between Fgf and Shh signaling is critical for patterned limb development, but the mechanisms underlying this feedback loop are not well-characterized. Implantation of Fgf beads in the proximal posterior limb bud can maintain *SHH* expression in the former ZPA domain (evident 3h after application), while prolonged exposure (24h) can induce *SHH* outside of this domain. Although temporally and spatially disparate, comparative analysis of transcriptome data from these different populations accentuated genes involved in SHH regulation. Comparative analysis identified 25 candidates common to both treatments with 8 linked to *SHH* expression or function. Furthermore, we demonstrated that LHX2, a Lim homeodomain transcription factor, is an intermediate in the FGF-mediated regulation of SHH. Our data suggest that LHX2 acts as a competency factor maintaining distal posterior *SHH* expression subjacent to the AER.

Introduction

Limb development generates a structure that has asymmetry along three coordinate axes: proximal-distal (PD), anterior-posterior (AP), and dorsal-ventral (DV). Each axis has its own signaling center that mediates patterning. The apical ectodermal ridge (AER), a thickening of ectoderm located along the distal rim of the limb, is a signaling center from which secreted Fibroblast growth factors (Fgfs) regulate PD patterning and outgrowth. Sonic hedgehog (Shh) is secreted from a cluster of cells in the distal posterior limb bud mesoderm called the zone of polarizing activity (ZPA) and regulates AP expansion and patterning. The dorsal ectoderm secretes Wnt7a, which dorsalizes the developing limb. During development crosstalk among these axes is required to coordinate proper limb patterning (Delgado & Torres, 2017).

Along the PD and AP axes, development is coordinated through crosstalk between Fgf and Shh in a reciprocal feedback loop (Laufer et al., 1994; Niswander et al., 1994). The mechanism by which Shh maintains Fgf in the AER is relatively well-characterized and includes Shh directing interactions between Formin, Gremlin, and Bone morphogenic proteins (Bmps) (Capdevila, Tsukui, Rodriquez Esteban, Zappavigna, & Izpisua Belmonte, 1999; Zeller et al., 1999; Zuniga, Haramis, McMahon, & Zeller, 1999). In the absence of Shh, the AER regresses and posterior elements of the developing limb are lost (Chiang et al., 2001; Ros et al., 2003). In contrast, the mechanism by which AER-secreted Fgfs regulate *Shh* is less clear. As the limb bud elongates, the ZPA (and *Shh* expression) persists distally subjacent to the AER. *Shh* expression in the more proximal posterior mesodermal cells, we refer to as the former ZPA domain, wanes as the cells move beyond the influence of the AER-FGFs (B. D. Harfe et al., 2004; Ingham &

Placzek, 2006).

FGF2 (Fallon et al., 1994; S. Li, Anderson, Reginelli, & Muneoka, 1996), FGF4 (Laufer et al., 1994; Niswander, Tickle, Vogel, Booth, & Martin, 1993; Vogel & Tickle, 1993), or FGF8 (Crossley, Minowada, MacArthur, & Martin, 1996; Vogel, Rodriguez, & Izpisua-Belmonte, 1996) is sufficient to recover *SHH* expression in the absence of the AER. When applied to the mid posterior half of the limb bud, nearly 24h is required to up-regulate *SHH* in cells outside of the ZPA or former ZPA domain (Fig 3A). Interestingly, in cells of the former ZPA domain (proximal posterior limb margin), *SHH* could be maintained in the presence of proximal FGF, evident within 3h of exposure. The former ZPA domain and the non-ZPA domain differ in context (maintenance versus induction, respectively); nevertheless, we suspected that the final pathway to *SHH* expression is common in both domains.

FGF2 is endogenously expressed in the chicken (Fallon et al., 1994; Savage et al., 1993) and human AER (Becic, Kero, Vukojevic, Mardesic, & Saraga-Babic, 2018), has broader effects than FGF4 and FGF8 (Harduf, Halperin, Reshef, & Ron, 2005), and interacts with greater affinity to the primary ZPA-related Fgf receptor (FGFr1cIII) (Sheeba, Andrade, Duprez, & Palmeirim, 2010; Verheyden, Lewandoski, Deng, Harfe, & Sun, 2005). Thus, we applied FGF2-laden beads to chicken limb buds to generate ZPA domain and the non-ZPA domain transcriptomes. Using comparative analysis, we identified genes that were common to both transcriptomes, genes we suspected would accentuate the FGF to SHH pathway. From our analysis, we were able to identify a number of candidates in the FGF to SHH pathway and confirm a role for Lim homeobox 2 (LHX2) as an intermediate in FGF-regulated *SHH* expression.

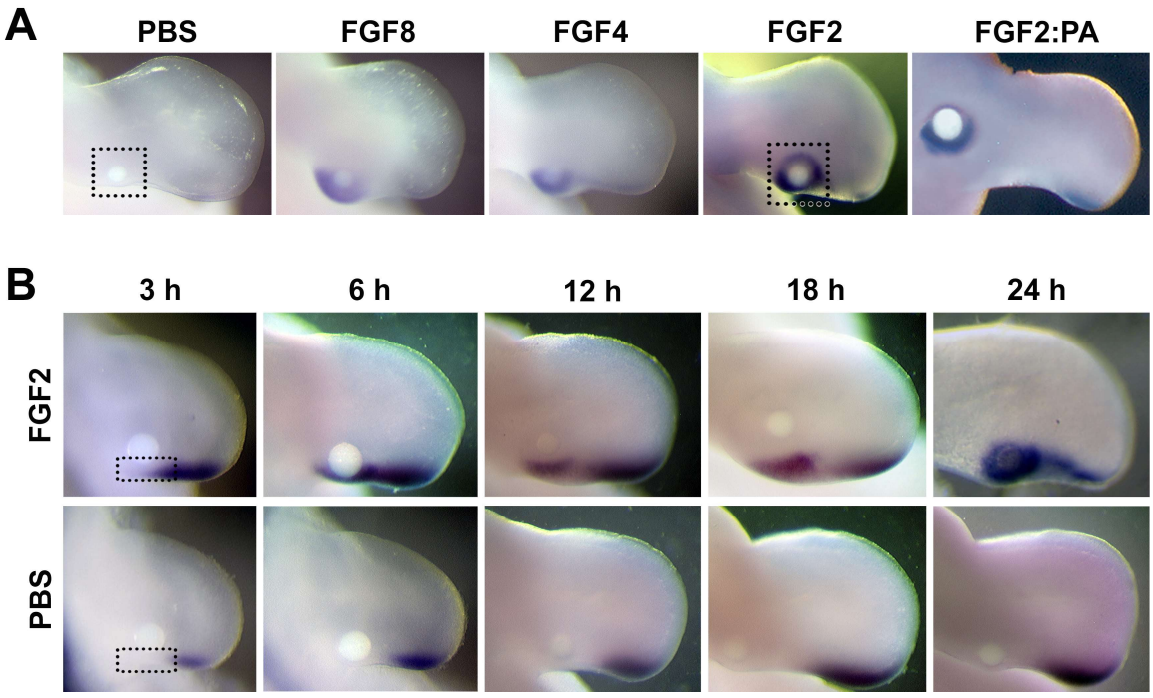


Figure 3. FGF has the Capacity to Maintain or Ectopically Induce *SHH* Expression. (A) SHH expression following 24h of exposure to FGF8, FGF4, or FGF2-soaked beads implanted into the posterior HH23 chicken wing mesoderm proximal to the ZPA (500 µm from the AER). Ectopic SHH expression is present around the FGF bead, but not the PBS control. This ectopic expression was noted in all embryos tested (n=3 for each FGF). The expression was present in cells of the former ZPA domain (along the posterior margin) and in cells that likely lacked prior ZPA exposure (anterior aspect of the bead). Induction of non-ZPA cells was accentuated when initial bead placement was more proximal (600 µm from the AER) and anterior (500 µm from posterior edge) (FGF2:PA). (B) Persistent SHH expression is evident in former ZPA domains over a 3-24h time course post-FGF bead implant. In addition, robust ectopic SHH expression is present around the FGF bead at 24h (top panel) as compared to the PBS control (bottom panel). For each time point, 5 embryos were assayed for response to FGF while 3 embryos were for expression in the presence of PBS (vehicle control). Two independent experiments were used to collect embryos and the pattern of ectopic SHH expression was consistent in all time-matched embryos. Tissue harvested for transcriptome analysis and RT-qPCR validation is indicated by black dotted lines.

Materials and Methods

FGF Bead Implants, WNT5a Cell Implants and Harvesting Embryos

Heparin acrylic beads (H5263 Sigma), about 150 μm in diameter, were soaked in 4 μl of 0.5 mg/ml recombinant human FGF2 (233-FB), FGF4 (235-F4) or FGF8 (423-F8) (R&D Systems, Minneapolis, MN) overnight at 4ºC or at least 1h at room temperature. For the control samples, the heparin acrylic beads were soaked in 4 μl 1XPBS before implantation. Using tungsten needle and forceps, FGF2 or PBS-soaked beads were implanted (*in ovo*) into the posterior forelimb bud mesoderm of Hamburger-Hamilton stage (HH) 23 chicken embryos about 500μm from the distal tip. Cells secreting Wnt5a (CRL-2814, ATCC®, Manassas, VA) were grown to confluence and then stained with neutral red. Strips of Wnt5a-secreting cells were then harvested and implanted into the posterior margin of HH23 right limb bud mesoderm as previously described (Oberg et al., 2002). Embryos were incubated at 39ºC in a humidified chamber for the experimental time (3-24h) and quickly harvested in PBS. Tissue from embryos for transcriptome and RT-qPCR analyses (dotted lines Fig. 1) was removed using a tungsten needle, flash frozen using liquid nitrogen and stored at -80ºC until further processing. Embryos for whole mount *in situ* hybridization (WMISH) were fixed in MEMFA (1M MOPS, 20mM EGTA, 10mM MgSO4, 38% Formaldehyde) overnight at 4ºC as described (Feenstra et al., 2012) then stored in 90% methanol at -20ºC until further processing.

RNA-seq Analysis

After 3h of incubation, the tissue directly posterior to the FGF2 or PBS bead was extracted, and RNA was isolated using the RNeasy Plus Micro Kit (Qiagen, Valencia,

CA). Three independent experiments were conducted and RNA within each sample group (FGF or PBS) from each experiment was pooled to decrease genetic variability (n=15-20 embryos per sample). RNA-seq was conducted by Norris Comprehensive Cancer Center, University of Southern California. RNA-seq differential gene expression and statistical analysis was conducted in R coding language with the systempipeR package tools. Reads were aligned with Hisat2 alignment algorithm to release-87 of *Gallus gallus* genome build 5. Fold changes were calculated, and statistical comparisons made with EdgeR. Genes exhibiting differential expression between FGF2- and PBS-treated embryos with $p<0.05$ were included in further analyses. RNA-seq data were submitted to Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) and can be located under series accession number GES114663.

DNA Microarray Analysis

RNA was isolated from tissue surrounding an FGF2 or PBS bead after 24h of incubation using the RNeasy kit (Qiagen). The RNA from 6 embryos was pooled for each treatment group to decrease genetic variability and the experiment was repeated 3 times on separate days. Microarray analysis was performed by University of California at Irvine Genomics High Throughput Facility as previously described (Feenstra et al., 2012). Samples were hybridized to the Affymetrix GeneChip® Chicken Genome Array (ThermoFisher Scientific, CA). The data was normalized using RMA and analyzed using the Comprehensive R and Bioconductor based web service for microarray data analysis. Only genes exhibiting differential expression between FGF and PBS treated embryos

with $p<0.05$ were included in further analyses. Microarray data were submitted to Gene Expression Omnibus and can be located under series accession number GES114663.

Gene Ontology

Differentially expressed genes at 3 and 24h post-FGF bead implantation were classified according to Gene Ontology biologic pathways using Ingenuity Pathway Analysis (IPA) software and database (Qiagen).

Gene Expression Analysis via Whole Mount In Situ Hybridization (WMISH)

To validate differential gene expression in response to 3 and 24h of FGF treatment, digoxigenin-labeled probes were generated, and embryos processed for WMISH as previously described (Feenstra et al., 2012; Yamada, Szendro, Prokscha, Schwartz, & Eichele, 1999). Primers used for probe generation are listed in Table S1; alternatively, probes were generated from plasmids received as gifts: *DKK1*, *WNT5a*, *PTCH2*, (Dr. Clifford Tabin); *FZD4* (Dr. Philippa Francis-West); *FGF8* (Dr. Eric Swindell); *PYST1/DUSP6* (Dr. Stephen Keyse). For all WMISH procedures, 3-5 embryos were examined for each gene per experiment and at least two independent experiments were performed.

RT-qPCR Validation of Transcriptome Data and Quantitation of LHX2 Overexpression/Knockdown Analysis

Further validation of the differential expression pattern of selected genes following 3 and 24h of FGF treatment was performed via RT-qPCR. RNA was extracted

from FGF2 or PBS treated tissue 3 and 24h after bead implantation. 7-10 embryos from each treatment group were pooled to minimize biologic variation. Primers used for RT-qPCR validation are listed in Table S1.

RT-qPCR was also used to detect changes in *SHH* and *PTCH2* expression following overexpression and knockdown of LHX2. For all samples, RNA extraction and on-column DNA digestion were performed using the RNeasy plus micro kit (Qiagen). The extracted RNA was converted to cDNA by reverse transcription using the iScript Advanced cDNA Synthesis kit (Bio-Rad). Primers used are listed in Table S1.

The RT-qPCR experiments were performed using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Irvine, CA) on the CFX96 ThermoCycler and analyzed using the CFX Manager 3.0 software (Bio-Rad). All RT-qPCR reactions were performed in triplicate for at least 2 independent experiments. Gene expression levels relative to a PBS-treated control were calculated using the $2^{-\Delta\Delta CT}$ method after values were normalized to the housekeeping gene *PGK1*. The resulting fold changes are plotted with error bars representing the standard deviation. The significance (p-value) was calculated using the Student's paired t-test.

Comparative Transcriptome Analysis

Integration of the RNA-seq and Microarray data into R coding platforms facilitated categorization of genes according to their fold change and p-values. This is visualized by the volcano plots and table in Fig. 4. We ranked the genes by dividing the fold change of each gene by their corresponding p-value. This narrowed down our target selection to LHX2.

Gene Overexpression and Knockdown via Electroporation

A plasmid encoding the mouse Lhx2 gene (pCGC-Lhx2; used at a final concentration of 1μg/μL) or the translation blocking anti-*LHX2* morpholino (1mM) was injected into HH23 chicken embryo forelimbs around the FGF bead (Fig. 6) or at the ZPA (Fig. 7). Confined micro-electroporation (CMEP) as previously described (Oberg et al., 2002) was used to introduce the plasmid or morpholino into the limb cells surrounding the injection site. Sterile mineral oil is used in our syringe and needle to facilitate accurate DNA delivery. After injection, a small amount of oil is typically introduced to occlude the site of injection and confine the DNA to the target region. An oil bubble may be seen in electroporated limbs and is marked with an "o" (e.g. Fig 7, bottom row, middle panel) to reduce confusion with an implanted bead.

The LHX2-GFP expression plasmid (pCGC-*Lhx2*) was received as a gift from Dr. Shubha Tole and used with the permission of Dr. Toshi Ohshima. The design and efficacy of the vector has been previously reported (Subramanian et al., 2011). Representative data from three independent experiments is shown in the results section with number of embryos assayed included in the figure legends.

A translation-blocking anti-*LHX2* morpholino (GeneTools, LLC, Philomath, OR) was designed against chicken *LHX2* (NCBI Reference Sequence: NM_204889.1). A negative control morpholino was generated from the anti-*LHX2* sequence incorporating 5 base mismatches to alter the sequence. A positive control anti-*SHH* morpholino was also generated against chicken *SHH* (NCBI Reference Sequence: NM_204821.1) A 3' carboxyfluorescein (green) tag was incorporated into the anti-c*LHX2* and anti-c*SHH* morpholinos while a lissamine (red) tag was incorporated on the 3' end of the negative

control morpholino (anti-c*LHX2*-5mis). The fluorescent tags were used to assess targeting of the injection and electroporation efficiency prior to harvesting embryos for WMISH or RT-qPCR analysis. Morpholino sequences are as follows: anti-c*SHH* MO: 5'-TTGTCAACAGCAGCATTTCGACCAT-3'; anti-c*LHX2* MO: 5'-ACAGGCTGTGGAAAAGCATCGCT-3'; anti-c*LHX2*-5mis: 5'-AgAGcCTGTcGAAAAcCATCGgT-3'. In the 5-base mismatch morpholino (anti-*cLHX2*-5mis), nucleotides in lower case specify the mismatches. All morpholinos were used at a concentration of 1 millimolar.

Controlling for Off-target Effects Using Morpholinos

To control for target specificity, we used 3 previously described approaches (Eisen & Smith, 2008; Moulton & Yan, 2008) including: 1) The use of a mismatched negative control morpholino that differed from the anti-target morpholino by 5 nucleotides. 2) Comparison of phenotypic changes with published reports. Our LHX2 knockdown was consistent with published reports of *Lhx2* mutants (Rodriguez-Esteban et al., 1998; Tzchori et al., 2009). 3) We used "rescue" experiments that showed overexpression of *Lhx2* was able to recover the expression of its downstream target, *SHH*. (Fig. 8).

Results

FGF Can Maintain and Induce SHH Expression in the Posterior Limb Bud

SHH expression persists in the former ZPA domain following implantation of an FGF-laden bead. *SHH* is detectable as early as 3h after application; at 24h, robust *SHH* expression is also induced around the bead (non-ZPA domain) (Fig. 3B). This spatial and

temporal difference in *SHH* up-regulation suggests that induction or reactivation of *SHH* expression outside the former ZPA domain (24h) requires additional FGF-mediated factors besides those required to maintain *SHH* in the former ZPA domain (3h). We analyzed the transcriptome along the posterior margin proximal to the ZPA 3h after FGF application and surrounding the FGF-laden bead after 24h to uncover molecules involved in FGF-mediated SHH expression (areas outlined by dotted lines in Fig. 3).

Brief (3h) FGF Exposure Affects Biologic Processes Associated with its Role in Gene Expression

We found 150 genes differentially regulated 3h after FGF exposure by RNA-seq analysis, with 102 targets up-regulated and 48 down-regulated ($p<0.05$). Four of the top biologic processes affected by FGF exposure were identified in our 3h dataset but not our 24h dataset: “Gene Expression”, “Cell death and survival”, “Cellular development”, and “Tissue morphology” (Fig. 4A). This collection of biologic processes was predicted to be downstream of FGF2, FGF8, in addition to some other growth factors. Moreover, limb-related functions formed subsets of many of these biological processes (listed in Table S2).

Approximately 30% of the targets (n = 42 out of 150) were associated with “Gene Expression”, with 36 categorized as transcription/translation regulators. Transcription factors *Early growth response 1* (*EGR1*), *Lim homeobox 2* (*LHX2*), and *Transcription factor AP2 gamma* (*TFAP2C*) were up-regulated 12.5, 3.9 and 3.4-fold, respectively. Their regulation in response to 3h FGF exposure was confirmed by WMISH and quantified by RT-qPCR (Figure 4B and C).

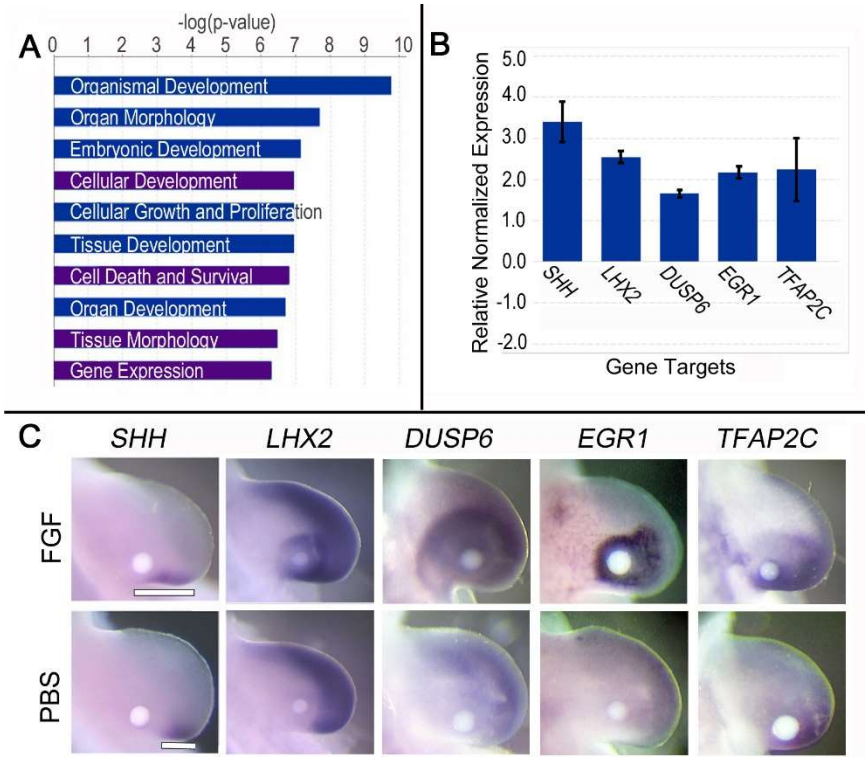


Figure 4. Brief (3h) FGF Treatment Promotes Transcription of Early Developmental Genes and Maintains *SHH* Expression in the Former ZPA Domain (A) Top pathways and biological processes affected 3h after FGF treatment. Purple bars indicate top pathways affected that differed from the 3h transcriptome analysis. (B) Fold change of selected targets treated with FGF for 3h compared to PBS treatment via RT-qPCR ($p<0.05$). Y-axis shows the relative normalized gene expression. Assays were performed in triplicate and at least two independent experiments were performed. Expression of each gene was compared to a PBS control ($p<0.05$ for each comparison). (C) WMISH validation of selected targets regulated by FGF after 3h exposure. Top panel shows up-regulation of respective genes while bottom panel consists of PBS-treated limbs. n=6 embryos per gene for FGF treatment and 3 embryos per gene for PBS. White bars highlight the proximal extension of the S*HH* expression domain in the FGF-treated limb.

Brief (3hr) FGF Exposure Regulates Genes Involved in FGF Feedback Inhibition, Distalization, and SHH Expression

After 3h of FGF treatment, *Dual specificity phosphatase 6* (*DUSP6*) and *Sprouty 2* (*SPRY2*), both negative regulators of FGF signaling, were up-regulated 2.6-fold and 3.4-fold, respectively, consistent with FGF feedback inhibition. The up-regulation of *DUSP6* was validated by WMISH and RT-qPCR (Fig 4B and C). Coupled with *DUSP6* and *SPRY2*, several other distally restricted genes were up-regulated in the former ZPA domain 3h after FGF exposure including *Bone morphogenetic protein 4 (BMP4;* 1.8-fold). BMP4 plays a role in AER maintenance and regression and cooperates with SHH in determining digit identity (Bastida, Sheth, & Ros, 2009; Norrie et al., 2014). In contrast, the proximally restricted *Pre-B-cell leukemia homeobox 1* (*PBX1*) was down-regulated 1.8-fold. *Pbx1*-deficient mice have malformations in proximal limb elements suggesting a role in proximal limb development (Capellini et al., 2006). Down-regulation of proximal markers by brief FGF exposure and the up-regulation of distal factors support a distal re-specification of the mid-proximal limb, which may be necessary for *SHH* expression.

Several targets known to be upstream of *SHH* expression including *Homeobox D9* (Matsubara et al., 2017; Xu & Wellik, 2011), *ELF2* (a member of the ETS family) and *HAND2* (a basic helix loop helix transcription factor) were up-regulated 2.3-fold, 1.9-fold and 1.6-fold, respectively. A complex interaction between HOX, PBX, ETS, and HAND2 has been implicated in the activation and localization of *SHH* expression to the ZPA (Osterwalder et al., 2014). These transcription factors coordinate their interactions on *SHH* expression through a conserved long range *cis*-regulatory module, the *ZPA*

regulatory sequence (*ZRS*) (L. Lettice, Heaney, & Hill, 2002). *LHX2* and *TFAP2C* are two additional distally restricted transcription factors with potential binding sites in the *ZRS* that were up-regulated after 3h of FGF treatment. Our dataset revealed a 2.6-fold increase in *SHH* expression after 3h of FGF exposure, which is supported by *in situ* and RT-qPCR data (Fig. 4B and C).

Prolonged (24hr) FGF Exposure Affects Cell Processes Related to Organ and Organismal Development

DNA microarray analysis identified 3434 differentially regulated targets after 24h of FGF exposure ($p<0.05$). In addition to *SHH*, 2152 targets were up-regulated, while 1281 were down-regulated. Of the differentially expressed targets, 1085 mapped to IPA-curated genes. IPA revealed the top 10 biological processes were related to growth and development. Four processes were found at 24h that are not present at 3h: "Skeletal Muscle System Development and Function", "Nervous Tissue Development and Function", "Organismal survival", and "Connective Tissue Development and Function" (Fig. 5A). IPA also predicted that the molecules, functions and biologic processes identified in the microarray data were downstream of FGF2, FGF4, and FGF8. Several limb-related functions form subsets of the biologic processes mined by IPA (Table S2). Of note, an abundance of differentially regulated genes encoding Wnt signaling proteins were detected (n=46). Wnt proteins are involved in limb initiation, outgrowth, cell migration, cell differentiation and patterning (Church & Francis-West, 2002; Geetha-Loganathan, Nimmagadda, & Scaal, 2008). This led us to question whether a Wnt pathway might be involved in the up-regulation of *SHH*. However, overexpression of the

highest up-regulated Wnt protein (*WNT5a*; 1.6-fold) in the posterior limb bud did not lead to ectopic *SHH* expression (Fig. S1).

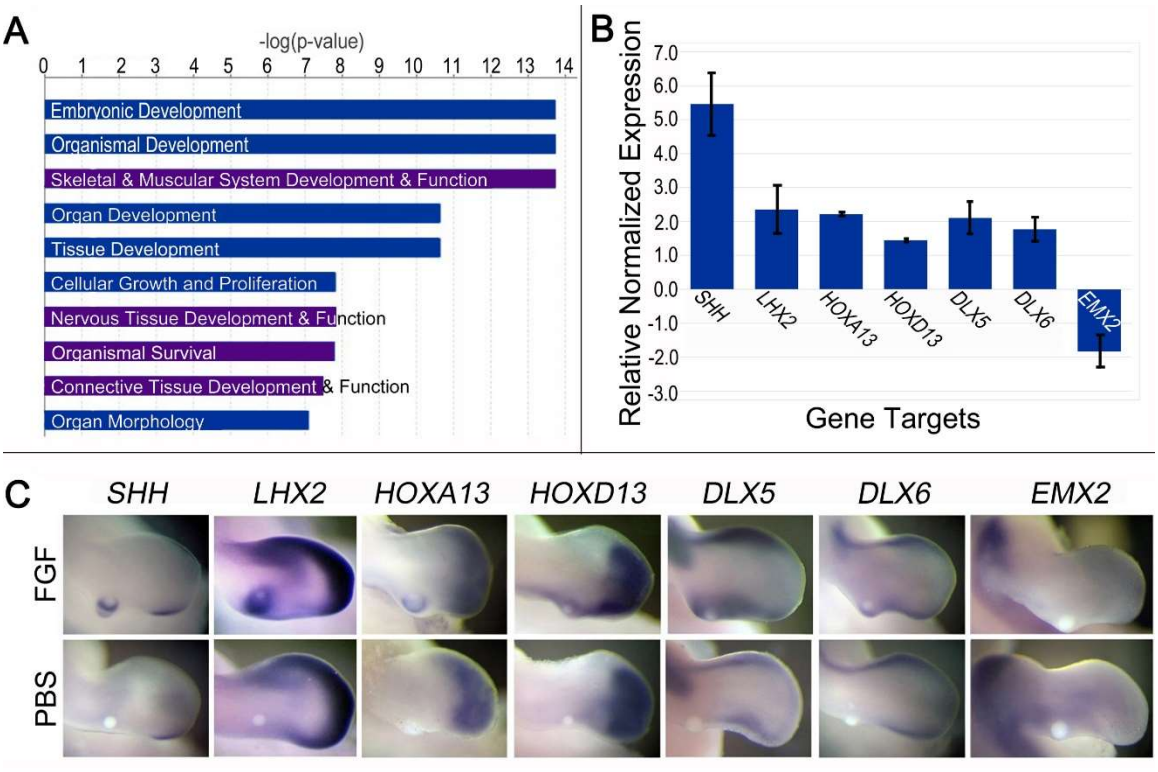


Figure 5. Prolonged (24hr) FGF Treatment in the Mid Proximal Limb Bud Promotes Distalization, Supports Processes Involved in Embryonic Development and Leads to *SHH* Induction. (A) Top pathways and biological processes affected by 24h of FGF treatment. Purple bars indicate top pathways affected that differed from the 3h transcriptome analysis. (B) RT-qPCR validation of FGF-mediated expression of target genes. Y-axis shows the relative normalized expression of selected target genes. Assays were performed in triplicate and at least two independent experiments were performed. Expression of each gene was compared to the same region on the contralateral limb buds ($p<0.05$ for each comparison). (C) WMISH of distally-restricted transcription factors confirm their regulation in response to 24h of FGF exposure. Top panel shows up-regulation/down-regulation in response to ectopic FGF when compared to a PBS-treated limb (control; bottom panel). n=6 embryos per gene for FGF treatment and 3 embryos per gene for PBS. Note that *EMX2* is strongly expressed proximally but has a weak distal expression that was reduced by FGF treatment after 24h.

Prolonged FGF (24hrs) Regulates Genes Associated with Dedifferentiation, Distalization, and SHH Regulation

Several transcription factors associated with cell dedifferentiation were up-regulated in response to prolonged FGF exposure: *Spalt Like Transcription Factor 4* (*SALL4*; 1.8-fold), *Muscle segment homeobox genes 1 and 2* (*MSX1*; 4.4-fold and *MSX2*; 3.0-fold), *LHX2* (5.6-fold), and *Matrix metallopeptidase 11* and *17* (*MMP11* and *MMP17*, each 1.4-fold). FGF treatment also down-regulated members of the collagen family, which are associated with limb differentiation and chondrocyte maturation (*COL12A1*, *COL6A1*, *COL6A3*, *COL21A1*; > 1.5-fold). Dedifferentiation of the mid-proximal limb bud may be a crucial step in re-programming this region to express *SHH*. Alternatively, FGF may be functioning to prevent differentiation and promote the maintenance of cells in an undifferentiated state. Another step could be the distal re-specification of the mid-proximal limb supported by the up-regulation of several distally restricted genes including *HOXA13* (13.0-fold), *HOXD13* (1.7 fold), *DLX5* (2.2 fold), *DLX6* (3.6-fold) and *LHX2* (5.6-fold) (Fig. 3B and 3C), while proximally restricted genes such as *Empty spiracles homeobox 2* (*EMX2*) and *MEIS2* were down-regulated 1.7- and 1.5-fold, respectively. Down-regulation of *EMX2* was validated by WMISH and RT-qPCR (Fig. 5B and C). Together, the data suggest that dedifferentiation and the distal re-specification of the mid-proximal limb bud mesoderm play a role in FGF-mediated *SHH* expression.

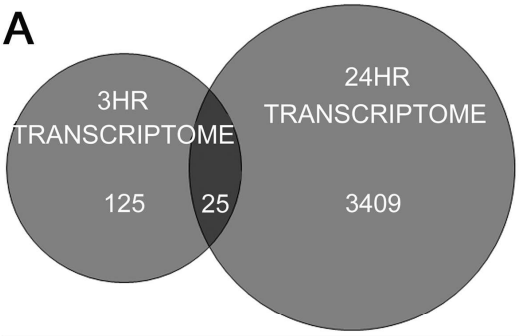
After 24h of FGF exposure, *SHH* is up-regulated 2.1-fold around the FGF bead. This correlated with RT-qPCR data (up-regulated 5.5-fold) (Fig. 5C). Genes associated with *SHH* expression include the distally restricted *HOXA13*, *HOXD13*, and *LHX2*. Other

up-regulated *SHH*-associated genes include *HOXA10* (1.4-fold), *HOXA11* (1.7-fold), *Gap junction protein, alpha 1* (*GJA1*; 1.3-fold) *ETS2* (1.3-fold), and *ETV5* (1.1-fold). Although, the fold change for *ETV5* might be less than typical cutoffs, it has a p value <0.05 and an established functional role in linking FGF signaling to the *ZRS* and *Shh* expression (Mao, McGlinn, Huang, Tabin, & McMahon, 2009; Z. Zhang, Verheyden, Hassell, & Sun, 2009). The *SHH* antagonist *Aristaless-like homeobox 4* (*ALX4*) was down-regulated 1.7-fold. ETS2, ETV5, and ALX4 contribute to the spatial localization of *SHH* expression (L. A. Lettice et al., 2012; Qu et al., 1997; Z. Zhang et al., 2009). Notably, our 24h dataset includes up-regulation of factors downstream of SHH signaling such as *PTCH1* (1.8-fold) and *PTCH2* (1.1-fold). Because the 24h transcriptome may include a number of downstream targets of SHH, we compared it with the 3h transcriptome to highlight common genes most likely to be upstream of *SHH*.

Common Pathways Enriched by Comparative Transcriptome Analysis

Six of the top IPA-curated pathways affected by FGF treatment were common between the 3 and 24h datasets including "Embryonic/Organismal Development" and "Growth and Proliferation" – hallmarks of FGF signaling (Fig. 4A and 5A). We also detected mutual limb-related functions associated with limb development and digit morphogenesis (see Table S2). Twenty-five (25) genes were common to both transcriptomes with 19 being up-regulated, 5 down-regulated, and 1 gene down-regulated at 3h but up-regulated at 24h (Fig. 6).

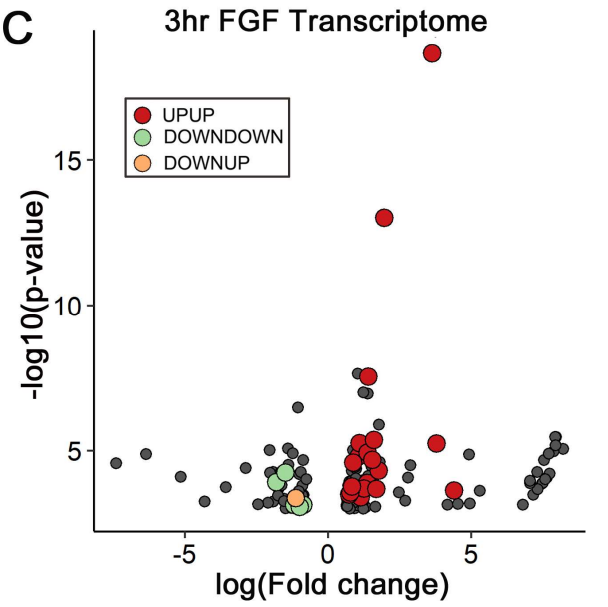
A



B

GENE		GROUP	RANK		
			OVERALL	3HR	24HR
EGR1	*	UPUP	1	1	10
LHX2	*	UPUP	2	2	1
DUSP6		UPUP	3	3	12
TCF21		UPUP	4	4	6
MYCN		UPUP	5	5	3
TFAP2C	*	UPUP	6	10	2
GJA1	*	UPUP	7	8	5
SHH		UPUP	8	7	8
IARS		UPUP	9	6	18
TOM1L1		UPUP	10	16	4
TAF4B		UPUP	11	9	15
TSKU		UPUP	12	11	19
APCDD1		UPUP	13	12	7
AMD1	*	UPUP	14	13	13
RANBP3		UPUP	15	14	17
MGAT4B	*	UPUP	16	15	16
DOK5		UPUP	17	17	11
PDPK1		UPUP	18	18	19
ADNP		UPUP	19	19	14
RSPO3	*	DOWNDOWN	1	1	1
TTC8	*	DOWNDOWN	2	2	2
SLC25A4		DOWNDOWN	3	3	3
HIC2		DOWNDOWN	4	4	4
DBNDD1		DOWNDOWN	5	5	5
PCDH10		DOWNUP	1	1	1

C



D

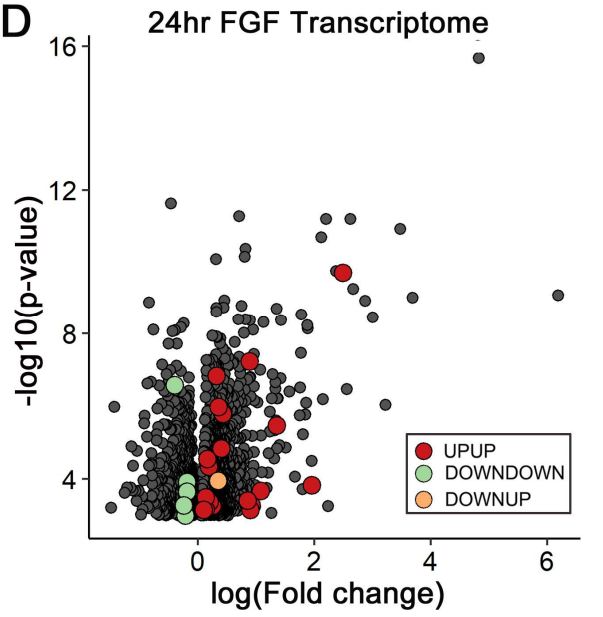


Figure 6. Common Transcripts Regulated by FGF2 During Maintenance and Induction of *SHH* Expression are Likely Candidates for FGF-mediated *SHH* Expression. (A) The number of FGF-regulated transcripts common to both 3 and 24h datasets is shown in the overlapping region of the Venn Diagram (n=25). (B) Table showing the 25 common targets from the 3 and 24h transcriptome that are likely candidates for FGF-mediated SHH expression. Targets are ranked based on differential expression (fold change) divided by significance (p-value). "*" denotes genes associated to *SHH* expression/function. Genes up-regulated at both 3 and 24h are designated with Group ID "UPUP": red (n=19). Genes down-regulated at both time points are designated "DOWNDOWN": green (n=5); while genes down-regulated at 3h and up-regulated at 24h are designated "DOWNUP": orange (n=1). (C) Distribution (by log10 p-value and log2 fold change) of the 150 genes differentially regulated after 3h FGF treatment with the 25 common targets highlighted in color ($p<0.05$). (D) Distribution (by log10 p-value and log2 fold change) of the 3434 genes differentially regulated by 24h FGF treatment with the 25 common targets highlighted in color ($p<0.05$).

Common Targets that Inhibit WNT Signaling

We identified 5 genes associated with the Wnt pathway within the shared 25 FGF-regulated genes. *APCDD1* (Shimomura et al., 2010) and *RANBP3* (Hendriksen et al., 2005) are Wnt signaling inhibitors that were up-regulated by FGF, while *R-spondin 3* (*RSPO3*), a secreted ligand that binds cell surface receptors and activates Wnt/β-Catenin or Wnt/Planar cell polarity signaling (Neufeld et al., 2012; M. Zhang et al., 2017), was down-regulated. One downstream target of Wnt signaling, SNAI2, was up-regulated while another, *TUSC3* (Gu et al., 2016), was down-regulated. Taken together, these data support a role for Fgf in the complicated regulation of Wnt signaling during limb development (Geetha-Loganathan et al., 2008; ten Berge, Brugmann, Helms, & Nusse, 2008).

Common Targets Associated with *SHH* Expression

Of the 25 common targets, 8 relate to *SHH* expression, function or signaling (highlighted by asterisks in Fig. 4B). Four genes associated with *SHH* expression were up-regulated: *EGR1*, *GJA1* (also known as *Connexin 43*), *LHX2*, and *TFAP2C*. *TTC8*, which is associated with ciliary-associated GLI processing, was down-regulated. Collectively, the regulation of these genes is expected to enhance SHH function/signaling. Paradoxically, *RSPO3*, a gene associated with *SHH* up-regulation, was down-regulated. *AMD1* and *MGAT4B* were both up-regulated and are decreased in Shh deficient mice (Probst et al., 2011). They could therefore be downstream of Shh signaling. Neither *AMD1* nor *MGAT4B* has been well-characterized, however, and require further investigation.

LHX2 as an Intermediate in FGF-regulated SHH Expression

Our screening identified LHX2 as a candidate for mediating FGF regulation of SHH. In the limb, *LHX2* is robustly up-regulated by FGF following 3 and 24h of exposure (Fig. 2 & 3). Additionally, *LHX2* is distally restricted subjacent to the AER overlapping the ZPA (Fig. 5A). Since Lhx9 appears to play a redundant role in regulating *Shh* expression in mice, we evaluated its expression pattern in the chicken model. In chicken wings, *LHX9* is restricted to the anterior margin of the developing limb mesoderm and does not overlap the ZPA. Moreover, *LHX9* was not significantly up-regulated in our transcriptome data and not convincingly up-regulated around applied FGF beads (Fig. 5B). Thus, in chicken, LHX2, but not LHX9, could function as an intermediate in the FGF to SHH pathway.

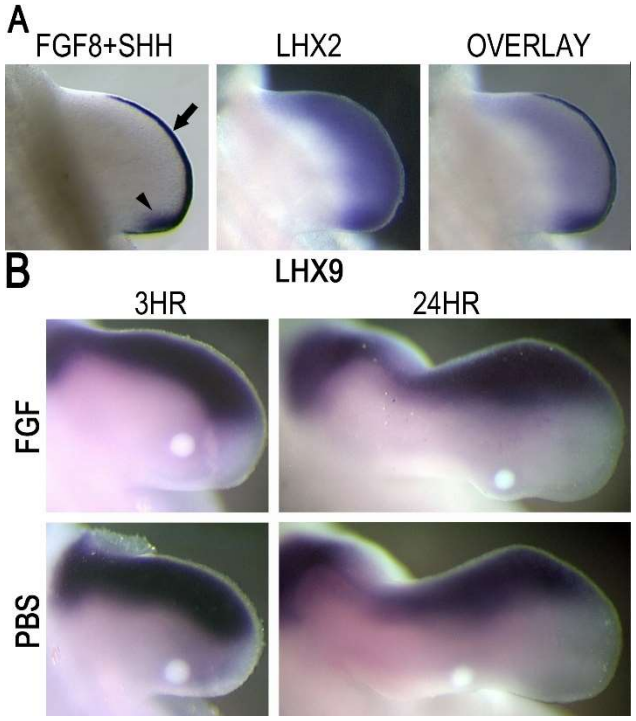


Figure 7. The *LHX2* Expression Pattern Supports a Role for Maintaining Distal Posterior *SHH* Expression During Progressive Limb Outgrowth (A) Left panel shows endogenous *FGF8* (arrow) and *SHH* (arrowhead) expression in a HH23 limb bud. Middle panel shows endogenous *LHX2* expression. Right panel is a composite of all 3 expression patterns. *LHX2* expression is restricted to the distal mesoderm in the developing limb subjacent to the *FGF8*-expressing AER. Importantly, *SHH* is expressed only within the posterior boundary of the *LHX2* expression domain. The overlapping expression pattern is consistent with a role for LHX2 in facilitating SHH expression in the developing limb. (B) After 3 and 24h of FGF2 exposure, no appreciable ectopic *LHX9* expression is observed (n=3 embryos per treatment group for each time point). PBS beads were used as controls. Note endogenous *LHX9* expression is restricted to the anterior mesoderm.

To determine the role of LHX2 in FGF-regulated *SHH* expression, we electroporated a mouse Lhx2 expression vector into the posterior limb mesoderm adjacent to an FGF or PBS soaked bead. Lhx2 was not sufficient to induce *SHH* independently. However, when combined with FGF, Lhx2 increased the expression of *SHH* (2.7-fold by RT-qPCR) and *PTCH2*, a downstream target of SHH signaling (Fig. 6). We also examined the loss of LHX2 function using an anti-*LHX2* morpholino (anti-c*LHX2* MO) designed to block translation of the chicken *LHX2* transcript. Electroporation of this morpholino around an FGF bead implant resulted in decreased *SHH* expression (35% by RT-qPCR) and a decrease in *PTCH2*. Targeted SHH knockdown gives a similar decrease in *PTCH2* expression (Fig. 6). Additionally, a decrease in LHX2 at the ZPA resulted in decreased limb outgrowth and reduced endogenous *SHH* expression after 24h (Fig. 7). This is consistent with a previous report that suggested competitive inhibition of Lhx2 activity inhibits limb outgrowth in chicken as well as interferes with the expression of distally restricted genes such as *SHH* (Rodriguez-Esteban et al., 1998).

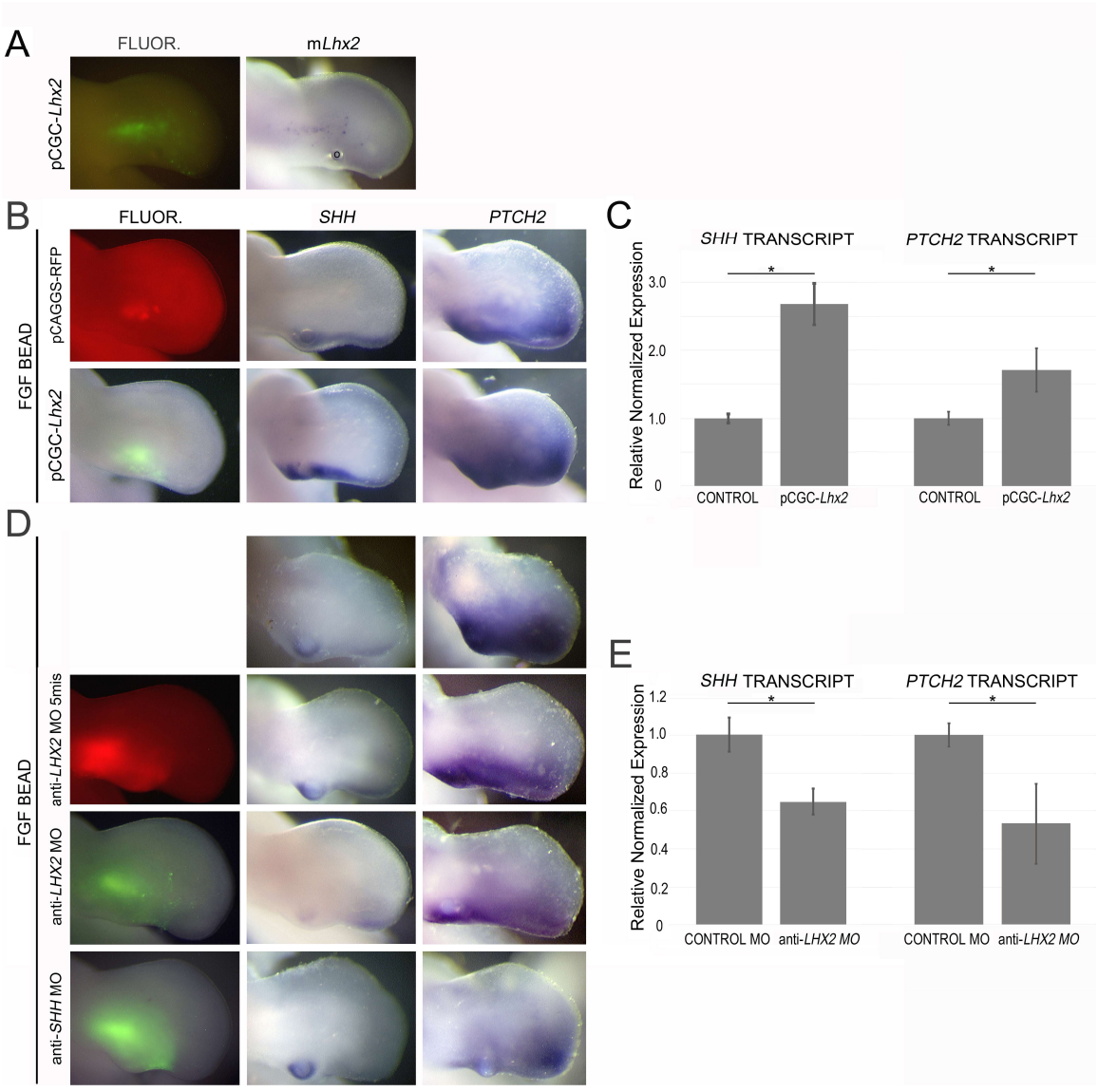

A
FLUOR.
mLhx2
pCGC-Lhx2
B
FLUOR.
SHH
PTCH2
FGF BEAD
pCAGGS-RFP
pCGC-Lhx2
C
SHH TRANSCRIPT
PTCH2 TRANSCRIPT
Relative Normalized Expression
3.0
2.0
1.0
0
CONTROL
pCGC-Lhx2
CONTROL
pCGC-Lhx2
D
FGF BEAD
anti-LHX2 MO 5mis
anti-LHX2 MO
anti-SHH MO
E
SHH TRANSCRIPT
PTCH2 TRANSCRIPT
Relative Normalized Expression
1.2
1.0
0.8
0.6
0.4
0.2
0
CONTROL MO
anti-LHX2 MO
CONTROL MO
anti-LHX2 MO

Figure 8. LHX2 Regulates SHH Expression and Function. (A) Fluorescence and WMISH pictures confirming ectopic mouse *Lhx2* following electroporation of an Lhx2 expression vector containing a GFP reporter (pCGC-*Lhx2*). "o" is used to identify the oil bubble which is occasionally present after the injection of a plasmid. (B) Increasing the level of local LHX2 by electroporation of pCGC-*Lhx2* around an FGF bead leads to an increase in the expression of *SHH* (n=12) and its downstream target *PTCH2* (n=8) when compared to an RFP vector. (C) RT-qPCR data from 2 independent experiments reveals a 2.7-fold up-regulation of FGF-induced *SHH* and a 1.7-fold up-regulation of *PTCH2* transcripts in the presence of additional LHX2 when compared to FGF alone ($p<0.05$). Tissue samples from 7-10 embryos were pooled for each treatment group and assayed in triplicate. (D) Transfection of an anti-*LHX2* morpholino decreases FGF-induced *SHH* expression (3rd row; n=7) when compared to limbs treated with FGF bead only (top row; n=5) or FGF with a negative control (5 base mismatch anti-*LHX2* MO; 2nd row; n=5). *PTCH2* expression was also decreased following treatment with the anti-*LHX2* morpholino (n=10) and mimics the *PTCH2* reduction observed with a knockdown of SHH using an anti-*SHH* MO (n=5). *SHH* expression is not affected by the electroporation of an anti-*SHH* morpholino designed to block translation of *SHH* transcript (bottom panel; n=3). (E) Following a knockdown of LHX2, *SHH* and *PTCH2* transcript levels were reduced by 35% and 53%, respectively, when compared to the anti-*LHX2* MO 5 mismatch control ($p<0.05$). Tissue samples from 7-10 embryos were pooled for each treatment group and assayed in triplicate for 2 independent RT-qPCR runs. * $p<0.05$.

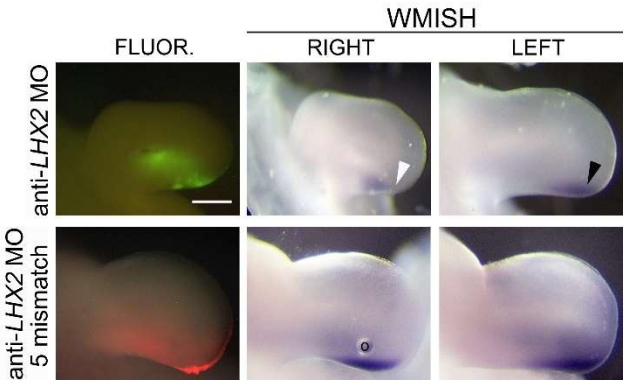


Figure 9. LHX2 Knockdown at the ZPA Decreases *SHH* Expression. 24h after electroporation of anti-*LHX2* MO at the ZPA, there is reduced *SHH* expression and retarded limb outgrowth when compared to the contralateral limb (LEFT) and to the negative control (anti-*LHX2* MO 5 mismatch). Reduced *SHH* expression and retarded limb outgrowth were observed in 9 out of 10 embryos from 2 independent experiments. Please note oil bubble "o" in bottom row (middle panel). The images of the left contralateral control limbs have been reversed for comparison. Scalebar = 0.25 inches.

Discussion

One of the roles of apical ectodermal ridge (AER)-secreted FGFs is the maintenance of *SHH* expression in the subjacent ZPA during limb outgrowth(Crossley et al., 1996). We were able to maintain the expression of *SHH* in the former ZPA domain by application of FGF proximal to the ZPA. Persistent *SHH* expression was evident within as little as 3h. FGF was also able to induce *SHH* in non-ZPA domain related mesoderm, although this required prolonged exposure (24h) (Fig. 1). Cells in the population responding to prolonged FGF are likely to include some prior SHH-expressing cells. Most of the prior SHH-expressing cells, however, are present within the autopod and prospective digits at a comparable stage of development (B.D. Harfe et al., 2004). Irrespective of their prior history, induction or reactivation of cells in the non-ZPA domain requires prolonged FGF exposure to induce *SHH* expression and a mechanism disparate from cells residing within the former ZPA domain. By comparing transcriptome data from these two populations of *SHH*-expressing cells, we were able to identify 25 common genes including 19 synexpressed with *SHH* and 5 down-regulated.

FGF Regulates Genes that Support SHH Expression

Eight of the 25 common targets differentially regulated by FGF exposure were associated with *SHH* expression, signaling or function (Fig.4). Consistent with our data, EGR1 has been reported to act downstream of FGF signaling (Han, Guerrero-Netro, Estienne, Cao, & Price, 2017; Lejard et al., 2011). Additionally, it serves as a direct transcriptional regulator of *SHH* in glioma cells (Sakakini et al., 2016). In the limb, EGR1 participates in FGF-induced tendon differentiation (Lejard et al., 2011), but its role

in FGF-mediated *Shh* expression has yet to be evaluated. FGF up-regulated *GJA1*, which is a gene that encodes Connexin 43, a gap junction protein. Gap junction proteins have been reported to relay FGF signals to neighboring cells; moreover, the conditional knockout of Connexin 43 in mice leads to reduced *Shh* expression, limb truncation, and patterning defects (Dobrowolski et al., 2009; Law, Lin, Becker, & Green, 2002). Thus, an increase in *GJA1* in both of our datasets suggests that FGF supports *SHH* expression by up-regulating intercellular communication. FGF also up-regulated *TFAP2C*. TFAP2C is an activating, enhancer-binding protein-2 (AP2) transcription factor expressed subjacent to the AER in normal limbs (Fig. 2C). The *ZPA regulatory sequence* (*ZRS*) contains a potential AP2 binding site. Interestingly, a mutation generating an extra AP2 binding site in the *ZRS* is associated with anterior ectopic *SHH* expression and preaxial polydactyly (Fuxman Bass et al., 2015). Although its expression pattern and role in misregulation of the *ZRS* are intriguing, a role for AP2 transcription factors in normal *SHH* expression has yet to be determined.

Tetratricopeptide Repeat Domain 8 (TTC8), also known as Bardet Biedl syndrome 8 (BBS8), was down-regulated in our analysis. BBS8 is part of the stable core protein complex of cilia involved with SMO ciliary trafficking, which processes Gli2 and Gli3 (Haycraft et al., 2005; Q. Zhang, Seo, Bugge, Stone, & Sheffield, 2012). Processed GLI transcription factors are truncated and repress SHH targets (Sheeba, Andrade, & Palmeirim, 2016). In humans, TTC8 mutations are associated with pre-and post-axial polydactyly consistent with activation of the hedgehog pathway(M'Hamdi, Ouertani, & Chaabouni-Bouhamed, 2014; Manouvrier-Hanu, Moerman, & Lefevre, 1999). Tayeh and colleagues showed that loss of BBS function in zebrafish increased fin/limb

expression of *SHH(Tayeh et al., 2008).* Thus, the regulation of TTC8 by FGF offers a mechanism to enhance the activation SHH that warrants further investigation.

R-spondin 3 (RSPO3) is down-regulated but potentially functions upstream of SHH. A *Rspo2/Rspo3* double mutant showed more severe limb defects than either single mutant with the most anterior and posterior digits missing, leaving 3 shortened middle digits (Neufeld et al., 2012). The fact that posterior elements in the forelimb are lost in this double mutant correlates well with the phenotype of *SHH* deficient limbs (Chiang et al., 2001; Kraus et al., 2001; Ros et al., 2003) and suggests that R-spondin genes could affect *SHH* expression/function. No limb expression patterns were found for *AMD1* and *MGAT4B,* but our data indicate that they are up-regulated by FGF signaling. Additionally, *Amd1* and the Mgat4b paralog *Mgat4a* are reported to be decreased in the limbs of Shh deficient mice (Probst et al., 2011) indicating that they may be downstream of SHH signaling.

FGF Regulates Factors that Localize SHH Expression

The *ZRS* is a conserved *cis*-acting regulatory element responsible for limb-specific *SHH* expression (L. Lettice et al., 2002; Ros et al., 2003; Sharpe et al., 1999; Smyth, Sreekumar, Coyle, & Bitgood, 2000). The *ZRS* houses multiple binding sites for the ETS/ETV family of transcription factors. At 3h, *ELF2* (*E74 like ETS transcription factor 2*) and *ETV6* were up-regulated while *ETS2* and *ETV5* were up-regulated at 24h. Lettice et al. reported that a balance between occupancy of the ETS/ETV binding sites within the *ZRS* contributes to the expression and location of *Shh* in the limb bud (L. A. Lettice et al., 2012). The up-regulation of members of the ETS family in our data

supports the notion that FGF regulates ETS/ETV transcription factors to modify *SHH* expression in the developing limb and highlights the possibility that family members other than ETS2 and ETV4/5 (L. A. Lettice et al., 2012; Z. Zhang et al., 2009) may be involved.

The TAATTA binding motif for LHX2 (Roberson, Schoderbek, Tremml, & Maurer, 1994) is found in the *ZRS* and is conserved across 16 vertebrate species including human, mouse and chicken (Kvon et al., 2016). Of note, other Hox proteins share this binding motif and 5' Hox genes have been reported to bind the ZRS (Capellini et al., 2006); therefore, LHX2 binding to this region needs to be confirmed.

LHX2, but not LHX9, Regulates FGF-mediated SHH Expression during Chicken Limb Development

Reports have suggested that LHX2 and a homolog LHX9 may be functionally redundant due to their overlapping expression patterns (Bertuzzi et al., 1999; Peukert, Weber, Lumsden, & Scholpp, 2011; Tzchori et al., 2009). Simultaneous knockout of *Lhx2* and *Lhx9* causes a marked reduction in *Shh* expression and altered limb growth and patterning characterized by oligodactyly, loss of digit morphology, and a shortened limb (Tzchori et al., 2009). In chicken, *LHX2* is expressed in the distal mesoderm subjacent to the AER in a pattern that overlaps the ZPA, while *LHX9* is restricted to the anterior and distal rim of the limb mesoderm distant from the ZPA (Fig. 5) (Nohno et al., 1997; Rodriguez-Esteban et al., 1998). Unlike the mouse model where an *Lhx2/Lhx9* double knockout was necessary to perturb limb outgrowth and *Shh* expression, Rodriguez-Esteban and coworkers showed that a retroviral LHX2-targeted repressor caused limb

truncations (Rodriguez-Esteban et al., 1998). We further show that targeted knockdown of LHX2 within the ZPA is sufficient to decrease *SHH* expression and disrupt limb outgrowth (Fig. 7). These collective findings indicate a species-specific difference in the function of Lhx family members but highlight Lhx2 as a common mediator of *Shh* expression.

Although the pattern of *Lhx2* and *Lhx9* expression in mice is consistent with regulation by FGF, Tzchori et al. suggested that Fgf4/8 did not control their expression (Tzchori et al., 2009). In contrast, Yang and colleagues showed that Fgf signaling was required for *Lhx9* expression in mouse limb explants(Yang & Wilson, 2015). In chicken, we demonstrate by transcriptome, WMISH, and RT-qPCR that FGF robustly up-regulates *LHX2* but not *LHX9*. *LHX2* overexpression or knockdown in the context of ectopic FGF-bead application also resulted in a robust increase or decrease in *SHH* expression, respectively. Our data indicate that LHX2 is a target of FGF signaling and an intermediate in the FGF to SHH regulatory loop.

Interestingly, Tzchori and colleagues demonstrated that Ldb1, a cofactor of LIM transcription factors, was required for FGF-mediated induction of *Shh. Ldb1* is ubiquitously expressed in the limb and is known to associate with a variety of LIM-domain genes (Jurata, Pfaff, & Gill, 1998; Tucker et al., 1999). The widespread expression of Ldb1 and its required presence to permit Fgf-mediated *Shh* expression points to a cofactor, such as Lhx2, as an intermediate in up-regulating *Shh*.

LHX2 as a Competency Marker for SHH Expression in the Limb

Our data corroborates other reports that indicate LHX2 is necessary for *SHH*

expression (Tzchori et al., 2009). Additionally, *LHX2* expression overlaps the ZPA and the proximal extent of *LHX2* expression corresponds to the proximal boundary of the ZPA; beyond this boundary, *SHH* expression wanes. Taken together, LHX2 is likely a competence factor for *SHH*, keeping its expression juxtaposed to the AER during progressive limb outgrowth. Other known competence factors such as *HoxB8* (H. C. Lu, Revelli, Goering, Thaller, & Eichele, 1997), *Hox9* paralogs (Xu & Wellik, 2011), and *Hand2* (Charite, McFadden, & Olson, 2000; Fernandez-Teran et al., 2000) display expression domains larger than, but inclusive of, the ZPA to permit *SHH* expression indicating a collective cooperation among competence factors.

Ectopic apical *Shh* expression subjacent to the AER in an *Lhx2*-like pattern has been reported (Y. Chen et al., 2004; Zhulyn et al., 2014). Interestingly, in both reports there was a reduction in *Gli3*. *Gli3* is expressed throughout the limb except within the distal posterior mesoderm (Abbasi et al., 2010) and, together with Alx4 (Qu et al., 1997), Twist (Z. Zhang et al., 2010), and the Etv proteins (Mao et al., 2009; Z. Zhang et al., 2010), plays a role in restricting *Shh* expression to this limited posterior domain. Reduction in the repressive activity of Gli3 results in the anterior expansion of Hand2, while *Lhx2* expression in *Gli3*-deficient limb buds remains unchanged (Yokoyama et al., 2017). The ectopic apical *SHH* expression pattern overlapped the expanded *Hand2* domain but was more distally restricted, suggesting that a distally restricted factor, such as Lhx2, was required. We suspect that Lhx2 is a transcription partner with Hand2 and other competency factors to regulate and maintain *Shh* transcription.

In conclusion, we identified a common set of genes regulated by FGF with potential to function as intermediates in limb-related FGF-mediated *SHH* expression.

Additionally, we have extended the role of LHX2 from previous reports providing evidence that LHX2 mediates the FGF to SHH regulatory loop during limb development.

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CHAPTER THREE

FIBROBLAST GROWTH FACTORS REGULATE LHX2 THROUGH THE RAS/MEK PATHWAY AND POTENTIAL CIS-REGULATORY MODULES

Abstract

Lim homeobox 2 (LHX2) is a transcription factor implicated in the development of many organs including the brain, eye, liver, lungs and limb. In the limb, *LHX2* is expressed in the distal mesoderm subjacent to the apical ectodermal ridge (AER). Fibroblast growth factors (FGFs) are secreted from the AER and control proximal to distal limb outgrowth and patterning. Loss of LHX2 results in truncation defects along this axis suggesting a link between FGFs and LHX2 during limb outgrowth. We set out to establish a link between FGF and LHX2 and investigate the molecular mechanisms driving this association. We found that LHX2 is a primary transcriptional target of FGF signaling and that removal of the AER as well inhibition of FGF signaling resulted in a loss of *LHX2* expression. Further, we screened and identified potential *cis*-regulatory modules (PCRMs) that house several FGF-related transcription factor binding sites. We tested the activity of 10 PCRMs and found that 3 exhibited activity coincident with *LHX2* expression in the developing limb and/or brain.

Introduction

The apical ectodermal ridge (AER) is a thickened epithelium that demarcates the dorsal-ventral boundary running along the antero-posterior axis at the distal tip of the limb bud. It serves as a signaling center from which Fibroblast growth factors (FGFs) and Wingless-related integration site (WNT) proteins are secreted. AER-related WNTS are responsible for AER initiation and maintenance while AER-related FGFs control proximal to distal limb outgrowth (Niswander et al., 1993; Tickle, 2002). In the absence of the AER, FGFs are sufficient to promote normal limb development. Additionally, complete ectopic limbs can be formed when FGF1, FGF2, or FGF4 are applied to the presumptive flank of the chicken embryo (Cohn, Izpisua-Belmonte, Abud, Heath, & Tickle, 1995) indicating that FGFs can drive the molecular machinery necessary for limb initiation and development.

The FGF ligands control cell proliferation and differentiation by activating four trans-membrane tyrosine kinase receptors, the fibroblast growth factor receptors (FGFR1-4) (Mohammadi et al., 1997). In mammals, there are 22 known FGF ligands with at least five of them being expressed in the embryonic limb. In chickens, FGF2, 4, 8, 9, and 17 are expressed in the AER and FGF2 and FGF10 in the limb mesoderm (Horakova et al., 2014). Three FGFRs are expressed during limb development and have been shown to be essential for skeletogenesis (De Luca & Baron, 1999): Fgfr1 is expressed in the developing limb mesoderm, Fgfr2 in mesodermal condensations, and Fgfr3 in developed growth plate cartilage (K. Peters, Ornitz, Werner, & Williams, 1993; K. G. Peters, Werner, Chen, & Williams, 1992).

FGF signaling is activated by a ligand-receptor interaction that results in the autophosphorylation of tyrosine residues in the intracellular region of an FGF receptor (FGFR) (Lanner & Rossant, 2010). The signal is further relayed through four distinct pathways: the Janus kinase/signal transducer and activator of transcription (Jak/Stat), phosphoinositide phospholipase C (PLCγ), phosphatidylinositol 3kinase (PI3K) and Ras/Mek/Erk pathways (Dailey, Ambrosetti, Mansukhani, & Basilico, 2005).

FGF signaling is tightly regulated and modulated at multiple levels, both intracellularly and extracellularly. For example, the bioavailability of secreted FGF ligands is regulated by their binding to extracellular heparan sulfate proteoglycans (HSPGs). (Ornitz & Itoh, 2001). Intracellularly, the FGF signaling cascade is further regulated by negative-feedback mechanisms at multiple levels by dual specificity phosphatases (DUSPs), similar expression to FGF (SEF), Spred and Sprouty proteins (Dailey et al., 2005). Several of these inhibitors are themselves downstream transcriptional targets of the FGF pathway. Other transcriptional targets of the FGF pathway drive mRNA transcription and expression of developmental genes based on the location and availability of their binding sites.

Transcription factors bind to *cis*-regulatory modules (CRMs), which are DNA sequences that have transcriptional activity. CRMs may act as promoters which bind the basal transcriptional machinery and delineates where transcription begins or as enhancers which control the spatio-temporal transcription of genes. Enhancers may be located at some distance from the transcriptional start site and they combinatorially bind multiple transcription factors (Davidson & Peter, 2015). Their distance from their target gene makes it difficult to associate genes and their respective enhancers. The importance of

this association cannot be understated however, as it provides insights into the architecture of the underlying gene regulatory networks which govern proper development.

We have previously reported the ability of FGFs to upregulate LHX2 in the context of FGF-mediated *SHH* expression (Watson et al., 2018). Here we explore the molecular mechanisms downstream of FGF signaling that result in the transcriptional regulation of *LHX2* in the developing limb.

Materials and Methods

Removal of the Apical Ectodermal Ridge (AER)

Fertilized white leghorn eggs were incubated at 39ºC in a humidified chamber for about 96 hours to embryonic stage HH23. The AER of the right limb was partially or completely removed *in ovo* using tungsten needles then the embryo was incubated for an additional 6, 12 or 24 hours before harvesting.

FGF Bead Implantation and Harvesting

Embryos were incubated for 3 1/2 to 4 days before FGF-soaked beads were implanted as outlined in the previous chapter. Briefly, heparin acrylic beads (H5263 Sigma) were soaked in 4 µl of 0.5 mg/ml recombinant human FGF2 (233-FB) (R&D Systems, Minneapolis, MN) overnight at 4ºC or at least 1h at room temperature. For the control samples, the heparin acrylic beads were soaked in 4 µl 1XPBS before implantation. Using tungsten needle and forceps, FGF2 or PBS-soaked beads were

implanted (*in ovo*) into the posterior forelimb bud mesoderm of Hamburger-Hamilton stage (HH) 23 chicken embryos about 500μm from the distal tip.

Cycloheximide (CHX) Treatment

After bead implantation, 100μL of CHX (1mg/mL in 1%DMSO) was added directly on top of the implanted limb. After an additional 3 hours of incubation, embryos were harvested and assessed via whole mount *in situ* hybridization for the expression *LHX2* as well as FGF downstream targets *DUSP6* and *DKK1*.

FGF Signaling Inhibitor Studies

PD173074 (FGFR1 inhibitor), PD325901 (MEK inhibitor), Salirasib (RAS inhibitor), MK2206 (AKT inhibitor) and U73122 (PLCγ inhibitor) were purchased from Selleck Chem and injected into the right wing of developing chicken embryos to inhibit signaling in the whole limb as previously described (Horakova et al., 2014). Additionally, inhibitor-soaked agarose beads were co-implanted with FGF beads in the chicken limb bud to assess gene expression following local inhibition of FGF signaling. Inhibitors were dissolved in DMSO which was also used as the negative control.

Identification of LHX2-associated PCRMs via Comparative Analyses

Chip-seq data on chromatin association marks H3K27ac, H3K27me3, H3Kme2, RNAP2 and Med12 were obtained as described by Haro et. al. (Haro et al., 2017). Comparative analysis of the LHX2 locus was performed by pairwise alignment (Vista browser; http://genome.lbl.gov/vista/) and on the UCSC browser. The UCSC browser

compares varied species and highlights potential functional regions of DNA based on alignment with conservation and active chromatin marks. A PCRM was considered conserved when it exhibited at least 70% homology with the human LHX2 locus. The conserved regions were given a score based on the number of chromatin activity marks that aligned within each region. PCRMs with the highest scores were deemed more likely to be active and were further assessed for activity.

Screening of PCRMs via Electroporation of Chicken Embryos

Each PCRM of interest was isolated from chicken genomic DNA and inserted into a minimal promoter-driven GFP reporter construct which was then electroporated into the chicken embryo at HH4, the anterior portion of the embryo at HH10, the presumptive limb region at HH14, or the distal limb mesoderm at HH23 as previously described by Pira and colleagues (Pira et.al. 2008). Co-electroporation of a β-actin promoter-driven RFP construct was used to determine transfection efficiency. Electroporation was performed using the CUY21 electroporation station (Protech International). Activity was determined by visualization of GFP expression.

Results

FGF Induces LHX2 Expression in the Limb

Previous work has demonstrated the ability of FGFs to upregulate LHX2 after brief and prolonged FGF treatment in the developing limb (Watson et al., 2018). At 24hrs, *LHX2* expression is more restricted around the FGF bead than at 3hrs. We performed a 24hr time course of *LHX2* expression and determined that the decrease in the LHX2 diffusion ring started at 18hrs after FGF bead implantation (Fig. 10). This could

indicate a time-dependent upregulation of targets that inhibit *LHX2* expression or could be explained by the fact that the concentration of FGF is halved after 24hrs. This would reflect a concentration-dependent threshold for *LHX2* induction.

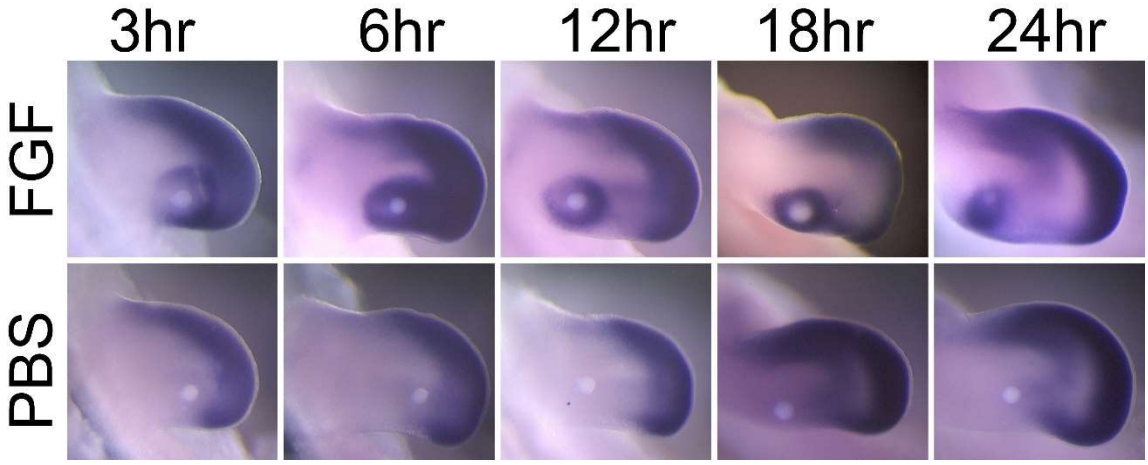

Figure 10. *LHX2* is Rapidly Upregulated in Response to FGF Treatment. After 3hrs of exposure to FGF, there is a robust upregulation of *LHX2* around the FGF bead which persisted for 24hrs. Beginning at 18hrs post-FGF bead implantation, *LHX2* is restricted around the bead when compared to earlier time points suggesting a time- or concentration-dependent regulation. PBS beads were used as control.

The AER is Required for LHX2 Expression

In the developing limb, *LHX2* is expressed in the distal mesoderm subjacent to the AER. This prompted us to test whether the presence of the AER is necessary for *LHX2* expression. As early as 6hrs after complete or partial removal of the AER from HH23 chicken limb bud, there is a decrease in *LHX2* expression in the underlying mesoderm (Fig. 11). Expression of *DUSP6*, an FGF downstream target, was also lost. We removed the AER at HH23 to eliminate the possibility that the observed loss of gene expression was due to apoptosis of cells subjacent to the AER. This is based on a study which demonstrated cell death in the distal mesoderm following AER removal at HH18-20 but no cell death after ridge removal at HH23 (Rowe, Cairns, & Fallon, 1982).

LHX2 is a Primary Transcription Target of FGF Signaling

Following FGF bead implantation, cycloheximide (CHX) was used to inhibit *de novo* protein synthesis. mRNA levels of LHX2 were not affected by CHX treatment indicating that FGF mediates LHX2 in in the absence of protein synthesis. Other targets of FGF signaling, DUSP6 and DKK1, were used as in-house controls. *DUSP6* expression was unaffected by CHX treatment while *DKK1* induction was inhibited (Fig. 12).

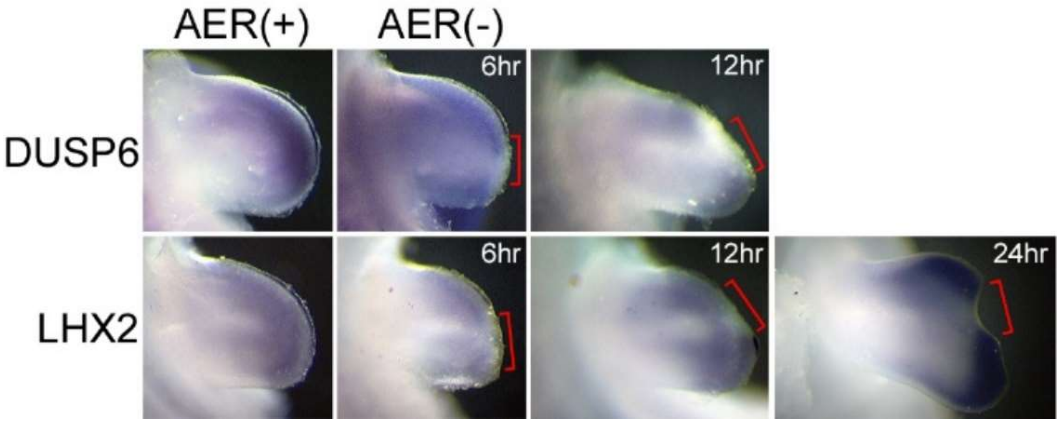

Figure 11. The AER is Necessary for LHX2 Expression. Intact AER is designated AER(+) and is stained using a probe for FGF8. Note the similar distally restricted endogenous expression pattern of *LHX2* and known FGF downstream transcriptional target *DUSP6*. After partial removal of the AER (indicated by region outlined by the red brackets) both *LHX2* and *DUSP6* expression decrease in the adjacent mesoderm. At 24hrs, *LHX2* expression is completely absent from the mesoderm adjacent to the missing AER.

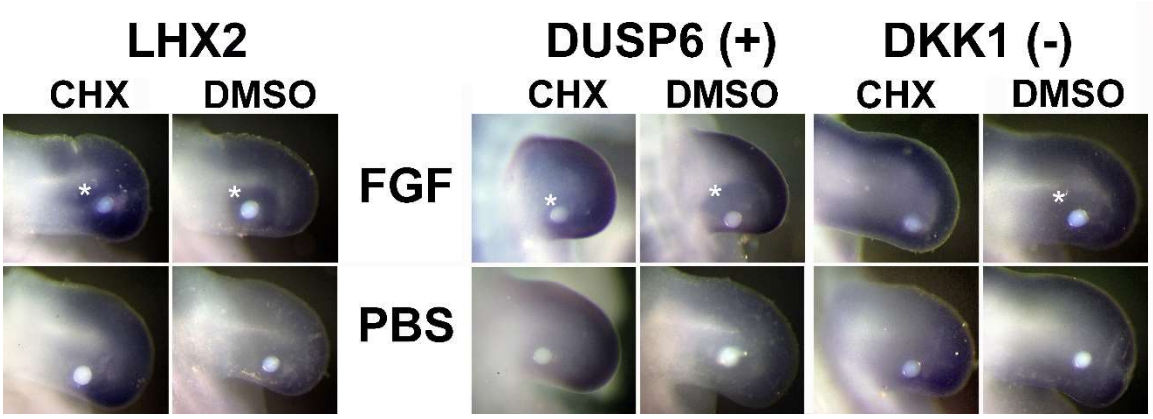

Figure 12. FGF Upregulates LHX2 in the Absence of *de novo* Protein Synthesis. Upregulation of *LHX2*, *DUSP6* and *DKK1* in response to FGF is indicated by white asterisks. Note that *DKK1* expression (the in-house negative control) is not induced in the presence of cycloheximide (CHX), an inhibitor of protein synthesis, indicating that one or more proteins need to be synthesized for FGF to regulate *DKK1* expression. *LHX2* and *DUSP6*, on the other hand are primary transcriptional targets of FGF signaling. PBS beads are used as vehicle controls for FGF.

The RAS-MEK/ERK Pathway is Necessary for LHX2 Expression in the Developing Limb

Inhibition of FGF function by injecting the FGFR1 inhibitor PD173074 as previously described (Horakova et al., 2014) abates limb outgrowth and leads to loss of *LHX2* and *DUSP6* expression. Similarly, inhibition of RAS from one of the main signaling pathways resulted in abated limb outgrowth and loss of *LHX2* and *DUSP6* expression while inhibition of PLCγ did neither (Figure 13A). Given these signal transduction pathways are downstream of other growth factors and ligands present in the limb, we retested this concept using FGF-soaked heparan sulfate beads and inhibitor-soaked agarose beads to associate these results to FGF signaling. This data was consistent with the inhibitor injection experiments with regards to the inhibition of *LHX2* and *DUSP6* expression (Figure 13B).

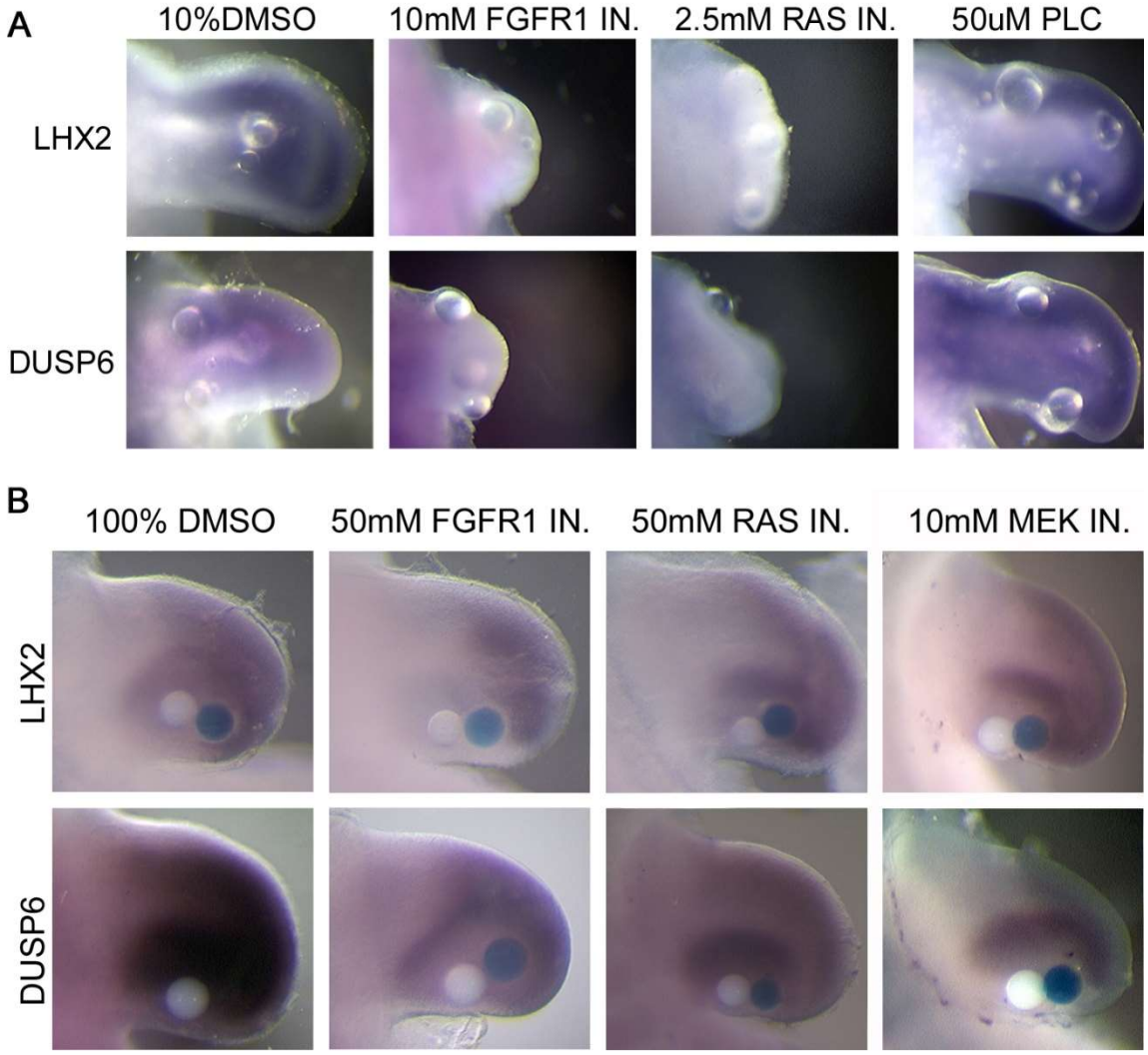

Figure 13. FGF Regulates *LHX2* Expression Through the RAS-MEK/ERK Pathway. A. Injection of the FGFR inhibitor as well as specific pathway inhibitors for RAS and PLCγ in the distal, anterior and posterior mesoderm of HH20-22 chicken limbs revealed that FGFR1 and RAS inhibition abate limb development and lead to the loss of *LHX2* and *DUSP6* expression. Oil bubbles roughly indicate the sites of injection. B. Co-implantation of an FGF-soaked bead with an FGF inhibitor-soaked bead showed local inhibition of *LHX2* and *DUSP6* confirming that both genes are being regulated by FGF through the RAS-MEK/ERK signaling pathway. Note the failure of the FGF bead to induce fulminant ectopic expression of *LHX2* and *DUSP6* in panels 2, 3 and 4 compared to the vehicle control bead (100% DMSO – panel 1). FGF beads: white; Inhibitor beads: blue.

Identification of Potential cis-Regulatory Modules (PCRMs) Associated with LHX2

Given that c*is*-regulatory modules that house binding sites for regulation of gene expression may be found near or at distant sites from their target gene, we examined a region roughly 300,000 bases in length upstream and downstream of the *LHX2* start site. To identify conserved PCRMs that were more likely to be functional we performed comparative analyses using the VISTA browser to detect homology across species. In our initial screen we identified 5 potential cis-regulatory modules (PCRMs 1-5) based on proximity to the *LHX2* start site and >70% homology across species. We then identified and characterized additional PCRMs based on conservation across species and overlap with chromatin regulatory marks from E11.5 mouse limbs (Figure 14). Next, we scored the additional PCRMs and selected 5 with the highest score which we associated with likelihood for activity in the limb (Table 1). PCRMs 8, -7, -12, -19, and 25 had the highest score and were selected for screening.

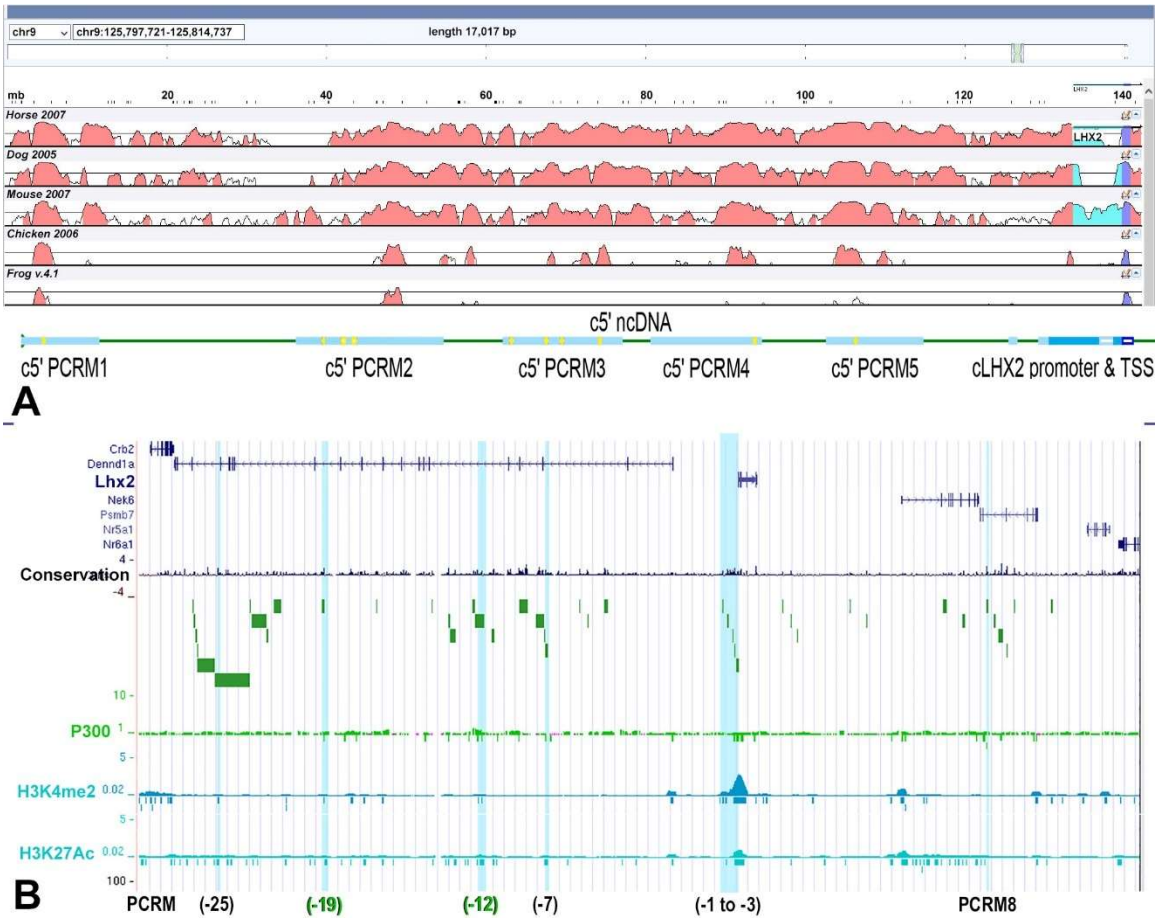

Figure 14. PCRM Selection Using Conservation and Chromatin Marks A) The initial PCRMS were identified less than 18,000 bases upstream of the LHX2 transcription start site (TSS) and were selected based on conservation between human and divergent species using VISTA pairwise analysis. The 5 PCRMs are shown as a light blue horizontal bar below each region. B) Subsequent analysis of the LHX2 locus (500,000 bases on either side of the LHX2 gene) using the UCSC genome browser, conservation, and a variety of chromatin markers including p300, H3K4me2 and H3K27ac revealed a larger subset of PCRMs. Conserved, non-coding DNA regions that possessed two or more overlapping chromatin marks are highlighted by light blue vertical bars.

Table 1. Summary of Chromatin Marks and Transcription Factor (TF) Binding Sites for the PCRMs with the Highest Scores. Scores were based on the number of chromatin marks that overlapped with the conserved regions/peaks.

Peak	Hum 2006	p300	H3KMe2	H3K27Ac	Pol II	Med12	H3K27Me3	ChrMrks	TF
-25	Chr9:125,239933-125,241,312	0.5	1	1				2.5	32
-19	Chr9:125,370,599-125,371,797	1	1	1	1			4	90
-12	Chr9:125,527,150-125,528,130	1	1	1	1			4	69
-7	Chr9:125,597,240-125,598,303	1		1	1	1		4	101
8	Chr9:126,162,777-126,164,318	1	0.5	1				2.5	145

Activity of PCRMs Overlap LHX2 Expression

PCRMs 1-5 were electroporated into the presumptive limb field of HH14 chick embryos to assess activity but none was detected in the limb 24hrs after electroporation. Since *LHX2* expression is not limited to the limb during development and enhancer activity may be tissue-specific, we electroporated HH4 chicken embryos to assess PCRM activity in the entire embryo. At HH10 (approximately 24hrs after HH4 electroporation), we detected activity of PCRM4 in the head of the developing embryo. Assessment of the head of a HH14 embryo following electroporation of PCRM4 at HH10, revealed that PCRM3, 4, and 5 were active in the forebrain. A representative figure shows that PCRM4 activity was consistent with *LHX2* expression in the forebrain (Figure 15). Activity of PCRMs 8, -7, -12, -19, and -25 were examined at the distal tip of HH23 limb buds. PCRM-12 and PCRM-19 exhibited activity coincident with *LHX2* expression in the limb (Figure 16).

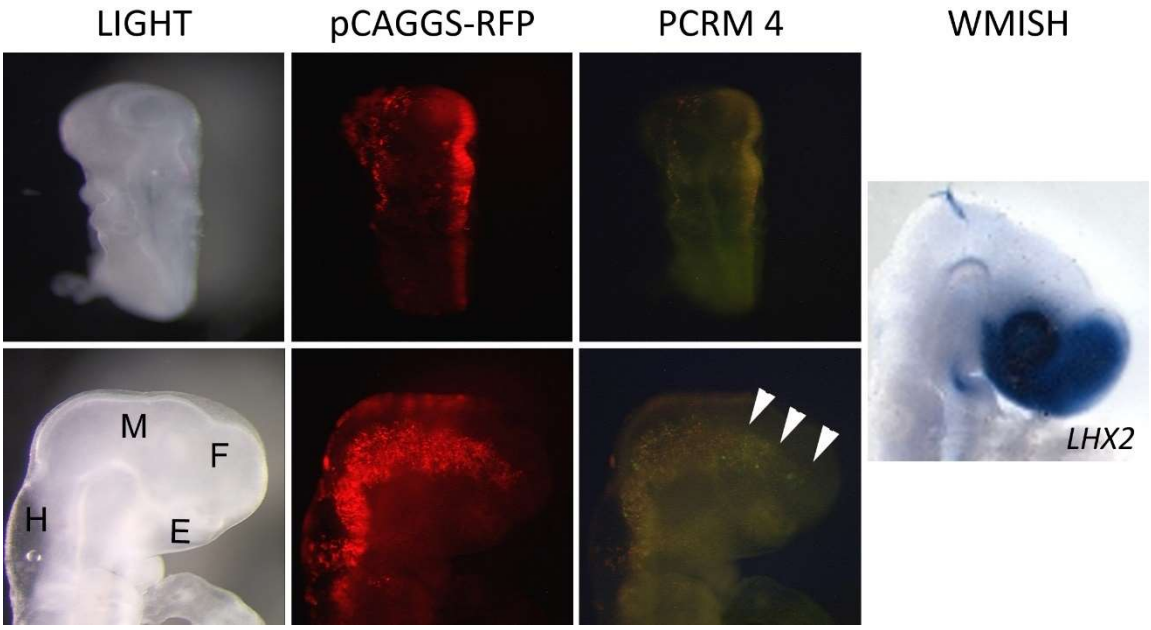

Figure 15. PCRM4 Exhibits Activity in the Brain Consistent with *LHX2* Expression. Top panel trio: Dorsal view of the head of a HH14 chicken embryo. The PCRM4 construct was co-electroporated with an RFP expression vector to assess transfection efficiency. Note stronger RFP expression laterally to the right. DNA constructs tend to migrate toward the positive electrode which was placed to the right of the embryo. Faint GFP expression also noted on the right. Bottom panel trio: Lateral section of the head shows PCRM4 activity in the forebrain indicated by white arrow heads. Right frame: *LHX2* expression in the developing head. H: Hindbrain; M: Midbrain; F: Forebrain; E: Eye

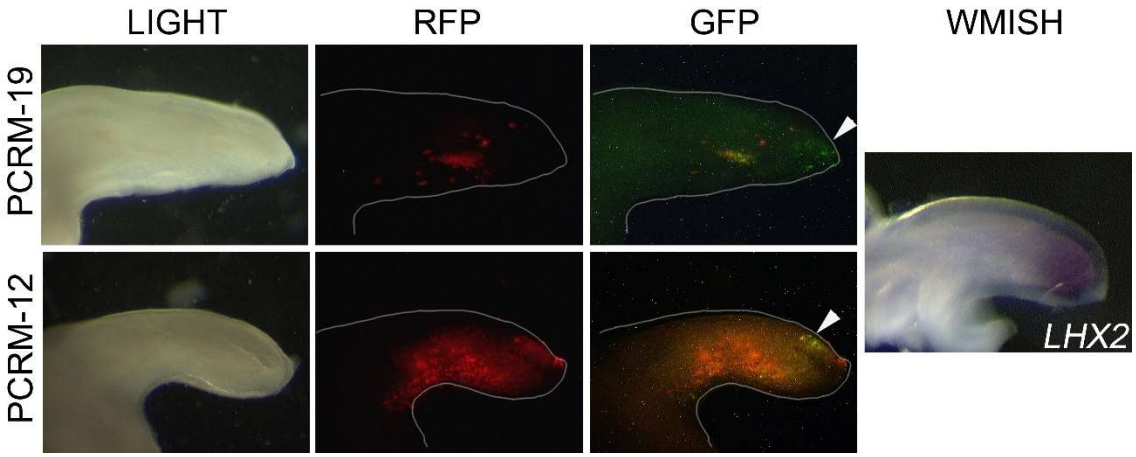

Figure 16. Activity of PCRMs is Consistent with LHX2 Expression in the Limb. Cross-section of limb transfected with PRM-12 and PCRM-19 reveal activity (GFP expression) at the distal tip of the limb bud (white arrowheads) consistent with *LHX2* expression (Right frame). RFP was used to assess electroporation efficiency.

Discussion

The role of the apical ectodermal ridge (AER) in embryonic limb outgrowth has long been established (J. W. Saunders, Jr., 1948; Summerbell, 1977). Since then, FGFs have emerged as the major functional proteins secreted from the AER that govern proper limb outgrowth and development via promoting cell growth, proliferation, and gene expression (Mariani, Ahn, & Martin, 2008; Martin, 1998). In the chicken limb, FGFs 2, 4, 8, 9, and 17 are expressed in the AER while FGFs 2 and 10 are expressed in the mesoderm.

AER-FGFs drive the regulation of many important developmental genes and transcription factors in the underlying limb mesoderm. One such gene is sonic hedgehog (SHH), a morphogen that directs anterior-posterior expansion and patterning and feeds back into regulating AER-FGFs. Lim homeobox 2, a distally expressed transcription factor, has recently been implicated in mediating FGF-related *SHH* expression although the mechanism has yet to be fully elucidated. Given that AER removal affects *SHH* expression (Niswander et al., 1994), we set out to determine whether the AER contributes to *LHX2* expression as well and found that ridge removal, in fact, resulted in loss of *LHX2* expression. We also determined from FGF bead implants that, much like SHH, FGF treatment in the posterior mesoderm resulted in ectopic *LHX2* expression around the bead. We further determined that LHX2 is a primary transcriptional target of FGF signaling since it was upregulated in the absence of *de novo* protein synthesis while FGF8 induction of *SHH* requires protein synthesis (Bastida et al., 2009). This lends support to our data in Chapter 2 which places LHX2 upstream of SHH during FGF signaling.

SHH was found to be downstream of the RAS signaling pathway (Bastida et al., 2009). To ascertain if this pathway was likely necessary for FGF-mediated LHX2 expression, we used PD173074 (FGFR1 inhibitor), Salirasib (RAS inhibitor) and PD325901 (MEK inhibitor) in the anterior, posterior and distal tip of HH20-22 embryonic limbs. All 3 abated limb outgrowth and led to the loss of expression of FGF downstream targets – *DUSP6* and *LHX2*. Results from the FGF inhibitor bead implantation also demonstrated local inhibition of *LHX2* with the all three inhibitors. This indicates that the RAS pathway is involved in FGF-mediated *LHX2* expression. This supports our recent finding that LHX2 acts upstream of SHH during FGF signaling in the limb by identifying a shared FGF signal transduction pathway for *LHX2* and *SHH* expression.

Although LHX2 is a primary transcription target of FGF signaling and does not require protein synthesis for its transcription to take place, existing transactivating factors are necessary for proper chromatin folding and gene expression. We therefore set out to identify c*is*-regulatory elements that could act as enhancers (long range) or promoters (nearby) to regulate gene expression by providing binding sites for one or more *trans*-acting factors. Usually *cis*-regulatory elements are inter- or intra-genomic regions of non-coding DNA (Wittkopp & Kalay, 2011) and may be found as far away as 1Mb away from the start site of the target gene as in the case of human *SHH* (L. A. Lettice et al., 2002). Additionally, conservation of these non-coding regions across species is taken to signify functional relevance (Ahituv, Prabhakar, Poulin, Rubin, & Couronne, 2005; Haro et al., 2017; Nora, Dekker, & Heard, 2013).

Our initial screen for potential *cis*-regulatory modules using proximity to the LHX2 start site and conservation across species yielded 5 PCRMs none of which exhibited activity in the limb. During development, *LHX2* expression is not solely restricted to the limb bud but is expressed in the forebrain and optic cup (Lee, Brenner, & Venkatesh, 2011). To ascertain whether these PCRMs exhibit activity that mimics *LHX2* expression in other tissues, we electroporated the entire embryo at earlier stages and hereby report that PCRM4 exhibits activity in the forebrain which overlaps *LHX2* expression. As previously reported however, Crumbs homolog 2 (CRB2) shares a locus with LHX2 and its expression overlaps that of LHX2 in the developing brain and eye (Lee et al., 2011). It is possible that PCRM4 could regulate both genes or one and not the other.

Our second round of screening was stricter. Although we widened the locus to 300kb, we incorporated chromatin associated marks which are used to predict activity of a genetic region (Hardison & Taylor, 2012). Of the 5 PCRMs thus selected, two (PCRM-12 and PCRM-19) exhibited activity at the distal tip of the limb coincident with *LHX2* expression. The fact that these enhancers overlap in their activity pattern could indicate that they are either redundant or both are necessary to ensure robust expression of their target gene, LHX2. A third alternative is that they regulate different genes within the LHX2 locus that are expressed in the distal tip of the limb. To determine which gene is being regulated by a specific PCRM, a process called chromosome conformation capture (3C) should be performed to predict their association (Barutcu et al., 2016). The other PCRMs that failed to exhibit activity could act as transcriptional repressors and not enhancers and would therefore not be detected using our minimal reporter construct.

Also, these “inactive” PCRMs could be active at different developmental stages than those tested.

Taken together, we have demonstrated that Fgfs are necessary for *LHX2* expression and that AER-related Fgfs are the most compelling source driving this expression. We also show that LHX2 is a primary transcription target of FGF signaling that acts through the RAS pathway. Further, we identified *LHX2*-associated PCRMs and tested 10 of them at different developmental stages. This revealed enhancer activity of PCRM4 in the developing brain at HH10 and HH14, and activity of PCRMs -12 and -19 at the distal tip of the limb at HH23 all coincident with *LHX2* expression. Additional work can be done to further elucidate the mechanism of FGF-mediated *LHX2* expression by mutating the transcription factor binding sites within the active PCRMs related to transcription factors downstream of FGF signaling.

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CHAPTER FOUR

DISCUSSION

Fibroblast Growth Factors (FGFs) Upregulate Sonic Hedgehog (SHH) During Limb Development

Limb development is often described along three interconnected axes – proximal-distal (PD), anterior-posterior (AP), and dorsal-ventral (DV). Crosstalk between the signaling centers of these axes is essential for normal outgrowth and patterning. Fibroblast growth factor (FGF) and sonic hedgehog (SHH), from the apical ectodermal ridge (AER) and Zone of polarizing activity (ZPA), respectively, regulate each other in a positive feedback loop which drives development along the PD and AP axes. From chicken studies using FGF bead implants, we learn that AER-FGFs can upregulate SHH in the posterior half of the limb bud (Kostakopoulou, Vogel, Brickell, & Tickle, 1996) and data from Bastida et. al. suggests this regulation is indirect (Bastida et al., 2009). We therefore set out to elucidate the molecular mechanisms surrounding FGF-mediated *SHH* expression in the limb.

FGF2-soaked beads implanted in the mid-proximal posterior mesoderm of chicken limbs resulted in ectopic *SHH* expression along the posterior margin of the limb at 3 hours and around the bead at 24 hours (Figure 3). We determined that transcriptome analysis of tissue from both time points would reveal FGF targets co-regulated with *SHH* and therefore likely intermediates in FGF-mediated *SHH* expression. By overlapping both data sets, we excluded extraneous FGF targets and highlighted those likely contributing to *SHH* expression (n=25) (Figure 6). Via literature review, we further focused on 8 genes which had an association with SHH at the transcript and/or protein

level. This finding supports our approach to uncovering molecules involved in FGF-mediated *SHH* expression and encourages assessment of the other 17 overlapping genes with which an association to SHH has not yet been made.

FGF Targets Associated with SHH Expression and Signaling/Function

Early growth response 1 (EGR1) is a transcription factor with notable involvement in tendon development (Lejard et al., 2011). In the chicken limb (HH23), EGR1 exhibits a low-level ubiquitous expression pattern; however, it is strongly induced in response to 3h FGF treatment (Fig. 3C). It has been described as an immediate-early responder that acts downstream of FGF signaling to upregulate FGF-downstream factors such as SPRY2 (Han et al., 2017). EGR1 is a direct transcriptional regulator of SHH in human glioma cells where it reportedly binds to intron 1 and increases *SHH* expression levels (Sakakini et al., 2016). As a transcription factor, the fact that EGR1 acts downstream of FGF and can upregulate SHH makes it a viable candidate for involvement in FGF-mediated *SHH* expression in the developing limb.

The upregulation of GJA1 highlights the importance of intercellular communication in FGF-mediated *SHH* expression. Gap junctions are membrane structures that permit direct cell–cell communication (Makarenkova & Patel, 1999). In the limb buds of both mouse and chick, gap junctions are present predominantly in the AER (Fallon & Kelley, 1977; Green, Bowles, Crawley, & Tickle, 1994; Makarenkova, Becker, Tickle, & Warner, 1997), and to a lesser extent in the limb mesoderm, colocalizing with the ZPA (Makarenkova et al., 1997; Wiens, Jensen, Jasper, & Becker, 1995). Conditional knockout of GJA1 in mice, which encodes Connexin43, leads to

syndactyly and a decrease in *Shh* expression. Additionally, Connexin43 has been shown to enhance *SHH* expression and has been specifically linked to the FGF-SHH loop (Dobrowolski et al., 2009; Law et al., 2002).

Tetratricopeptide Repeat Domain 8 (TTC8), also known as Bardet Biedl syndrome 8 (BBS8), was downregulated in our analysis. Disruption of BBS proteins impairs movement of the Smoothened (SMO) transmembrane protein involved in Hedgehog signaling from the ciliary membrane (Haycraft et al., 2005; Huangfu et al., 2003). This interrupts activation and processing of the GLI transcription factors which mediate SHH signaling (Q. Zhang et al., 2012). GLI1 and 2 function as transcriptional activators while processed GLI3 acts as a repressor of SHH signaling (Sheeba et al., 2016). Thus, downregulating TTC8/BBS8 could enhance FGF-mediated SHH signaling by preventing GLI3 from being processed and truncated into its repressor form.

R-spondins are WNT/β-catenin signaling agonists that are essential for embryonic development. Our data also indicate that they are FGF-responsive genes. Of the 4 R-spondin genes, the most noticeable expression patterns with regards to the FGF to SHH crosstalk belong to RSPO2 (expressed in the AER) and RSPO3 (expressed in the distal posterior mesoderm overlapping the ZPA) (Neufeld et al., 2012). A *Rspo2/Rspo3* double mutant showed more severe limb defects than either single mutant with the most anterior and posterior digits missing, leaving 3 shortened middle digits (Neufeld et al., 2012). The fact that posterior elements in the forelimb are lost in this double mutant, indicates that the effect of R-spondin genes on *SHH* expression/function needs to be assayed. - Additionally, the difference in regulation between *RSPO2* (upregulated in our 24hr

dataset) and *RSPO3* (downregulated in both datasets) suggests a role for balancing the overall contribution of R-spondins during normal limb development.

There were 2 genes downregulated after both 3 and 24h FGF treatment: *AMD1* and *MGAT4B*. Adenosylmethionine decarboxylase 1 (AMD1) is an enzyme that participates in polyamine synthesis. Polyamines are polycations that interact with negatively charged molecules such as RNA and DNA and are crucial to the growth and proliferation of mammalian cells (Minois, Carmona-Gutierrez, & Madeo, 2011). Mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase, isozyme B (MGAT4B) is linked to glycan synthesis (Hinske, Galante, Kuo, & Ohno-Machado, 2010). *Amd1* and the *Mgat4b* paralog *Mgat4a* are decreased in Shh deficient mice (Probst et al., 2011) and are therefore thought to be downstream targets of Shh. However, the fact that these genes could participate in a feedback loop and therefore operate both up-and downstream of SHH cannot not be discounted. Since these genes have not been well-characterized in the limb, further investigation is required. The other two genes, LHX2 and TFAP2C, will be discussed in the upcoming subsections.

The Role of the ZRS in SHH Expression

The ZPA regulatory sequence (ZRS) is a conserved *cis*-acting regulatory element found in intron 5 of the *LMBR1* gene located 1Mb upstream of the *SHH* gene in humans.(L. A. Lettice et al., 2002) It is responsible for limb-specific *SHH* expression and mutations in this region may result in the following phenotypes that are characteristic of *SHH* functional absence and misexpression: oligozeugodactyly (Ros et al., 2003; Smyth et al., 2000) and pre-axial polydactyly (PPD) (Sharpe et al., 1999), respectively.

Oligozeugodactyly (*ozd*) is a spontaneous limb malformation reported in chickens that results from a deletion in noncoding DNA that contains the ZRS. As a result of this mutation, both fore- and hindlimbs in these chicks lack the posterior element in the zeugopod (ulna/fibula) and all digits except digit 1 in the leg (Ros et al., 2003). This phenotype mimics that of *Shh*$^{-/-}$ mice (Chiang et al., 2001; Kraus et al., 2001; Ros et al., 2003). In *ozd* chicks, *SHH* expression and activity are absent in the limb while present in other normally-developed organs (Ros et al., 2003). Consistent with this observation is the fact that transcripts of downstream effectors of SHH signaling (*Ptc1* and *Gli1*) are undetected in the limb (Ros et al., 2003). The *ozd* phenotype can be rescued by application of recombinant SHH or ZPA grafts; however, in the presence of *SHH* inducers such as endogenous FGFs, *SHH* remains undetected (Ros et al., 2003). The insufficiency of FGFs to induce *SHH* in chicks with deficient ZRS highlights the requirement for this sequence in FGF-related *SHH* expression in the limb. In mouse mutants exhibiting PPD, a translocation breakpoint and a transgenic insertion site were identified in the ZRS (L. A. Lettice et al., 2002) which explained the misexpression or ectopic anterior expression of Shh resulting in PPD (Sharpe et al., 1999).

Mutations (translocations, insertion or single nucleotide polymorphisms) in the ZRS can lead to the loss or gain of a transcription factor binding site. Transcription factor AP2 gamma (TFAP2C) binding motifs are found within the ZRS. AP-2 factors bind to the consensus sequence 5'-GCCNNNGGC-3' and activate genes involved in a large spectrum of important biological functions including proper eye, face, body wall, limb and neural tube development. The human ZRS mutation termed “Dutch” creates a potential AP2 binding site which leads to preaxial polydactyly (PPD). A luciferase assay

confirmed that TFAP2B can bind to this mutated site (Fuxman Bass et al., 2015). Although not confirmatory, this suggests a role for members of the AP2 family of transcription factors (such as TFAP2C from our transcriptome analyses) to regulate *SHH* expression.

The ZRS also houses multiple binding sites for the ETS family of transcription factors. Although no single member appeared in both transcriptome data, members of this family were upregulated at each time point. At 3hrs, ELF2 and ETV6 were slightly upregulated while ETS2 and ETV5 were upregulated at 24hrs. Lettice et al reported that a balanced between occupancy of the ETS binding sites between ETS1 and ETV4/5 within the ZRS might control the expression level of *Shh* in the limb bud. A human PPD mutation in an Australian family (AUS) converts one of the ETV4/5 binding sites into an additional ETS1 binding site. This mutation not only results in an anterior ectopic domain of *Shh*, but also a posterior expansion of the ZPA (L. A. Lettice et al., 2012). This suggests that FGF-dependent regulation of ETS transcription factors not only participate in the posterior restriction of *SHH* expression (Mao et al., 2009; Z. Zhang et al., 2009) but could also enhance endogenous expression.

LHX2 belongs to a family of transcription factors that have been identified as proteins that integrate signaling events along all three axes of limb development and whose absence leads to growth and patterning defects (Tzchori et al., 2009). Simultaneous knockout of Lhx2 and its homolog, Lhx9, leads to defects along the proximal distal and anterior posterior axes as well as a reduction in *Shh* expression (Tzchori et al., 2009). The TAATTA binding motif for LHX2 (Roberson et al., 1994) is found in the *ZRS* and is conserved across 16 vertebrate species including human, mouse

and chicken (Kvon et al., 2016). Further work is needed to evaluate whether LHX2 binds to this region or whether mutations at this site affect *SHH* expression.

LHX2 as an Intermediate in FGF-mediated SHH Expression

Lim homeobox 2 (Lhx2) is expressed in an FGF-responsive region - the distal mesoderm directly adjacent to the AER and it overlaps the ZPA where *SHH* is expressed (Figure 8). It has been suggested that LHX2 and LHX9 may be functionally redundant due to their overlapping expression patterns in some developmental domains (Bertuzzi et al., 1999). During limb development in chicks however, *LHX2* is expressed in the distal mesoderm while *LHX9* is limited to the anterior mesoderm (Nohno et al., 1997). Given the divergent expression domains of *LHX2* and *LHX9*, it is unlikely that during chick limb development, *LHX9* would co-operatively or instead of *LHX2* mediate FGF signaling to *SHH* in the posterior limb mesoderm. Additionally, at 3 and 24hrs post FGF bead implantation, no ectopic *LHX9* expression was detected. Tzchori et al reported that neither Lim homeobox genes were regulated by FGF signaling; however, we have shown that *LHX2* is present in both 3 and 24hr FGF transcriptomes, and an increase in its expression can be detected via WMISH and RT-qPCR in response to FGF treatment.

Using a morpholino (MO) that blocks translation of LHX2, we were able to show a delay in limb outgrowth and a decrease in *SHH* expression (Figure 10). This is consistent with data from Rodriguez-Esteban that showed also showed an arrest in limb outgrowth and a decrease in expression of distally restricted genes, for example, *SHH, MSX1, WNT3a,* and *FGF8* (Rodriguez-Esteban et al., 1998). To assess the effect of LHX2 on SHH in the context of FGF signaling, we electroporated the anti-LHX2 MO

around an FGF bead and noted a reduction in *SHH* expression which was quantified by RT-qPCR as a 40% decrease in transcript level. Accordingly, an increase in *LHX2* around the FGF bead using an expression vector resulted in a local increase in ectopic *SHH* expression which was quantified as a 3.5-fold increase. Further, the SHH downstream target *PTCH2* was decreased when LHX2 translation was blocked and increased with an overexpression of LHX2 (Figure 9).

LHX2 as a Competency Marker for SHH Expression in the Limb

Prior to SHH expression in the developing limb antero-posterior (AP) polarity is set up by a mutually antagonistic gradient of Hand2 (in the posterior mesoderm) and Gli3 (in the anterior mesoderm) (te Welscher et al., 2002) which restricts SHH to the ZPA. Ectopic SHH expression can be detected in response to FGF treatment within the posterior half of the limb; that is, within the expression domain of HAND2. Although LHX2 can alter SHH expression in the context of FGF signaling, unlike FGF, overexpression of LHX2 on its own is not sufficient to upregulate *SHH* in the HAND2-rich posterior limb domain within 24 hours. Therefore, rather than being an inductive factor we propose LHX2 is a competency marker for SHH expression in the limb. In addition to LHX2 overlapping the ZPA, we noted reports of ectopic distal SHH expression underlying the AER in a LHX2-like pattern (Y. Chen et al., 2004; Zhulyn et al., 2014). Each case involved dysregulation of the Gli family of transcription factors which are downstream regulators of SHH signaling. Full length Gli3 is constitutively being processed into its shorter repressive form, Gli3R. SHH blocks this processing leading to an increasing gradient of Gli3R from the posterior to anterior mesoderm.

Generally, Gli1 and Gli2 function as transcriptional activators while Gli3 functions as a repressor (Sheeba et al., 2016).

Chen and colleagues reported an extensive ectopic Shh expression pattern that spans the entire distal mesenchyme subjacent to the AER in a compound hemizygous *Tg-Hoxd12;Gli3*$^{+/-}$ mouse model (Y. Chen et al., 2004). Since neither of the single mutants affected SHH expression, the pattern in the compound model, suggests that a concentration-dependent interaction between a homeobox-containing gene (Hoxd12) and Gli3 leads to SHH expression along the distal margin of the developing limb. Another report discusses the precocious activation of SHH signaling using a *Prx1-Cre;Ptch1*$^{f/-}$ mouse model which resulted in failure to properly establish the AER or ZPA (Zhulyn et al., 2014). Widespread expression of Gli1 and Hand2 were detected along with reduced Gli3 and an ectopic SHH expression restricted to the distal mesoderm underlying the AER. Zhulyn and colleagues also created *Kif7*$^{-/-}$;*Gli3*$^{-/-}$ double mutants that similarly resulted in an apical shift of the SHH expression domain. Both *Kif7* and *Gli3* are required for Gli3R formation and their absence results in elevated *Gli1* mRNA and Gli2 protein levels. To explain the complex interactions leading to distal ectopic *Shh* expression, we reviewed the reduction of Gli3 repressor which was common in all three settings. Reduction of the repressive activity of Gli3 in the limb usually results in an ectopic *Shh* expression restricted to the anterior mesodermal boundary of the AER. An additional increase in widespread Hoxd12 and Hand2 (Y. Chen et al., 2004; Zhulyn et al., 2014), markers for SHH competency, appears to extend this ectopic SHH expression, albeit in a distally restricted pattern dissimilar to either marker. Therefore, while contributory, this explanation seems incomplete. We propose that LHX2 is the distal marker conferring a

basal level of competency for SHH expression and allowing for *SHH* to remain juxtaposed to the AER with progressive distal outgrowth. Additionally, when there is a reduction in repressive factors from the anterior mesoderm, ectopic *SHH* expression along the entire distal rim of the limb bud within the *LHX2* expression domain is possible. Further work will need to be done to ascertain whether LHX2 forms a transcriptional complex with Hand2, Hoxd12, or any other transcription factor in conferring *SHH* competence in developing limbs. Mechanistically, we also wanted to investigate how FGF regulates *LHX2* expression.

FGF Signaling in the Limb

In the developing limb, FGFs promote the expression of the polarizing morphogen, SHH, which is secreted from a discrete population of cells in the posterior-distal mesenchyme and controls antero-posterior expansion and patterning. We recently showed that LHX2 is an intermediate in FGF-mediated SHH expression and here set out to identify the molecular mechanisms surrounding LHX2 regulation in the limb.

Fibroblast growth factor (FGF) signaling is integral to a wide range of normal physiological processes including cell growth, proliferation, differentiation, migration, and survival. FGFs are also capable of acting on a wide range of cell types and eliciting a cascade of subsequent signaling events through 4 main signaling pathways.

Given the association we have made between LHX2 and SHH downstream of FGF signaling we first determined that the AER was necessary for LHX2 expression as it is for SHH. Next, we demonstrated that FGFs were the likely AER protein inducing LHX2 by performing a time course of LHX2 expression following FGF bead implants.

The rapid response of *LHX2* to FGF led us to test whether LHX2 was a primary transcriptional target of FGFs or if the synthesis of other proteins were required. Additional protein synthesis was not required.

To identify which of the signal transduction pathways were involved in FGF-mediated *LHX2* expression, we first tested the RAS pathway since that pathway has been identified as necessary for *SHH* expression and found it also necessary for *LHX2*. We have identified inhibitors for other pathways and believe they should also be assessed for their involvement in *LHX2* expression, so a complete picture can be had.

Potential cis-Regulatory Modules (PCRMs) Associated to LHX2 Expression

Conservation of non-coding regions of DNA suggests function. Our approach of using comparative analyses of chromatin marks in the limb to identify conserved regions of DNA within the LHX2 locus that could serve as *cis*-regulatory modules was fruitful. The fact that PCRM4 from our initial screen did not exhibit activity in the limb but did in the forebrain, suggests that this PCRM may be tissue-specific and therefore only contribute to LHX2 expression in the brain. This is not an uncommon occurrence with a major example being the ZRS which is specific to *SHH* expression in the limb.

Coupling our initial approach of conservation and proximity with chromatin marks in the limb, yielded more favorable results with regards to limb expression. The overlapping activity patterns of PCRMs -12 and -19 suggest that they are redundant enhancers with each contributing to fulminant activity or it could indicate that they are associated with different genes in this locus such as CRB2 or DENND1A with expression in the forebrain (Lee et al., 2011). To determine which gene each of the PCRMs are

associated with Chromosome conformation capture (3C) should be performed. This technique measures the interaction frequency of two loci based on their spatial proximity in the three-dimensional nucleus (Barutcu et al., 2016). After validating their interaction with the LHX2 promoter region the next steep that can be taken is to mutate binding sites within the PCRMs to determine which transcription factors are likely involved in regulating *LHX2*.

In sum, FGF and SHH are two major morphogens that control limb outgrowth and patterning. Cross-talk between these two molecules is important since each has downstream targets that support the expression and function of the other. Consequently, loss of either results in a failure in limb development. Chapter 2 presents the most robust list to date of possible intermediaries in the FGF to SHH feedback loop which governs growth and patterning along the PD and AP axes and provides some functional support for the transcription factor, LHX2, as a necessary intermediate. Further we believe it's role is as a competency factor, coupling SHH to the AER during outgrowth, given its expression pattern and that of *SHH*. Chapter 3 reveals a signal transduction pathway for FGF-mediated LHX2 upregulation and uncovers novel enhancer elements for *LHX2* expression in the limb and brain. Thus, we have identified and characterized a major target of FGF-mediated *SHH* expression during limb development.

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SUPPLEMENTARY DATA

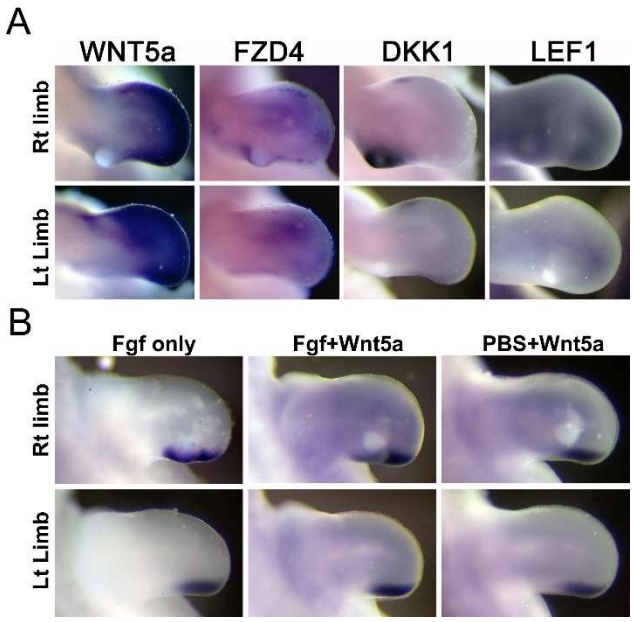

Figure S1. Wnt5a is not Sufficient to Drive *SHH* Expression in the Posterior Limb Mesoderm. (A) Up-regulation of members of the Wnt signaling pathway after 24h of FGF treatment. Compare ectopic expression to the contralateral or PBS-treated limb (bottom panel). (B) Wnt5a-expressing cells grafted (around a PBS bead) in the posterior limb mesoderm failed to induce *SHH* expression 6h post implantation. Contralateral limbs used as control.

Table S1. Primers Used for RT-qPCR Validation of Selected Genes from the 3hr and 24hr Transcriptome Analyses.

PRIMER SEQUENCES

Gene	Probe		qPCR	
	Fwd Primer 5'-3'	**Rev Primer 5'-3'**	**Fwd Primer 5'-3'**	**Rev Primer 5'-3'**
SHH	CATGGTCGAAATGCTGCTGTTG	ACGCTGCCACTGCGTTTTCTGC	GTGGCAGAGAAGACCCTAGG	GAGTCATCAGTCTGTCAGCTCC
LHX2	GGATTTCTGCCTCTGAAATGG	CATCAGAAAAGATTTGTGAGAGTTGTC	GGATTTCTGCCTCTGAAATGG	GATGAGAGTCTCGAAATGGAGG
EGR1	GATGAACTAACTCGTCACATTCG	CAGTACCTAAAGGAAAGCAAAACC	CAAACTGGAGGAGATGATGC	TAGCTGGTTTCTGGCATGG
TFAP2C	CGCTCGTGACTTCGGATACG	CTTCGTACACTTCCATGAGAAACC	CATGGCCCATCAGATAGAGG	GGAGCAGAACACTTCGTTGG
DUSP6	CCAAAACCTGTCTCAGTTCTTCC	CACCTGGTATACATTCTGGTTGG	CGAGTTCAAGTACAAGCAGATCC	GAGCTTCTGCATGAGGTAGG
HOXA13	CCGTCATGTTCCTCTACGAC	GCTTCTCCGCGGTTAATCC	GGAACGGGCAAGTGTACTG	GTAGGGCACTCGCTTCTTCC
HOXD13	GGTGTACTGTGCCAAAGATCAG	CCGACCAATACACCAAATCG	CCCTACCAGCACGTTCC	GAGCTCTGTGTCTGATCTTTGG
DLX5	GACTTCCAGCCGCCTTTCC	CCATCCGCATTCCTTTCG	GTACGGCATGAACGGCTCC	GTTCACCATCCTCACCTCG
DLX6	GCAGGACTCCTCCAAATCC	CTCGGACAGTTCACATCATCTG	CCATTTACTCCAGCCTTCAGC	CTTCAGCAGCTTCTTAAACTTGG
EMX2	CCGAGAGTTTCCTTTTGCACAACGC	GTTGCTATACTCTGCCTACAAACGTAACTG	GCTTCCTCCTGCACAACG	GTGTGCCAGCTGCTTTCTC
PTCH2			GCTGTTCCCCTGTGTGATCC	CTCCTCCATCAGCTGCAAAGG
PGK1			CCATGCCTGACAAGTTCTCC	GGTTCTCTAGCAGGATGACGG
LEF1	GGATTCCTACCATGACAAGG	GGATAAAGCTGCATGTGTAGC		
LHX9	CAAAGGGGGACAGATGAACG	GGATTGGTCAGGTCTGTTAAAGTG		
LHX2 (mouse)	CACCTCAACTGCTTCACATGC	GCGAGTCATTAGAAAAGGTTGG		

Table S2. IPA-based Gene Ontology Table Outlining Limb-related Functional Annotations within the Main Categories Regulated by Brief and Prolonged FGF Treatment. The number of molecules and major molecules associated with each function are included.

BRIEF (3HR) FGF TREATMENT				
CATEGORY	ANNOTATION	P-VALUE	MOLECUES	# OF MOLECULES
ED; OD; SMSDF	Limb development	6.95E-05	ASPH, BMP4, CTGF, DLX5, GJA1, HAND2, HOXD10, HOXD9, MYCN, PBX1, PTCH1, RAMP2, SHH, ZNF521	14
ED; OD; SMSDF	Abnormal morphology of limb	2.06E-04	BMP4, CD47, CTGF, EYA1, HAND2, HOXD10, HOXD9, MTF2, PBX1, SFRP1, SHH, SPRY2, ZNF521	13
ED; OD; SMSDF	Morphogenesis of limb	1.21E-05	ASPH, BMP4, DLX5, GJA1, HAND2, HOXD10, HOXD9, MYCN, PBX1, PTCH1, SHH	11
ED; OD; SMSDF	Morphogenesis of embryonic limb	3.15E-04	BMP4, DLX5, HOXD10, HOXD9, PBX1, PTCH1, SHH	7
ED; OD; SMSDF	Morphogenesis of hindlimb	2.95E-04	BMP4, HOXD10, HOXD9, PTCH1, SHH	5
PROLONGED (24HR) FGF TREATMENT				
ED; OD; SMSDF; TD; CTDF; OM	Limb development	2.39E-11	ALX4, DLX5, DLX6, FGF10, GPC3, HOXA13, HOXD13, MSX1, MSX2, PRKG2, RDH10, SALL1, SALL3, SALL4, SHH, SP8, WNT5A	17
ED; OD; SMSDF; TD; CTDF; OM	Morphogenesis of limb	1.74E-14	ALX4, DLX5, DLX6, FGF10, GPC3, HOXA13, HOXD13, MSX1, MSX2, RDH10, SALL1, SALL3, SALL4, SHH, SP8, WNT5A	16
ED; OD; SMSDF; TD; CTDF; OM	Abnormal morphology of limb	1.49E-09	ALX4, COL12A1, ERCC6, FGF10, GPC3, HOXA13, HOXD13, MSX2, PRKG2, RDH10, ROR1, SALL4, SHH, SP8, WNT5A	15
ED; OD; SMSDF; TD; CTDF; OM	Morphogenesis of embryonic limb	1.77E-13	ALX4, DLX5, DLX6, GPC3, HOXA13, HOXD13, MSX1, MSX2, RDH10, SALL4, SHH, SP8, WNT5A	13
ED; OD; SMSDF; TD; CTDF; OM	Morphology of limb bone	7.55E-08	ALX4, COL12A1, FGF10, HOXA13, HOXD13, MSX2, PRKG2, SHH, SP8, WNT5A	10
ED; OD; SMSDF; TD; CTDF; OM	Morphogenesis of forelimb	7.59E-10	ALX4, HOXA13, MSX1, MSX2, RDH10, SALL1, SALL3, SHH	8
ED; OD; SMSDF; TD; CTDF; OM	Morphogenesis of hindlimb	2.03E-08	ALX4, GPC3, MSX1, MSX2, SALL1, SALL3, SHH	7
ED; OD; SMSDF; TD; CTDF; OM	Morphogenesis of embryonic forelimb	1.77E-07	ALX4, HOXA13, MSX1, MSX2, RDH10, SHH	6
ED; OD; SMSDF; TD; CTDF; OM	Morphogenesis of embryonic hindlimb	3.70E-06	ALX4, GPC3, MSX1, MSX2, SHH	5
ED; OD; SMSDF; TD; CTDF; OM	Morphology of hindlimb	1.01E-05	COL12A1, FGF10, GPC3, SHH, SP8	5
ED; OD; SMSDF; TD; CTDF; OM	Morphology of forelimb	9.33E-04	FGF10, SHH, SP8	3
ED; OD; SMSDF; TD; CTDF; OM	Abnormal morphology of hindlimb	2.05E-03	COL12A1, GPC3, SP8	3
ED; OD; SMSDF; TD; CTDF; OM	Formation of limb bud	1.13E-03	FGF10, SHH	2
ED; OD; SMSDF; TD; CTDF; OM	Lack of forelimb	1.65E-04	FGF10, SHH	2
ED; OD; SMSDF; TD; CTDF; OM	Lack of hindlimb	5.45E-04	FGF10, SHH	2

ED: Embryonic Development; OD: Organismal Development; SMSDF: Skeletal and Muscular System Development and Function; TD: Tissue Development; CTDF: Connective Tissue Development and Function; OM: Organ Morphology