STUDYING ENHANCER FUNCTION USING THE *TBX4* LOCUS AS A MODEL

by

ALEXANDRA GABRIELA MIHALA

(Under the Direction of Douglas Menke)

ABSTRACT

Key developmental processes in embryogenesis are modulated by transcription factors and signaling molecules whose spatio-temporal expression patterns need to be tightly regulated by *cis*-regulatory elements or enhancers. I first set out to investigate enhancer redundancy in mice using the *Tbx4* locus. I show that the two hindlimb specific regulatory elements of *Tbx4*, HLEA and HLEB, although have overlapping expression patterns in this tissue, do serve distinct roles and are therefore not redundant. Further investigation of the active appendage regulome has uncovered a large overlap between enhancers active in limbs and those active in the genital tubercle. One of these regulatory elements is HLEB which, besides gene expression in the hindlimbs, also drives robust expression in the genital tubercle. I show that this enhancer is not only active in two distinct appendage types, but also is functionally important for the proper development of both tissues.

INDEX WORDS: Enhancer, *Cis*-regulatory element, Hindlimb, Appendage, Genital tubercle, Tbx4, HLEA, HLEB

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ALEXANDRA GABRIELA MIHALA

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ALEXANDRA GABRIELA MIHALA

Major Professor: Douglas Menke

Committee: Nancy Manley
Brian Condie
Jim Lauderdale
Haini Cai

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
May 2014

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Chapter 1

INTRODUCTION AND LITERATURE REVIEW

Why study limbs?

The limb has become a great model for studying embryonic developmental processes such as growth and patterning. The growing limbs are located on the exterior of the embryo, away from the main body axis, which makes them quite easy to access and manipulate surgically. Also, conditional knockdown of genes crucial for the development of vital embryonic processes can be performed in limbs without causing embryonic lethality. This versatility allowed the limb to become a structure of intense focus, and over the years many of the genetic networks and signaling cascades involved in limb outgrowth and patterning have been discovered.

Adult limbs develop from embryonic limb buds that first appear as bulges on either side of the main body axis. These bulges initially form when mesenchymal cells migrate from the lateral plate mesoderm (LPM) to form the growing limb buds. They are comprised of three segments: the stylopod (the humerus and femur) the zeugopod (radius and ulna or tibia and fibula) and the autopod (the hand or foot) (Niswander, 2003; Stopper and Wagner, 2005). The morphologically homogenous mesenchymal cells and the underling ectoderm of limb buds need to integrate signals of growth and patterning along three axes: antero-posterior (A-P), proximo-distal (P-D) and dorso-ventral (D-V) (Niswander, 2003). Therefore, throughout development a myriad of complex signaling cascades as well as regulatory and genetic

networks shape the limb buds into morphologically complex structures such as the legs and arms. Here, I will review what is known today about the main events that drive growth and patterning in the early stages of limb development.

Initiation and Early Limb Bud Outgrowth across the P-D Axis

Wnt signaling mediates limb bud initiation at precise locations along the flanks of the main body. In chick it was discovered that expression of *Wnt2b* in the presumptive forelimb field correlates very well with the location of forelimb initiation while the initiation of hindlimb outgrowth correlates with *Wnt8c* expression (Kawakami et al., 2001). Nevertheless, Wnt signaling in the LPM induces expression of *Fgf10* which is a crucial step in the initiation of limb formation and outgrowth (Kawakami et al., 2001). In turn, Fgf10 induces expression of *Fgf8* in a signaling center located at the distal tip of the bud called the apical ectodermal ridge (AER) (Sun et al., 2000). This sequence of events establishes a positive feedback loop where Fgf8 from the AER and Fgf10 from the mesenchyme regulate each other's expression which in turn maintains the outgrowth of the limb bud (Kawakami et al., 2001; Min et al., 1998).

The importance of the AER in limb outgrowth was first noted by Saunders in 1948 when he observed an almost complete loss of all limb elements after its removal from chick limbs (Saunders, 1948). He also noticed that removal of AER at earlier time points caused sequential loss of more proximal structures indicating that AER is involved in the growth and patterning of the limb along the P-D axis. Unfortunately, as of now, the exact mechanisms by which AER supports cell proliferation and P-D patterning are still open for debate (Towers and Tickle, 2009).

Limb Growth and Patterning along the A-P Axis

Limb growth and patterning across the A-P axis is governed mainly by the zone of polarizing activity (ZPA) located at the posterior end of the autopod (Towers and Tickle, 2009). It was first discovered by Saunders and Gasseling in 1968 when they noticed that this region, when grafted to the anterior part of the chick limb bud, had a polarizing effect causing mirror like duplication of digits along the A-P axis (Saunders, 1968). It was later shown that the polarizing effect of the ZPA is carried out by Shh, a morphogen expressed and excreted by the ZPA (Riddle et al., 1993). This observation was further confirmed when genetic disruption of Shh expression in the mouse caused the loss of all skeletal elements along the A-P axis (Chiang et al., 1996).

Interestingly, conditional inactivation of *Shh* in the limbs showed that Shh has a dual role, one early in development when digit identity is specified and another growth promoting role later in development (Zhu et al., 2008). In fact, genetic manipulations of *Shh* in limbs and other tissues have shown that growth and patterning are intimately linked processes (Seifert et al., 2010; Towers et al., 2008). For example, in limbs, Shh was shown to regulate the expression of proteins important in cell cycle regulation (Towers et al., 2008). Furthermore, when the expression of these proteins was disrupted in the limb, it caused phenotypes similar to the ones observed in *Shh* mutants (Towers et al., 2008). Taken together this data strongly suggested that proliferation and patterning should be viewed in developmental biology as fundamentally linked processes.

Limb Patterning across the D-V Axis

The limb dorso-ventral (D-V) patterning is coordinated by the underlying ectoderm (MacCabe et al., 1974). When the ectoderm is rotated so that the dorsal ectoderm is in direct contact with the ventral mesenchyme and vice-versa, the resulting identity of all limb elements changes to correspond to the polarity of the underlying ectoderm (MacCabe et al., 1974). Wnt signaling, Wnt7a specifically, has been shown in both chicken (Rodriguez-Esteban et al., 1997) and mice (Kimmel et al., 2000) to specify dorsal identity. Wnt signaling specifies dorsal identity by activating mesenchymal expression of *Lmx-1* in the chick (Riddle et al., 1995) and *Lmx-1b* in the mouse (Loomis et al., 1998) dorsal limb compartments. In contrast, ventral identity is specified by *Engrailed 1* (*En-1*) which is expressed in the ventral ectoderm and restricts *Wnt7a* expression to dorsal domains (Chen and Johnson, 1999; Kimmel et al., 2000; Loomis et al., 1996).

The involvement of Hox Genes in Limb Development

The most 5' Hox genes of the A and D clusters (11-13) are crucial in the development and patterning of the limb (Nelson et al., 1996; Zakany and Duboule, 2007). Early in limb development Hox genes are expressed in a collinear fashion similar to what is observed in the development of the main body axis (Tarchini and Duboule, 2006; Zakany and Duboule, 2007). Later in development, gene expression of HoxA and HoxD clusters become very dynamic eventually being restricted to the autopod in nested domains (Zakany and Duboule, 2007).

Identifying the function of any particular Hox gene has been difficult due to functional redundancy among them (Zakany and Duboule, 2007). However, the phenotypes of several double mutants have shown that Hox genes indeed carry out complex roles in limb

development. For example, the limbs of *Hoxa11* and *Hoxd11* double mutants lack the structures associated with the zeugopod (Davis et al., 1995) suggesting that these genes are involved in the growth and pattering of the limb across the P-D axis. In contrast, *Hoxa13* and *Hoxd13* double mutants display gross abnormalities of the digits across the A-P axis, similar to what is observed when expression of *Shh* is disrupted (Zakany et al., 1997). Furthermore, anterior misexpression of *Hoxd11*, *Hoxd12* and *Hoxd13* results in ectopic expression of *Shh* and results in digit abnormalities (Zakany et al., 2004). This data supports the idea that these particular Hox genes function upstream of *Shh* in mediating A-P growth and patterning.

Integrating Signals of Growth and Patterning

The complex gene regulatory networks and signaling pathways that are involved in limb development need to be integrated for a harmonious morphological outcome. For example, the growth and patterning across the P-D axis, is under the regulation of Fgfs expressed from the AER (Niswander et al., 1993; Saunders, 1948; Sun et al., 2000; Sun et al., 2002) while the ZPA expressing Shh regulates limb development across the A-P axis (Chiang et al., 1996; Saunders, 1968; Zhu et al., 2008). However, Fgf signaling from the AER and Shh from the ZPA were shown to regulate each other's expression creating positive feedback loop (Niswander et al., 1994). Furthermore, *Fgf8* mutants display digit abnormalities consistent with disruption of *Shh* expression (Sun et al., 2002). Taken together these results show that the limb patterning and growth along the P-D and A-P axes are coordinated by a Shh-Fgf feedback loop.

This positive feedback loop was shown to be initiated by mesenchymal expression of BMP which in turn upregulates expression of its own antagonist, *Gremlin* (Nissim et al., 2006). Gremlin is a signal intermediate between Shh and the AER (Nissim et al., 2006; Zuniga et al.,

1999) such that loss of Gremlin in the limb mesenchyme reduces *Fgf4* expression and stops the Shh-Fgf feedback loop (Zuniga et al., 1999).

Interestingly, the boundary between the dorsal and ventral compartments of the limb is essential for AER positioning (Tanaka et al., 1997). Furthermore, genetic disruption of *En-1* expression, which specifies ventral identity, disrupts AER formation (Loomis et al., 1998). Also, removal of the dorsal ectoderm or genetic disruption of *Wnt7a* expression, causes disruption of *Shh* expression (Parr and McMahon, 1995). Taken together these results suggest that signals necessary for specification of dorsal and ventral fates are also involved in the regulation of other genetic networks involved in overall limb development.

Tbx4 Plays a Crucial Role in Limb Development

The first T-box containing locus was discovered by Nadine Dobrovolskaia-Zavadskaia in 1927 when she noticed that some of the offspring of X-ray irradiated mice exhibited short tails (Korzh and Grunwald, 2001). Since then, other genes have been discovered in many metazoan organisms that are related to the T gene by a well conserved DNA binding domain called T-box (Papaioannou, 2001). In mammals many of these genes serve important developmental functions in heart (Plageman and Yutzey, 2005), lung (Acquaah-Mensah et al., 2012), somitogenesis (Tanaka and Tickle, 2004) umbilical chord formation as well as limb development (Naiche, 2003). For example, *Tbx4* has been linked to human syndromes that affect the skeletal development of the legs such as Small Patella Syndrome (Bongers et al., 2004) and Clubfoot (Alvarado et al., 2010). Moreover, Tbx4 was thought to be a transcription factor involved in the acquisition of hindlimb identity due absence of expression in the forelimbs where Tbx5 is active

(Ohuchi et al., 1998; Rodriguez-Esteban et al., 1999; Takeuchi et al., 1999), generating an intensive focus on the role of these genes in limb development.

Interestingly, experiments using *Tbx4* knockout mutant mice showed that *Tbx4* is indeed necessary for hindlimb outgrowth, however it is dispensable for the initiation of this appendage (Minguillon et al., 2005; Naiche, 2003). Disrupting *Tbx4* expression at different time points revealed multiple roles of this transcription factor in limb development (Naiche and Papaioannou, 2007). Early disruption caused appendage outgrowth to be severely impaired while later disruption caused skeletal abnormalities such as hypoplasia of the pelvis, the femur and the fibula as well as partially fused digits (Naiche and Papaioannou, 2007). Besides skeletal phenotypes, conditional disruption of *Tbx4* results in significant muscle and tendon abnormalities (Hasson et al., 2010). Taken together these experiments uncovered multiple roles that *Tbx4* is playing in limb development. Early on, *Tbx4* is necessary for proper hindlimb outgrowth while later, this transcription factor is involved in the patterning of skeletal elements.

In mice, *Tbx4* expression is initiated in the presumptive limb field of the LPM at stage E9.5 before limb bud induction (Gibson-Brown et al., 1996). Afterwards, *Tbx4* continues to be expressed throughout the mesenchyme of the growing limb bud until after stage E11.5 when expression levels decrease in dynamically restricted domains (Gibson-Brown et al., 1996). These patterns of gene expression suggested that *Tbx4* may play an important role in early hindlimb development. Upon removal of AER or ZPA from the chick limb bud, *Tbx4* expression remained unaffected (Gibson-Brown et al., 1998), showing that gene expression initiates and is maintained independent from the signals generated by these domains. However, gain and loss

of function experiments in chick uncovered a potential feedback loop between Tbx4, Wnt, BMP and Fgf signaling (Rodriguez-Esteban et al., 1999) supporting the idea that *Tbx4* is involved in hindlimb outgrowth. Indeed, misexpression of a dominant negative form of *Tbx4* resulted in downregulation of both Wnt and Fgf signaling (Takeuchi, 2003). Furthermore, misexpression of *Tbx4* in the LPM resulted in the upregulation of Wnt and FGF signaling as well as the induction of a hindlimb-like appendage (Takeuchi, 2003). Taken together these results indicate that in chick, *Tbx4* plays an important role in hindlimb outgrowth.

A potential upstream target of *Tbx4* was uncovered when misexpression of *Pitx1* in the chick wing, induced expression of *Tbx4*(Takeuchi et al., 1999). Just like Tbx4, Pitx1 is a transcription factor expressed in the mesenchyme of the hindlimb but not the forelimb (Logan et al., 1998). Furthermore, disruption of *Pitx1* expression in the chick hindlimb results in downregulation of *Tbx4* expression, while misexpression of *Pitx1* in the forelimb results in upregulation of *Tbx4* in this appendage (Szeto et al., 1999). Consistent with this finding in chick, *Pitx1* knockout mutant mice also display significant downregulation of *Tbx4* expression in embryonic hindlimb as well as severe skeletal abnormalities in the adult leg (Lanctot et al., 1999; Szeto et al., 1999).

Does the Tbx4 Locus Serve a Function in the Acquisition of Hindlimb Specific Morphology?

Tbx4 shares a close evolutionary relationship with another T-box transcription factor, *Tbx5* (Agulnik et al., 1996). In fact *Tbx4* and *Tbx5* arose from an ancient duplication event sometimes during the early vertebrate lineage (Agulnik et al., 1996). Because of their particular limb expression, *Tbx5* and *Tbx4* have been thought to specify forelimb and hindlimb identity respectively (Ohuchi et al., 1998; Rodriguez-Esteban et al., 1999; Takeuchi et al., 1999). More

specifically, *Tbx4* limb expression is restricted to the hindlimbs while *Tbx5* expression is restricted to the forelimbs in avians (Ohuchi et al., 1995), mammals (Gibson-Brown et al., 1996), amphibians (Takabatake et al., 2000) and fish (Ruvinsky et al., 2000; Tanaka et al., 2002).

Indeed, misexpression experiments in chick support the hypothesis that *Tbx4* and *Tbx5* can specify the identities of hindlimbs and forelimbs respectively (Rodriguez-Esteban et al., 1999; Takeuchi et al., 1999). In contrast, misexpression experiments in mice have shown that the Tbx4 protein can rescue forelimb like morphology in *Tbx5* mutant mice (Minguillon et al., 2005). This shows that in mice, the protein coded by the *Tbx4* locus is capable to carry out the function of Tbx5 in the forelimb. It does not disprove the possibility that the mouse *Tbx4* locus may still be implicated in modulating hindlimb specific morphology.

Amphioxus is a fish-like chordate whose earliest common ancestor with mice or chick, has diverged before the *Tbx4/Tbx5* ancestral gene duplication (Horton et al., 2008). Interestingly, the amphioxus lacks paired appendages and presumably also any appendage specific regulatory elements (Horton et al., 2008). Interestingly, misexpression of the amphioxus *Tbx4/Tbx5* gene product in *Tbx5* mutant mice rescues limb outgrowth, showing that the protein itself has the potential to undertake limb specific function, however it may be missing appendage specific regulatory elements (Minguillon et al., 2009).

Some of the limb specific *cis*-regulatory elements of *Tbx4* and *Tbx5* have been discovered in mice and are indeed distinct (Menke et al., 2008; Minguillon et al., 2012). This raises the possibility that the two loci have divergent functions in modulating limb type morphology through distinct regulatory networks. For example, Pitx1 has been shown both *in vitro* and *in vivo* to bind a hindlimb specific enhancer of *Tbx4* (Infante et al., 2013). *Pitx1* is

expressed specifically in the hindlimbs and has been shown to be involved in the acquisition of hindlimb like morphology in mice (DeLaurier et al., 2006). Therefore, this particular *Pitx1-Tbx4* genetic interaction may be one of the many mechanisms by which *Pitx1* may be modulating hindlimb like morphology. Therefore, in order to understand how paired-like appendages such as the limbs evolved distinct morphologies it would be useful to focus instead on their active regulome.

Cis-Regulatory Elements at the Heart of Development and Evolution

I have discussed the roles of many key transcription factors and signaling pathways important for the developing limb. Their overall expression patterns are for the most part unique and can be restricted both spatially and temporally within distinct compartments of the limb. Ectopic expression or missexpression of many of these genes can give rise to serious phenotypic consequences that are distinct from what is observed in wild type individuals (Kimmel et al., 2000; Loomis et al., 1998; Suzuki et al., 2004; Zakany and Duboule, 2007). The phenotypic consequences of such alterations in gene expression are for the most part deleterious; however, they could provide a mechanism for the evolution of traits if a novel developmental phenotype happens to provide an evolutionary advantage than can be selected for. Given that the precise spatio-temporal gene expression is under the regulation of *cis*-regulatory elements, alterations in the sequence or function of these elements may be of importance for the development and evolution of traits.

Relatively recent functional work in Drosophila has shed some light into the importance of *cis*-regulatory elements in the evolution of phenotypic differences between species. In 2007, McGregor and colleagues identified 3 independent enhancers driving expression of a gene

important in tricome morphogenesis, called *shavenbaby* (*svb*) (McGregor et al., 2007). With the use of transgenics they investigated the function of these enhancers from both *D*. *sechellia* as well as *D*. *melanogaster*, two closely related fruit fly species that differ substantially in the number of tricomes. They noticed that the enhancer copies from *D*. *sechellia* do not drive the same expression pattern as the ones from *D. melanogaster*, which raised the possibility that the phenotypic differences in tricome number between these two species may be due to functional differences in these enhancers. To investigate this possibility, they performed high resolution gene mapping and showed that the region that overlaps all three enhancers of *svb* is required for the *D. sechellia* tricome pattern. These results were significant because they showed a functional link between alterations in enhancer function and morphological output. But is the regulation of *svb* a unique example?

In 2008 Jeong and colleagues uncovered a similar link between enhancer function and phenotypic outcome of another gene, *tan* (Jeong et al., 2008). They investigated the variation in abdominal pigmentation between two related species of fruit fly, *D*. *yakuba* (pigmented) and *D*. *santomea* (no abdominal pigment). They linked the loss of abdominal pigmentation in *D*. *santomea* to a loss of *tan* expression in the abdominal epidermis of developing larvae. By comparing *tan* cDNA from both species they showed no alterations in the sequence of the protein product. This indicated that instead of coding sequence alterations, the cause of the loss of pigmentation and *tan* gene expression may be due to differences in regulatory elements. By using a series of transgenic reporter constructs, they isolated the *tan cis*-regulatory element and further went on to show through a phenotypic rescue experiment, that the loss of *tan* gene expression in the abdominal epidermis of *D*. *santomea* flies is indeed due to mutations in this

element. Using similar approaches, high resolution genetic mapping and transgenic assays, Rebeiz and colleagues showed that variations in the enhancer region sequence of the *ebony* locus are responsible for the phenotypic differences in pigmentation observed between two populations of D. *melanogaster* (Rebeiz et al., 2009). But are these observations specific only to fruit flies?

Evolution of pelvic fin loss in stickleback fish has been shown to occur through mechanisms similar to those that underlie evolution of certain traits in fruit flies (Alvarado et al., 2010; Shapiro et al., 2004). Many freshwater stickleback populations have lived in genetic isolation for more than 10,000 years, when marine poplulations invaded freshwater lakes that formed due to glacial recession (Levine, 2010). Such isolation has allowed traits, such as the pelvic fin, to be independently lost in several populations. This has allowed robust comparative studies as well as high resolution genetic mapping to be possible. The loss of the pelvic fin, which is considered to be homologous to the hindlimb in other vertebrates, has been shown by Chan and colleagues to be caused by deletions in an enhancer of *Pitx1*. *Pitx1,* as discussed previously, is a gene known to be very important in the development of hindlimbs so the involvement of *Pitx1* in the evolution of hindfin loss makes intuitive sense. What is special about this outcome is that *Pitx1* expression, which is also involved in the development of other important tissues such as the pituitary (Levine, 2010), is altered only in the region that would give rise to the pelvic fin and is normal in the other tissues. This implies the existence of multiple distinct tissue specific enhancers of *Pitx1.* In time, these distinct regulatory elements can independently accumulate base pair alterations that could account for morphological alterations in specific tissues. This mechanism has allowed sticklebacks evolve pelvic fin loss

without impacting pituitary development and points to the versatility of enhancers not only in development but also evolution. However, these studies have focused on the evolution of trait loss. How can novel traits emerge? Do they require another mechanism, such as alterations in protein function, or are enhancers versatile enough to evolve and acquire novel morphologies?

Frankel and colleagues took this line of research a few steps further and uncovered more details about the function of regulatory elements (Frankel et al., 2011). Investigating the *svb* locus, they uncovered specific base pair changes in one of the enhancer elements that alter its function, both spatially and temporally. They also showed that this particular change in gene expression causes observable phenotypic consequences. In other words, they linked base pair alterations in a regulatory element to changes in when, where, and for how long a gene can be expressed, to altered morphology. This is significant because it is a mechanism by which novel morphologies can be acquired. For example, if alterations in enhancer sequence worked as an on/off switch only, the phenotypic consequences could be restricted to either loss or (less likely) gain of traits but would restrict the possibility to modify an existing trait.

For example, bats did not evolve a new set of appendages to replace their forelimbs in order to take flight. Instead, they modified their existing forelimbs by incorporating many changes such as elongation of the phalanges. In 2008, Cretekos and colleagues set out to investigate the evolutionary and developmental mechanism of trait acquisition using the bat wing as a model (Cretekos et al., 2008). They investigated an enhancer of *Prx1*, a gene important in limb development, and showed that when the endogenous mouse copy of this enhancer is replaced by the bat copy, the resulting forelimbs of mutant mice are significantly longer than controls. This study showed that base pair alterations in enhancers can cause

phenotypic modifications of existing traits. The subtlety of the phenotype observed in these mutant mice suggests the possibility that complex alterations in morphology might be attained by many such subtle effects caused by sequence variations in many regulatory elements.

Interest in the development and evolution of complex structures, such as the limb, has pushed the field to develop more global approaches to understand gene expression and regulation at the genomic level. Sequencing of whole genomes has become a routine method, allowing us to peer into the complex genomic architecture of many different organisms. Chromatin immunoprecipitation followed by massive parallel sequencing is another global approach that is aimed at understanding chromatin architecture and how that architecture varies between different tissues or different organisms. These tools have proved immensely successful at allowing us to better understand the genetic mechanisms of development and even the evolution of different tissues. I will continue by making a brief overview of how these global approaches have been used to understand limb development.

Modern tetrapod limbs evolved from paired fins during the water to land transition and the mechanisms by which such a transformation may have occurred are of great interest for understanding how evolution and development interplay. Amemiya and colleagues were interested in the fin to limb transition and thus sequenced the genome of a coelacanth specimen, *Latimeria chalumnae*, that is thought to be the relative of an ancient lobe-finned fish that may be a link in the evolution of limbs from fins (Amemiya et al., 2013). Genome wide comparisons between the coelacanth and several tetrapod species as well as several teleosts uncovered 44,200 conserved noncoding elements (CNEs) that they believed originated after the divergence teleosts from the coelacanth lineage. They compared these CNEs to putative

enhancers previously identified in the mouse embryo by ChIP-seq using an antibody against p300 and detected a seven fold enrichment for their candidate CNEs in this data set. This implied that indeed, the detected CNE's are enriched for regulatory elements.

Next, they associated the set of candidate CNEs with gene function and noted that most of them are associated with the perception of smell, morphogenesis and cellular differentiation. To uncover potential CNEs potentially involved in appendage evolution they looked at loci known to be involved in limb morphogenesis in other organisms. The HoxD cluster in particular is important in the development of the digits in tetrapods, and the digits are a novel innovation of tetrapods. The expression of the HoxD cluster in the autopod has been shown to be under the regulation of six independent regulatory elements out of which three are conserved between modern tetrapods and the coelacanth. Furthermore, one of these enhancers is capable of driving limb specific expression in mouse transgenic assays implying the possibility that the fin to limb transition may have occurred by modifications of already existing fin enhancers.

Although very expansive, this particular data set is limited in the types of analyses one can make. For example, given genome wide comparisons one cannot tell when and especially where such putative enhancers may be active. If one is interested specifically in limb development and evolution, they need to identify enhancers that are active only in this tissue. ChIP-seq experiments are therefore more suitable to answer such questions since they are based on data collected from carefully dissected tissues.

Visel and colleagues used an antibody against p300 to detect active enhancers in different tissues of the mouse embryo, including pooled forelimbs and hindlimbs from stage

E11.5 (Visel et al., 2009). They detected 2105 limb specific putative active enhancers. They functionally tested 25 putative limb enhancers using transgenic constructs and noticed positive activity in the limb from 20 (80%) constructs suggesting that this type of global approach is very effective at uncovering tissue specific regulatory elements. This was amongst the first global efforts at identifying active limb enhancers, however the morphologies of forelimbs and hindlimbs can vary substantially and that could be reflected in their regulatory networks as well.

To uncover possible regulatory differences between the two sets of limbs, Cotney and colleagues performed ChIP-seq on separate forelimb and hindlimb tissues from mouse E10.5 and E11.5 embryos (Cotney et al., 2012). They used an antibody against a different mark for active enhancers, histone H3K27ac, which has been previously shown to be an excellent mark for identifying such features (Creyghton et al., 2010). Cotney and colleagues also performed RNA-seq on the same tissues to be able to associate regions of active enhancers with gene expression. They found that few genes are differentially expressed between the two stages of development. Surprisingly they also found that the transcriptomes of the two sets of appendages are incredibly similar as well, with only 186 genes being differentially expressed. However, the forelimb is developmentally more advanced than the hindlimb at the stages they tested, therefore some of these differences may be due to developmental delay. Thus, the real number of differentially expressed genes may be smaller. They also found that histone signatures of 1121 regions are differentially marked between the two sets of appendages at E10.5 and are therefore putative enhancers that may be responsible for modulating forelimb and hindlimb specific morphology. However, very few regions have been found to be

associated with this histone mark in only one set of appendages compared to the other. Overall the surprising find of this study has been the incredible similarity of gene expression and histone signatures of putative active enhancers between forelimbs and hindlimbs.

Interested in how limb enhancers have evolved to acquire different morphologies in different species, specifically the human lineage, the same group generated H3K27ac ChIP-seq datasets from human, rhesus monkey and mouse limbs at different time points in development corresponding to E10.5-E13.5 mouse developmental stages (Cotney et al., 2013). They compared the human derived putative active enhancers with those previously published that have been identified from other tissues to find sites that are very limb specific. They determined that these highly limb specific sites correlate well with genes known to be expressed in limbs or to be important in limb development. The HoxD cluster regulatory region as well as *Pitx1* and *Tbx5* are similarly marked in all three species studied.

When they compared signal strengths of these putative enhancers between human and the other two species they found many more signal gains than losses. There are 2,175 promoters and 2915 putative enhancers with increased signal strength in human at one or more time points that have been validated using ChIP-qPCR (21 out of 28 tested regions). These signals are enriched near genes associated with proliferation, growth, bone morphogenesis and connective tissue. In contrast, only 74 orthologous regions displayed any signal loss in the human lineage.

91% of human gained promoters display H3K27ac signatures in the other three species suggesting that the mechanism of gain may be due to sequence divergence. 6% of human gained promoters are not marked in the limbs of mouse or rhesus but they are marked in

another tissue suggesting co-option as a mechanism of gain, while 3% do not show marking in any tissue examined suggesting that they may have gained signal *de-novo.* When they did the same analysis using putative human active enhancers they noticed a much higher proportion of regions that may have gained signal via co-option (18%), while 20% may have been gained *de-novo* and 62% through sequence alterations.

However expansive these genome wide enhancer data sets are, they do have limitations. Enhancers are DNA sequences recognized by a slew of transcription factors that modulate enhancer-promoter interactions to generate a particular expression output. Transcription factor binding can be inferred computationally once the enhancers are identified but many binding motifs are shared between different closely related factors or are still unknown. Other studies have undergone investigations into limb regulatory networks by performing ChIP-seq using antibodies against key transcription factors known to be crucial for limb developmental processes such as *Gli3* (Vokes et al., 2008) and *Pitx1* (Infante et al., 2013).

Vokes and colleagues used this method to indentify binding regions of *Gli3* and were able to identify potential downstream genes of this transcription factor. Interestingly among them were *Tbx2* and *Tbx3*, genes that were shown previously to be upstream of *Gli3* implying reciprocal regulation. They functionally tested several putative *Gli3* limb enhancers and showed limb specific expression as well as identifying a regulatory element of *Hand2* that acts as a repressor in this tissue.

A similar approach was used by Infante and colleagues to identify binding sites and downstream targets of *Pitx1*, a transcription factor not expressed in the forelimbs and implicated in the acquisition of hindlimb morphology. They determined that *Pitx1* binding is

enriched in hindlimb specific compared to forelimb specific regulatory elements that were identified by Cotney et al. via H3k27ac histone mark. However *Pitx1* also binds limb enhancers known to have similar levels of activity in both sets of appendages. Therefore they concluded that *Pitx1* may modulate the acquisition of hindlimb morphology by two mechanisms: binding to hindlimb specific enhancers and by associating to a generic limb enhancer and modulating its function in a hindlimb specific manner.

To understand overall limb development and evolution in the future, there may be a need to analyze all these genome wide datasets together. Each can tell a part of this large and complex story such as which enhancers are active in a particular tissue such as the limb, what transcription factors bind to these enhancers and how the expression of downstream genes is affected. By comparing datasets between different species one can uncover how sequence alterations can affect specific transcription factor binding and possibly enhancer function as well as how these changes relate to the differences in morphologies we see between these species.

References

Abbasi, A.A., Paparidis, Z., Malik, S., Bangs, F., Schmidt, A., Koch, S., Lopez-Rios, J., Grzeschik, K.H., 2010. Human intronic enhancers control distinct sub-domains of Gli3 expression during mouse CNS and limb development. BMC developmental biology 10, 44.

Acquaah-Mensah, G.K., Malhotra, D., Vulimiri, M., McDermott, J.E., Biswal, S., 2012. Suppressed expression of T-box transcription factors is involved in senescence in chronic obstructive pulmonary disease. PLoS computational biology 8, e1002597.

Agulnik, S.I., Garvey, N., Hancock, S., Ruvinsky, I., Chapman, D.L., Agulnik, I., Bollag, R., Papaioannou, V., Silver, L.M., 1996. Evolution of mouse T-box genes by tandem duplication and cluster dispersion. Genetics 144, 249-254.

Ahituv, N., Zhu, Y., Visel, A., Holt, A., Afzal, V., Pennacchio, L.A., Rubin, E.M., 2007. Deletion of ultraconserved elements yields viable mice. PLoS biology 5, e234.

Alvarado, D.M., Aferol, H., McCall, K., Huang, J.B., Techy, M., Buchan, J., Cady, J., Gonzales, P.R., Dobbs, M.B., Gurnett, C.A., 2010. Familial isolated clubfoot is associated with recurrent chromosome 17q23.1q23.2 microduplications containing TBX4. American journal of human genetics 87, 154-160.

Barna, M., Pandolfi, P.P., Niswander, L., 2005. Gli3 and Plzf cooperate in proximal limb patterning at early stages of limb development. Nature 436, 277-281.

Barolo, S., 2012. Shadow enhancers: frequently asked questions about distributed cis-regulatory information and enhancer redundancy. Bioessays 34, 135-141.

Bongers, E.M., Duijf, P.H., van Beersum, S.E., Schoots, J., Van Kampen, A., Burckhardt, A., Hamel, B.C., Losan, F., Hoefsloot, L.H., Yntema, H.G., Knoers, N.V., van Bokhoven, H., 2004. Mutations in the human TBX4 gene cause small patella syndrome. American journal of human genetics 74, 1239-1248.

Cande, J., Goltsev, Y., Levine, M.S., 2009. Conservation of enhancer location in divergent insects. Proceedings of the National Academy of Sciences of the United States of America 106, 14414-14419.

Chen, H., Johnson, R.L., 1999. Dorsoventral patterning of the vertebrate limb: a process governed by multiple events. Cell and tissue research 296, 67-73.

Chiang, C., Litingtung, Y., Lee, E., Young, K.E., Corden, J.L., Westphal, H., Beachy, P.A., 1996. Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. Nature 383, 407-413.

Cobb, J., Duboule, D., 2005. Comparative analysis of genes downstream of the Hoxd cluster in developing digits and external genitalia. Development 132, 3055-3067.

Cotney, J., Leng, J., Oh, S., DeMare, L.E., Reilly, S.K., Gerstein, M.B., Noonan, J.P., 2012. Chromatin state signatures associated with tissue-specific gene expression and enhancer activity in the embryonic limb. Genome research 22, 1069-1080.

Cretekos, C.J., Wang, Y., Green, E.D., Martin, J.F., Rasweiler, J.J.t., Behringer, R.R., 2008. Regulatory divergence modifies limb length between mammals. Genes & development 22, 141-151.

Creyghton, M.P., Cheng, A.W., Welstead, G.G., Kooistra, T., Carey, B.W., Steine, E.J., Hanna, J., Lodato, M.A., Frampton, G.M., Sharp, P.A., Boyer, L.A., Young, R.A., Jaenisch, R., 2010. Histone H3K27ac separates active from poised enhancers and predicts developmental state. Proceedings of the National Academy of Sciences of the United States of America 107, 21931-21936.

Davis, A.P., Witte, D.P., Hsieh-Li, H.M., Potter, S.S., Capecchi, M.R., 1995. Absence of radius and ulna in mice lacking hoxa-11 and hoxd-11. Nature 375, 791-795.

Degenhardt, K.R., Milewski, R.C., Padmanabhan, A., Miller, M., Singh, M.K., Lang, D., Engleka, K.A., Wu, M., Li, J., Zhou, D., Antonucci, N., Li, L., Epstein, J.A., 2010. Distinct enhancers at the Pax3 locus can function redundantly to regulate neural tube and neural crest expressions. Developmental biology 339, 519-527.

DeLaurier, A., Schweitzer, R., Logan, M., 2006. Pitx1 determines the morphology of muscle, tendon, and bones of the hindlimb. Developmental biology 299, 22-34.

DiLeone, R.J., Russell, L.B., Kingsley, D.M., 1998. An extensive 3' regulatory region controls expression of Bmp5 in specific anatomical structures of the mouse embryo. Genetics 148, 401-408.

Dollé, P., Dierich, A., LeMeur, M., Schimmang, T., Schuhbaur, B., Chambon, P., Duboule, D., 1993. Disruption of the Hoxd-13 gene induces localized heterochrony leading to mice with neotenic limbs. Cell 75, 431-441.

Dunn, N.R., Winnier, G.E., Hargett, L.K., Schrick, J.J., Fogo, A.B., Hogan, B.L., 1997. Haploinsufficient phenotypes in Bmp4 heterozygous null mice and modification by mutations in Gli3 and Alx4. Developmental biology 188, 235-247.

Ekici, A.B., Strissel, P.L., Oppelt, P.G., Renner, S.P., Brucker, S., Beckmann, M.W., Strick, R., 2013. HOXA10 and HOXA13 sequence variations in human female genital malformations including congenital absence of the uterus and vagina. Gene 518, 267-272.

Epner, E., Reik, A., Cimbora, D., Telling, A., Bender, M.A., Fiering, S., Enver, T., Martin, D.I.K., Kennedy, M., Keller, G., Groudine, M., 1998. The Globin LCR Is Not Necessary for an Open Chromatin Structure or Developmentally Regulated Transcription of the Native Mouse Globin Locus. Molecular cell 2, 447-455.

Ernst, J., Kheradpour, P., Mikkelsen, T.S., Shoresh, N., Ward, L.D., Epstein, C.B., Zhang, X., Wang, L., Issner, R., Coyne, M., Ku, M., Durham, T., Kellis, M., Bernstein, B.E., 2011. Mapping and analysis of chromatin state dynamics in nine human cell types. Nature 473, 43-49.

Frankel, N., Davis, G.K., Vargas, D., Wang, S., Payre, F., Stern, D.L., 2010. Phenotypic robustness conferred by apparently redundant transcriptional enhancers. Nature 466, 490-493.

Frankel, N., Erezyilmaz, D.F., McGregor, A.P., Wang, S., Payre, F., Stern, D.L., 2011. Morphological evolution caused by many subtle-effect substitutions in regulatory DNA. Nature 474, 598-603.

Gibson-Brown, J.J., Agulnik, S.I., Chapman, D.L., Alexiou, M., Garvey, N., Silver, L.M., Papaioannou, V.E., 1996. Evidence of a role for T-box genes in the evolution of limb morphogenesis and the specification of forelimb/hindlimb identity. Mechanisms of development 56, 93-101.

Gibson-Brown, J.J., Agulnik, S.I., Silver, L.M., Niswander, L., Papaioannou, V.E., 1998. Involvement of T-box genes Tbx2-Tbx5 in vertebrate limb specification and development. Development 125, 2499-2509.

Goodman, F.R., Bacchelli, C., Brady, A.F., Brueton, L.A., Fryns, J.P., Mortlock, D.P., Innis, J.W., Holmes, L.B., Donnenfeld, A.E., Feingold, M., Beemer, F.A., Hennekam, R.C., Scambler, P.J., 2000. Novel HOXA13 mutations and the phenotypic spectrum of hand-foot-genital syndrome. Am J Hum Genet 67, 197-202.

Haraguchi, R., Suzuki, K., Murakami, R., Sakai, M., Kamikawa, M., Kengaku, M., Sekine, K., Kawano, H., Kato, S., Ueno, N., Yamada, G., 2000. Molecular analysis of external genitalia formation: the role of fibroblast growth factor (Fgf) genes during genital tubercle formation. Development 127, 2471-2479.

Harding, S.D., Armit, C., Armstrong, J., Brennan, J., Cheng, Y., Haggarty, B., Houghton, D., Lloyd-MacGilp, S., Pi, X., Roochun, Y., Sharghi, M., Tindal, C., McMahon, A.P., Gottesman, B., Little, M.H., Georgas, K., Aronow, B.J., Potter, S.S., Brunskill, E.W., Southard-Smith, E.M., Mendelsohn, C., Baldock, R.A., Davies, J.A., Davidson, D., 2011. The GUDMAP database--an online resource for genitourinary research. Development 138, 2845-2853.

Harfe, B.D., 2011. Keeping up with the zone of polarizing activity: New roles for an old signaling center. Developmental dynamics : an official publication of the American Association of Anatomists 240, 915-919.

Hasson, P., DeLaurier, A., Bennett, M., Grigorieva, E., Naiche, L.A., Papaioannou, V.E., Mohun, T.J., Logan, M.P., 2010. Tbx4 and tbx5 acting in connective tissue are required for limb muscle and tendon patterning. Developmental cell 18, 148-156.

Hobert, O., 2010. Gene regulation: enhancers stepping out of the shadow. Current biology : CB 20, R697-699.

Hong, J.W., Hendrix, D.A., Levine, M.S., 2008. Shadow enhancers as a source of evolutionary novelty. Science 321, 1314-1314.

Horton, A.C., Mahadevan, N.R., Minguillon, C., Osoegawa, K., Rokhsar, D.S., Ruvinsky, I., de Jong, P.J., Logan, M.P., Gibson-Brown, J.J., 2008. Conservation of linkage and evolution of developmental function within the Tbx2/3/4/5 subfamily of T-box genes: implications for the origin of vertebrate limbs. Development genes and evolution 218, 613-628.

Infante, C.R., Park, S., Mihala, A.G., Kingsley, D.M., Menke, D.B., 2013. Pitx1 broadly associates with limb enhancers and is enriched on hindlimb cis-regulatory elements. Developmental biology 374, 234-244.

Izpisua-Belmonte, J.C., Brown, J.M., Crawley, A., Duboule, D., Tickle, C., 1992. Hox-4 gene expression in mouse/chicken heterospecific grafts of signalling regions to limb buds reveals similarities in patterning mechanisms. Development 115, 553-560.

Jeong, S., Hahn, Y., Rong, Q., Pfeifer, K., 2007. Accurate quantitation of allele-specific expression patterns by analysis of DNA melting. Genome research 17, 1093-1100.

Jeong, Y., El-Jaick, K., Roessler, E., Muenke, M., Epstein, D.J., 2006. A functional screen for sonic hedgehog regulatory elements across a 1 Mb interval identifies long-range ventral forebrain enhancers. Development 133, 761-772.

Juan, A.H., Ruddle, F.H., 2003. Enhancer timing of Hox gene expression: deletion of the endogenous Hoxc8 early enhancer. Development 130, 4823-4834.

Kaku, Y., Ohmori, T., Kudo, K., Fujimura, S., Suzuki, K., Evans, S.M., Kawakami, Y., Nishinakamura, R., 2013. Islet1 deletion causes kidney agenesis and hydroureter resembling CAKUT. Journal of the American Society of Nephrology : JASN 24, 1242-1249.

Kawakami, Y., Capdevila, J., Buscher, D., Itoh, T., Rodriguez Esteban, C., Izpisua Belmonte, J.C., 2001. WNT signals control FGF-dependent limb initiation and AER induction in the chick embryo. Cell 104, 891-900.

Kawakami, Y., Marti, M., Kawakami, H., Itou, J., Quach, T., Johnson, A., Sahara, S., O'Leary, D.D., Nakagawa, Y., Lewandoski, M., Pfaff, S., Evans, S.M., Izpisua Belmonte, J.C., 2011. Islet1-mediated activation of the beta-catenin pathway is necessary for hindlimb initiation in mice. Development 138, 4465-4473.

Kimmel, R.A., Turnbull, D.H., Blanquet, V., Wurst, W., Loomis, C.A., Joyner, A.L., 2000. Two lineage boundaries coordinate vertebrate apical ectodermal ridge formation. Genes Dev 14, 1377-1389.

Kobayashi, A., Kwan, K.M., Carroll, T.J., McMahon, A.P., Mendelsohn, C.L., Behringer, R.R., 2005. Distinct and sequential tissue-specific activities of the LIM-class homeobox gene Lim1 for tubular morphogenesis during kidney development. Development 132, 2809-2823.

Kobayashi, A., Shawlot, W., Kania, A., Behringer, R.R., 2004. Requirement of Lim1 for female reproductive tract development. Development 131, 539-549.

Korzh, V., Grunwald, D., 2001. Nadine Dobrovolskaia-Zavadskaia and the dawn of developmental genetics. Bioessays 23, 365-371.

Kuijper, S., Beverdam, A., Kroon, C., Brouwer, A., Candille, S., Barsh, G., Meijlink, F., 2005a. Genetics of shoulder girdle formation: roles of Tbx15 and aristaless-like genes. Development 132, 1601-1610.

Kuijper, S., Feitsma, H., Sheth, R., Korving, J., Reijnen, M., Meijlink, F., 2005b. Function and regulation of Alx4 in limb development: complex genetic interactions with Gli3 and Shh. Developmental biology 285, 533-544.

Kuschert, S., Rowitch, D.H., Haenig, B., McMahon, A.P., Kispert, A., 2001. Characterization of Pax-2 regulatory sequences that direct transgene expression in the Wolffian duct and its derivatives. Developmental biology 229, 128-140.

Lanctot, C., Moreau, A., Chamberland, M., Tremblay, M.L., Drouin, J., 1999. Hindlimb patterning and mandible development require the Ptx1 gene. Development 126, 1805-1810.

Levine, M., 2010. Transcriptional enhancers in animal development and evolution. Current biology : CB 20, R754-763.

Lin, C., Yin, Y., Bell, S.M., Veith, G.M., Chen, H., Huh, S.H., Ornitz, D.M., Ma, L., 2013. Delineating a conserved genetic cassette promoting outgrowth of body appendages. PLoS genetics 9, e1003231.

Lin, C., Yin, Y., Veith, G.M., Fisher, A.V., Long, F., Ma, L., 2009. Temporal and spatial dissection of Shh signaling in genital tubercle development. Development 136, 3959-3967.

Logan, M., Simon, H.G., Tabin, C., 1998. Differential regulation of T-box and homeobox transcription factors suggests roles in controlling chick limb-type identity. Development 125, 2825-2835.

Loomis, C.A., Kimmel, R.A., Tong, C.X., Michaud, J., Joyner, A.L., 1998. Analysis of the genetic pathway leading to formation of ectopic apical ectodermal ridges in mouse Engrailed-1 mutant limbs. Development 125, 1137-1148.

Lopez-Martinez, A., Chang, D.T., Chiang, C., Porter, J.A., Ros, M.A., Simandl, B.K., Beachy, P.A., Fallon, J.F., 1995. Limb-patterning activity and restricted posterior localization of the amino-terminal product of Sonic hedgehog cleavage. Current biology : CB 5, 791-796.

Lufkin, T., Mark, M., Hart, C.P., Dolle, P., LeMeur, M., Chambon, P., 1992. Homeotic transformation of the occipital bones of the skull by ectopic expression of a homeobox gene. Nature 359, 835-841.

MacCabe, J.A., Errick, J., Saunders, J.W., Jr., 1974. Ectodermal control of the dorsoventral axis in the leg bud of the chick embryo. Developmental biology 39, 69-82.

Malashichev, Y., Borkhvardt, V., Christ, B., Scaal, M., 2005. Differential regulation of avian pelvic girdle development by the limb field ectoderm. Anatomy and embryology 210, 187-197.

Malashichev, Y., Christ, B., Prols, F., 2008. Avian pelvis originates from lateral plate mesoderm and its development requires signals from both ectoderm and paraxial mesoderm. Cell and tissue research 331, 595-604.

Menke, D.B., Guenther, C., Kingsley, D.M., 2008. Dual hindlimb control elements in the Tbx4 gene and region-specific control of bone size in vertebrate limbs. Development 135, 2543-2553.

Min, H.S., Danilenko, D.M., Scully, S.A., Bolon, B., Ring, B.D., Tarpley, J.E., DeRose, M., Simonet, W.S., 1998. Fgf-10 is required for both limb and lung development and exhibits striking functional similarity to Drosophila branchless. Genes & development 12, 3156-3161.

Minelli, A., 2002. Homology, limbs, and genitalia. Evolution & development 4, 127-132.

Minguillon, C., Del Buono, J., Logan, M.P., 2005. Tbx5 and Tbx4 are not sufficient to determine limb-specific morphologies but have common roles in initiating limb outgrowth. Developmental cell 8, 75-84.

Minguillon, C., Gibson-Brown, J.J., Logan, M.P., 2009. Tbx4/5 gene duplication and the origin of vertebrate paired appendages. Proceedings of the National Academy of Sciences of the United States of America 106, 21726-21730.

Minguillon, C., Nishimoto, S., Wood, S., Vendrell, E., Gibson-Brown, J.J., Logan, M.P., 2012. Hox genes regulate the onset of Tbx5 expression in the forelimb. Development 139, 3180-3188.

Miyagawa, S., Moon, A., Haraguchi, R., Inoue, C., Harada, M., Nakahara, C., Suzuki, K., Matsumaru, D., Kaneko, T., Matsuo, I., Yang, L., Taketo, M.M., Iguchi, T., Evans, S.M., Yamada, G., 2009. Dosage-dependent hedgehog signals integrated with Wnt/beta-catenin signaling regulate external genitalia formation as an appendicular program. Development 136, 3969-3978.

Miyamoto, N., Yoshida, M., Kuratani, S., Matsuo, I., Aizawa, S., 1997. Defects of urogenital development in mice lacking Emx2. Development 124, 1653-1664.

Morgan, E.A., 2003. Loss of Bmp7 and Fgf8 signaling in Hoxa13-mutant mice causes hypospadia. Development 130, 3095-3109.

Murakami, R., Mizuno, T., 1986. Proximal-distal sequence of development of the skeletal tissues in the penis of rat and the inductive effect of epithelium. Journal of embryology and experimental morphology 92, 133-143.

Naiche, L.A., 2003. Loss of Tbx4 blocks hindlimb development and affects vascularization and fusion of the allantois. Development 130, 2681-2693.

Naiche, L.A., Arora, R., Kania, A., Lewandoski, M., Papaioannou, V.E., 2011. Identity and fate of Tbx4-expressing cells reveal developmental cell fate decisions in the allantois, limb, and external genitalia. Developmental dynamics : an official publication of the American Association of Anatomists 240, 2290-2300.

Naiche, L.A., Papaioannou, V.E., 2007. Tbx4 is not required for hindlimb identity or post-bud hindlimb outgrowth. Development 134, 93-103.

Nakada, Y., Parab, P., Simmons, A., Omer-Abdalla, A., Johnson, J.E., 2004. Separable enhancer sequences regulate the expression of the neural bHLH transcription factor neurogenin 1. Developmental biology 271, 479-487.

Nelson, C.E., Morgan, B.A., Burke, A.C., Laufer, E., DiMambro, E., Murtaugh, L.C., Gonzales, E., Tessarollo, L., Parada, L.F., Tabin, C., 1996. Analysis of Hox gene expression in the chick limb bud. Development 122, 1449-1466.

Nissim, S., Hasso, S.M., Fallon, J.F., Tabin, C.J., 2006. Regulation of Gremlin expression in the posterior limb bud. Developmental biology 299, 12-21.

Niswander, L., 2003. Pattern formation: old models out on a limb. Nature reviews. Genetics 4, 133-143.

Niswander, L., Jeffrey, S., Martin, G.R., Tickle, C., 1994. A positive feedback loop coordinates growth and patterning in the vertebrate limb. Nature 371, 609-612.

Niswander, L., Tickle, C., Vogel, A., Booth, I., Martin, G.R., 1993. FGF-4 replaces the apical ectodermal ridge and directs outgrowth and patterning of the limb. Cell 75, 579-587.

Nobrega, M.A., Zhu, Y., Plajzer-Frick, I., Afzal, V., Rubin, E.M., 2004. Megabase deletions of gene deserts result in viable mice. Nature 431, 988-993.

Ohuchi, H., Nakagawa, T., Yamauchi, M., Ohata, T., Yoshioka, H., Kuwana, T., Mima, T., Mikawa, T., Nohno, T., Noji, S., 1995. An Additional Limb Can Be Induced from the Flank of the Chick-Embryo by Fgf4. Biochem Bioph Res Co 209, 809-816.

Ohuchi, H., Takeuchi, J., Yoshioka, H., Ishimaru, Y., Ogura, K., Takahashi, N., Ogura, T., Noji, S., 1998. Correlation of wing-leg identity in ectopic FGF-induced chimeric limbs with the differential expression of chick Tbx5 and Tbx4. Development 125, 51-60.

Panman, L., Drenth, T., Tewelscher, P., Zuniga, A., Zeller, R., 2005. Genetic interaction of Gli3 and Alx4 during limb development. The International journal of developmental biology 49, 443-448.

Papaioannou, V.E., 2001. T-box genes in development: from hydra to humans. International review of cytology 207, 1-70.

Pappu, K.S., Ostrin, E.J., Middlebrooks, B.W., Sili, B.T., Chen, R., Atkins, M.R., Gibbs, R., Mardon, G., 2005. Dual regulation and redundant function of two eye-specific enhancers of the Drosophila retinal determination gene dachshund. Development 132, 2895-2905.

Parr, B.A., McMahon, A.P., 1995. Dorsalizing signal Wnt-7a required for normal polarity of D-V and A-P axes of mouse limb. Nature 374, 350-353.

Pedersen, A., Skjong, C., Shawlot, W., 2005. Lim 1 is required for nephric duct extension and ureteric bud morphogenesis. Developmental biology 288, 571-581.

Perriton, C.L., Powles, N., Chiang, C., Maconochie, M.K., Cohn, M.J., 2002. Sonic hedgehog signaling from the urethral epithelium controls external genital development. Developmental biology 247, 26-46.

Perry, M.W., Boettiger, A.N., Bothma, J.P., Levine, M., 2010. Shadow enhancers foster robustness of Drosophila gastrulation. Current biology : CB 20, 1562-1567.

Peterson, R.L., Jacobs, D.F., Awgulewitsch, A., 1992. Hox-3.6: isolation and characterization of a new murine homeobox gene located in the 5' region of the Hox-3 cluster. Mechanisms of development 37, 151-166.

Plageman, T.F., Jr., Yutzey, K.E., 2005. T-box genes and heart development: putting the "T" in heart. Developmental dynamics : an official publication of the American Association of Anatomists 232, 11-20.

Pomikal, C., Streicher, J., 2010. 4D-analysis of early pelvic girdle development in the mouse (Mus musculus). Journal of morphology 271, 116-126.

Qu, S., Niswender, K.D., Ji, Q., van der Meer, R., Keeney, D., Magnuson, M.A., Wisdom, R., 1997. Polydactyly and ectopic ZPA formation in Alx-4 mutant mice. Development 124, 3999-4008.

Riddle, R.D., Johnson, R.L., Laufer, E., Tabin, C., 1993. Sonic hedgehog mediates the polarizing activity of the ZPA. Cell 75, 1401-1416.

Riddle, R.D., Nelson, C., Tsuchida, T., Jessell, T.M., Tabin, C., 1995. Induction of the LIM Homeobox Gene Lmx7 by WNT7a Establishes Dorsoventral Pattern in the Vertebrate Limb. Cell 83, 631-640.

Rodriguez-Esteban, C., Schwabe, J.W., De La Pena, J., Foys, B., Eshelman, B., Izpisua Belmonte, J.C., 1997. Radical fringe positions the apical ectodermal ridge at the dorsoventral boundary of the vertebrate limb. Nature 386, 360-366.

Rodriguez-Esteban, C., Tsukui, T., Yonei, S., Magallon, J., Tamura, K., Izpisua Belmonte, J.C., 1999. The T-box genes Tbx4 and Tbx5 regulate limb outgrowth and identity. Nature 398, 814-818.

Rosa-Molinar, E., Burke, A.C., 2002. Starting from fins: parallelism in the evolution of limbs and genitalia: the fin-to-genitalia transition. Evolution & development 4, 124-126.

Ruvinsky, I., Oates, A.C., Silver, L.M., Ho, R.K., 2000. The evolution of paired appendages in vertebrates: T-box genes in the zebrafish. Development genes and evolution 210, 82-91.

Saunders, J.W., Jr., 1948. The proximo-distal sequence of origin of the parts of the chick wing and the role of the ectoderm. The Journal of experimental zoology 108, 363-403.

Saunders, J.W.a.G., M. T. Saunders, 1968. Ectodermal-mesenchymal interactions in the origin of limb symmetry. Mesenchymal-Epithelial Interactions (ed. R. Fleichmeyer and R. E. Billingham), pp. 78-97.

Schaffner, G., Schirm, S., Mullerbaden, B., Weber, F., Schaffner, W., 1988. Redundancy of Information in Enhancers as a Principle of Mammalian Transcription Control. J Mol Biol 201, 81-90.

Seifert, A.W., Zheng, Z., Ormerod, B.K., Cohn, M.J., 2010. Sonic hedgehog controls growth of external genitalia by regulating cell cycle kinetics. Nature communications 1, 23.

Shou, S., Scott, V., Reed, C., Hitzemann, R., Stadler, H.S., 2005. Transcriptome analysis of the murine forelimb and hindlimb autopod. Developmental dynamics : an official publication of the American Association of Anatomists 234, 74-89.

Spitz, F., Gonzalez, F., Duboule, D., 2003. A global control region defines a chromosomal regulatory landscape containing the HoxD cluster. Cell 113, 405-417.

Stopper, G.F., Wagner, G.P., 2005. Of chicken wings and frog legs: a smorgasbord of evolutionary variation in mechanisms of tetrapod limb development. Developmental biology 288, 21-39.

Sun, X., Lewandoski, M., Meyers, E.N., Liu, Y.H., Maxson, R.E., Jr., Martin, G.R., 2000. Conditional inactivation of Fgf4 reveals complexity of signalling during limb bud development. Nat Genet 25, 83-86.

Sun, X., Mariani, F.V., Martin, G.R., 2002. Functions of FGF signalling from the apical ectodermal ridge in limb development. Nature 418, 501-508.

Suzuki, K., Bachiller, D., Chen, Y.P., Kamikawa, M., Ogi, H., Haraguchi, R., Ogino, Y., Minami, Y., Mishina, Y., Ahn, K., Crenshaw, E.B., 3rd, Yamada, G., 2003. Regulation of outgrowth and apoptosis for the terminal appendage: external genitalia development by concerted actions of BMP signaling [corrected]. Development 130, 6209-6220.

Suzuki, T., 2013. How is digit identity determined during limb development? Development, growth & differentiation 55, 130-138.

Szeto, D.P., Rodriguez-Esteban, C., Ryan, A.K., O'Connell, S.M., Liu, F., Kioussi, C., Gleiberman, A.S., Izpisua-Belmonte, J.C., Rosenfeld, M.G., 1999. Role of the Bicoid-related homeodomain factor Pitx1 in specifying hindlimb morphogenesis and pituitary development. Genes Dev 13, 484-494.

Takabatake, Y., Takabatake, T., Takeshima, K., 2000. Conserved and divergent expression of T-box genes Tbx2-Tbx5 in Xenopus. Mechanisms of development 91, 433-437.

Takahashi, M., Tamura, K., Buscher, D., Masuya, H., Yonei-Tamura, S., Matsumoto, K., Naitoh-Matsuo, M., Takeuchi, J., Ogura, K., Shiroishi, T., Ogura, T., Izpisua Belmonte, J.C., 1998. The role of Alx-4 in the establishment of anteroposterior polarity during vertebrate limb development. Development 125, 4417-4425.

Takeuchi, J.K., 2003. Tbx5 and Tbx4 trigger limb initiation through activation of the Wnt/Fgf signaling cascade. Development 130, 2729-2739.

Takeuchi, J.K., Koshiba-Takeuchi, K., Matsumoto, K., Vogel-Hopker, A., Naitoh-Matsuo, M., Ogura, K., Takahashi, N., Yasuda, K., Ogura, T., 1999. Tbx5 and Tbx4 genes determine the wing/leg identity of limb buds. Nature 398, 810-814.

Tanaka, M., Munsterberg, A., Anderson, W.G., Prescott, A.R., Hazon, N., Tickle, C., 2002. Fin development in a cartilaginous fish and the origin of vertebrate limbs. Nature 416, 527-531.

Tanaka, M., Tamura, K., Noji, S., Nohno, T., Ide, H., 1997. Induction of additional limb at the dorsal-ventral boundary of a chick embryo. Developmental biology 182, 191-203.

Tanaka, M., Tickle, C., 2004. Tbx18 and boundary formation in chick somite and wing development. Developmental biology 268, 470-480.

Tarchini, B., Duboule, D., 2006. Control of Hoxd genes' collinearity during early limb development. Developmental cell 10, 93-103.

Tickle, C., Summerbell, D., Wolpert, L., 1975. Positional signalling and specification of digits in chick limb morphogenesis. Nature 254, 199-202.

Torres, M., Gomez-Pardo, E., Dressler, G.R., Gruss, P., 1995. Pax-2 controls multiple steps of urogenital development. Development 121, 4057-4065.

Towers, M., Mahood, R., Yin, Y., Tickle, C., 2008. Integration of growth and specification in chick wing digit-patterning. Nature 452, 882-886.

Towers, M., Tickle, C., 2009. Growing models of vertebrate limb development. Development 136, 179-190.

Valasek, P., Evans, D.J., Maina, F., Grim, M., Patel, K., 2005. A dual fate of the hindlimb muscle mass: cloacal/perineal musculature develops from leg muscle cells. Development 132, 447-458.

Verhofstede, C., Fransen, K., Marissens, D., Verhelst, R., van der Groen, G., Lauwers, S., Zissis, G., Plum, J., 1996. Isolation of HIV-1 RNA from plasma: evaluation of eight different extraction methods. Journal of virological methods 60, 155-159.

Visel, A., Blow, M.J., Li, Z., Zhang, T., Akiyama, J.A., Holt, A., Plajzer-Frick, I., Shoukry, M., Wright, C., Chen, F., Afzal, V., Ren, B., Rubin, E.M., Pennacchio, L.A., 2009. ChIP-seq accurately predicts tissue-specific activity of enhancers. Nature 457, 854-858.

Warot, X., Fromental-Ramain, C., Fraulob, V., Chambon, P., Dolle, P., 1997. Gene dosage-dependent effects of the Hoxa-13 and Hoxd-13 mutations on morphogenesis of the terminal parts of the digestive and urogenital tracts. Development 124, 4781-4791.

Wright, E., Hargrave, M.R., Christiansen, J., Cooper, L., Kun, J., Evans, T., Gangadharan, U., Greenfield, A., Koopman, P., 1995. The Sry-related gene Sox9 is expressed during chondrogenesis in mouse embryos. Nat Genet 9, 15-20.

Xiong, N., Kang, C., Raulet, D.H., 2002. Redundant and unique roles of two enhancer elements in the TCRgamma locus in gene regulation and gammadelta T cell development. Immunity 16, 453-463.

Zakany, J., Duboule, D., 2007. The role of Hox genes during vertebrate limb development. Current opinion in genetics & development 17, 359-366.

Zakany, J., Fromental-Ramain, C., Warot, X., Duboule, D., 1997. Regulation of number and size of digits by posterior Hox genes: a dose-dependent mechanism with potential evolutionary implications. Proceedings of the National Academy of Sciences of the United States of America 94, 13695-13700.

Zakany, J., Kmita, M., Duboule, D., 2004. A dual role for Hox genes in limb anterior-posterior asymmetry. Science 304, 1669-1672.

Zhou, X., Sigmund, C.D., 2008. Chorionic enhancer is dispensable for regulated expression of the human renin gene. American journal of physiology. Regulatory, integrative and comparative physiology 294, R279-287.

Zhu, J., Nakamura, E., Nguyen, M.T., Bao, X., Akiyama, H., Mackem, S., 2008. Uncoupling Sonic hedgehog control of pattern and expansion of the developing limb bud. Developmental cell 14, 624-632.

Zuniga, A., Haramis, A.P., McMahon, A.P., Zeller, R., 1999. Signal relay by BMP antagonism controls the SHH/FGF4 feedback loop in vertebrate limb buds. Nature 401, 598-602.

Chapter 2

TBX4 LIMB EXPRESSION IS MODULATED BY TWO ENHANCERS WITH DISTINCT FUNCTIONS[1]

[1] Alexandra G. Mihala, Sungdae Park and Douglas Menke. To be submitted to Developmental Biology.

Abstract

The exact spatio-temporal expression of genes is often under the regulation of multiple *cis*-regulatory elements, also called enhancers, which have largely been considered to be functionally redundant (Barolo, 2012; Hong et al., 2008). Recently this view has come under question, in part due to transgenic work (Abbasi et al., 2010; Frankel et al., 2010; Frankel et al., 2011). However, few functional studies have been performed to understand the roles of enhancers in their endogenous context. To better understand enhancer redundancy in mice, we have focused on the locus of *Tbx4*, a transcription factor necessary for hindlimb development that has been shown to be regulated by two distinct limb enhancers with overlapping patterns of activity: HLEA and HLEB (Naiche, 2003; Menke et al., 2008). Menke and colleagues have shown that knocking out HLEA in mice results in a significant decrease in *Tbx4* expression, a reduction in the size of hindlimb long bones, and other abnormal phenotypes. In comparison to the HLEA, HLEB shows a higher degree of sequence conservation (conserved from fish to mammals), but a fairly similar pattern of activity during early hindlimb development. In order to understand whether or not this second enhancer is redundant for proper *Tbx4* expression, we deleted HLEB from the *Tbx4* locus. We observed that hindlimb buds of different embryonic stages display dynamic patterns of *Tbx4* expression that vary between the HLEA and HLEB knockout mutants, suggesting the two enhancers may have distinct roles in modulating *Tbx4* expression. Indeed, careful measurements of adult mutant mice show significant alterations in the size of the hindlimb bones and the pelvic girdle that are strikingly different between the two mutants. We also show that the hindlimb expression of *Alx4*, a transcription factor known to be involved in the proper patterning of the pubic bone, is

significantly altered by the deletion of HLEB but not HLEA. Taken together this data suggests that HLEA and HLEB have distinct activities and non redundant functions in the developing hindlimb.

Introduction

Regulatory elements, or enhancers, are important non-coding sequences involved in the proper spatio-temporal transcriptional regulation of gene expression (Levine, 2010). Many genes have been shown to be under the regulation of multiple enhancer elements, with a subset of the enhancers for a given gene often exhibiting similar or overlapping patterns of activity within particular expression domains. Some investigators have suggested that gene enhancers with overlapping activity may be largely redundant (Ahituv et al., 2007; Barolo, 2012; Degenhardt et al., 2010; Jeong et al., 2006; Nakada et al., 2004; Nobrega et al., 2004; Pappu et al., 2005; Schaffner et al., 1988; Zhou and Sigmund, 2008).

Recently, the concept of simple enhancer redundancy has come under question. For example, research on *Drosophila* has shown that many such "redundant" enhancers may serve an important developmental function in buffering gene expression during adverse conditions such as extreme temperatures (Frankel et al., 2010; Hobert, 2010; Perry et al., 2010). When Stern's group deleted two regulatory elements of *svb*, they noticed minimal phenotypes in the trichomes of Drosophila embryos raised at optimum lab temperatures (25^{o}C). However, when the same embryos were allowed to develop under extreme temperatures (17^{o}C or 32^{o}C), they developed significantly fewer trichomes than controls. This observation indicates that some enhancers may serve a function in buffering gene expression during sub-optimal conditions (Frankel et al., 2010). A similar observation was made by Levine's group. When they performed

rescue experiments for a *Drosophila* line deficient in *snail* using BAC constructs, they noted that either one of the two *snail* enhancers is sufficient to rescue the *snail*-specific gastrulation phenotype under normal laboratory temperature. In contrast, when grown under suboptimal temperatures, or placed on a sensitive genetic background, full rescue could not be attained (Perry et al., 2010). Taken together, this data indicates that multiple enhancers may not be redundant despite their overlapping patterns of activity. Enhancers with overlapping activity have also recently been shown to be very important for driving precise spatial patterns of gene expression. Sequence alterations within these secondary enhancers results in changes in expression patterns between closely related species (Abbasi et al., 2010; Cande et al., 2009; Frankel et al., 2011).

Although previous experiments have been designed to elucidate potential roles that multiple enhancers of a gene may have in development and evolution, very few have addressed this question through functional investigations of “redundant” enhancers in their endogenous context (Barolo, 2012). Due to many logistical constraints, the most frequent means of understanding enhancer function is through reporter assays (Degenhardt et al., 2010; Jeong et al., 2006; Nakada et al., 2004) or though transgenic constructs harboring deletions of such enhancers (Zhou and Sigmund, 2008). Some groups adressed the question of enhancer function by deleting the endogenous regulatory element and scoring phenotipic changes (Cretekos et al., 2008; Epner et al., 1998). Certain enhancer deletions show no observable phenotype (Ahituv et al., 2007; Cretekos et al., 2008) while others do not fully recapitulate the phenotypes of the gene knockout suggesting other enhacers are involved in generating full and faithful gene expression (Epner et al., 1998; Juan and Ruddle, 2003). Despite these studies, very little is

known about the alterations in gene expression and morphological phenotypes associated with the deletion of multiple endogeous enhancers of a certain gene.

To better adress the very important question of enhancer redundancy we have chosen to focus on the *Tbx4* gene which codes for a T-box transcription factor necessary for hindlimb outgrowth and development. The *Tbx4* locus has been shown to be under the regualtion of two independent enhancers, hindlimb enhancer A (HLEA) and hindlimb enhancer B (HLEB) (Menke et al., 2008). Via reporter assays, these two hindlimb enhancers were shown to drive robust and overlapping LacZ expression during embryonic hindlimb development in mice. Menke and colleagues only characterized the functional role of HLEA in modulating *Tbx4* expression in the hindlimb. When this particular enhancer is deleted from the mouse genome, hindlimb bones are significantly reduced in size and characteristic malformations of the patella and ankle bones are observed (Menke et al., 2008).

At first glance it may be tempting to assume that, given their overlapping hindlimb expression profiles, HLEA and HLEB serve as redundant limb enhancers of *Tbx4*. However, because the role of Tbx4 in patterning the bones of the mouse hindlimb is largely complete by E11.5, this suggests that the embryonic limb bud at this time point is not homogenous but quite complex (Hasson et al., 2010; Naiche and Papaioannou, 2007). Therefore, although HLEA and HLEB are both hindlimb enhancers of *Tbx4* with overlapping activity patterns at E9.5-E11.5, it is possible that enough spatio-temporal differences exist between their activities within the developing hindlimb, that they make different contributions to the formation of particular hindlimb bones or soft tissues such as the muscles. To test for enhancer redundancy in this system, we deleted the endogenous copy of the HLEB enhancer in mice and performed careful

phenotypic measurements of the mutants. Here we show that the relative importance of HLEA and HLEB in driving *Tbx4* expression differs across developmental stages and within different compartments of the hindlimbs. Furthermore, the two mutants display distinct skeletal phenotypes, suggesting that these two enhancers serve non-redundant functions within the developing hindlimb bud.

Results

Deletions of either HLEA or HLEB have distinct phenotypic consequences

First, we measured the bones of the hindlimbs of the same adult mice (Fig 2.3). As Menke and colleagues previously reported, the length of the femur, patella and the width of the tibia are all reduced significantly in $Tbx4^{\Delta HLEA/\Delta HLEA}$ mutants as compared to wild type (3.2%, 16.1% and 18.3%, respectively) (Figure 2.3, A). In contrast, $Tbx4^{\Delta HLEB/\Delta HLEB}$ mutants show no significant difference in the measurements of the patella or the tibia width, although the femur is reduced by a similar magnitude as in $Tbx4^{\Delta HLEA/\Delta HLEA}$ mutants (2%) (Fig 2.3, B). Previously unreported, the length of the tibia is also reduced in $Tbx4^{\Delta HLEA/\Delta HLEA}$ mutants by 1.7%, in contrast in our $Tbx4^{\Delta HLEB/\Delta HLEB}$ mutants the tibia is longer by 1.6% compared to controls (Fig 2.3, B). Overall, only the femur length is impacted in the same manner and with a similar magnitude in both homozygous mutant mice. We detected patella size and tibia width reductions as well as fusions in the ankle bones only in $Tbx4^{\Delta HLEA/\Delta HLEA}$ mutants (Fig2.3). In summary only the femur, the proximal long bone of the hindlimb, is affected in equal magnitude in both mutants, while the more distant bones of the hindlimb display different phenotypes between ΔHLEA and ΔHLEB homozygotes.

The pelvic bone harbored alterations in bone size as well. Although the total length of the pelvis was not affected (Fig 2.4,C), significant alterations occurred throughout the posterior part of the pelvis in *Tbx4*$^{\Delta HLEB/\Delta HLEB}$ mutants. The posterior pelvis is made up of the ischium, the pubis and the bridge between them called ischiopubic ramus (IPR) (Fig 2.4 A). Compared to wild type, the pubic bone was reduced by 7.7% in *Tbx4*$^{\Delta HLEB/\Delta HLEB}$ mutants, while in ΔHLEA homozygotes it was not significantly different from wild type. The ischiopubic ramus is also affected in homozygous mutants. Somewhat surprisingly this structure is thicker by 15.9% in *Tbx4*$^{\Delta HLEA/\Delta HLEA}$ mice while in *Tbx4*$^{\Delta HLEB/\Delta HLEB}$ animals it is reduced by 22.7%. Although the ischium length is not affected in either mutant, the anterior width of this bone is altered in *Tbx4*$^{\Delta HLEA/\Delta HLEA}$ showing a 4.3% increase, while the posterior width of the ischium is altered only in *Tbx4*$^{\Delta HLEB/\Delta HLEB}$ mutants showing a decrease of 10.5% (Fig 2.4, C). This reveals that alterations in the final conformation of the pubic bone differ between *Tbx4*$^{\Delta HLEA/\Delta HLEA}$ and *Tbx4*$^{\Delta HLEB/\Delta HLEB}$ mice.

To further investigate these conformational changes, we measured the area of each bone of the posterior pelvis (Fig 2.5). The total area of all the posterior structures (ischium, pubis, IPR) is increased by about 6% in *ΔHLEA* homozygous mutants, but reduced by the same magnitude in *Tbx4*$^{\Delta HLEB/\Delta HLEB}$ mutants. The area of the pubis is significantly altered only in ΔHLEB homozygotes (reduced by 5.9%), while the ischium bone is not significantly altered in either mutant. The area of the ischiopubic ramus is significantly altered in both mutants, being increased by 17.2% in ΔHLEA homozygotes and reduced by 12.2% in *Tbx4*$^{\Delta HLEB/\Delta HLEB}$ mutants (Figure 2.5, B). Together this data indicates that deleting either one of the *Tbx4* hindlimb

enhancers causes a change in the total area of the posterior pelvis, but this change is not evenly distributed amongst the three structures, with the IPR being the most affected.

HLEA and HLEB serve distinct spatio-temporal functions within the developing limb

Having found significant alterations in many bones of the hindlimb in these mutants, we wondered if there were associated alterations in *Tbx4* expression in early embryonic stages of development. *In situ* hybridization was performed on homozygous mutant embryos (*Tbx4*$^{\Delta HLEA/\Delta HLEA}$ and *Tbx4*$^{\Delta HLEB/\Delta HLEB}$) as well as wild type littermates from stages E9.5 to E11.5 (Fig 2.6). At E9.5 expression levels of *Tbx4* from both *Tbx4*$^{\Delta HLEA/\Delta HLEA}$ and *Tbx4*$^{\Delta HLEB/\Delta HLEB}$ hindlimb fields are reduced compared to wild type. At E10.5 the reduction seen by *in situ* hybridization in *Tbx4*$^{\Delta HLEB/\Delta HLEB}$ mutant embryos is different from what can be seen in ΔHLEA homozygotes, there being a compartment within the proximal area of the hindlimb with diminished levels of expression compared to the distal portion that has comparable staining with wild type (Fig 2.6 compare D,E,F red arrows). However, the reduction of gene expression seen in *Tbx4*$^{\Delta HLEA/\Delta HLEA}$ embryos is not uniform either, exhibiting a strong anterior reduction not seen in the posterior part of the hindlimb (Fig 2.6, E). This anterior-posterior bias is maintained in E11.5 *Tbx4*$^{\Delta HLEA/\Delta HLEA}$ mice (Fig 2.6, G,H).

In order to quantify any *Tbx4* expression differences observed between the two mutants alleles and the wildtype *Tbx4* allele, we performed allele-specific expression on RNA extracted from dissected tissues (Fig 2.7). By allele specific expression (ASE), the gene expression driven by the ΔHLEA allele in the E9.5 hindlimb field is reduced to 85% of wild type allele levels (Fig2.7, A). In comparison, the ΔHLEB allele reduces *Tbx4* expression to by a slightly greater magnitude (74%) at this time point (Fig2.7,A). At E10.5, *Tbx4* expression from the ΔHLEA allele from the

anterior hindlimb is reduced to only 11% that of wild type while in the posterior part of the limb expression is reduced to 48% (Fig 2.7,A). In contrast, the pattern of *Tbx4* expression in the hindlimbs of $Tbx4^{\Delta HLEB/\Delta HLEB}$ embryos does not display an anterior-posterior bias but as seen from *in situ* experiments, in these mutants at E10.5 there is a proximal pocket that displays a lowered level of expression. This particular compartment was dissected as part of a larger tissue (pelvic field) to be used for ASE, therefore the reduction in this particular pocket is not fully represented in the ASE due to the *Tbx4* expression in surrounding tissue. Together this data shows that the relative importance of HLEA and HLEB in driving gene expression in different compartments of the limb differs and these differences may underlie the different phenotypes observed in the bone size of these mutants.

HLEA and HLEB are important for the proper development of distal and proximal skeletal elements, respectively

We wanted to more stringently assess the functional impact of deleting HLEA or HLEB. In order to do so, we obtained *Tbx4* null mice that lack exon 5, coding for a non-functional transcript (Naiche, 2003). We then bred these mice to either *ΔHLEA/ΔHLEA* or *ΔHLEB/ΔHLEB* mutant mice to obtain $Tbx4^{\Delta HLEA/-}$ or $Tbx4^{\Delta HLEB/-}$ mice, respectively. We then measured the bones of the hindlimbs of the newly generated adult mice to uncover any other phenotypes. The morphology of the leg bones of $Tbx4^{\Delta HLEB/-}$ adult mice looked grossly normal, in contrast $Tbx4^{\Delta HLEA/-}$ mice showed clear abnormalities in the distal part of the hindlimb (Fig 2.8). The tibia and fibula failed to fuse properly in 19 out of a total of 24 $Tbx4^{\Delta HLEA/-}$ hindlimbs. Moreover, all 24 $Tbx4^{\Delta HLEA/-}$ ankles showed extensive fusion of tarsal bones. In addition, fusion of the metatarsals of the first two digits was apparent in 4 out of 24 hindlimbs (Fig 2.8).

Measurements of each individual bone showed a similar trend as reported in homozygous mutants (Table 2.1).

In contrast to the apparently normal distal hindlimb structures observed in *Tbx4*$^{\Delta HLEB/-}$ mice, all mice of this genotype showed substantial abnormalities in the posterior part of their pelvic bones. This posterior region of the hip bone is also the most impacted one in *ΔHLEA* and *ΔHLEB* homozygous mutants. In *Tbx4*$^{\Delta HLEB/-}$ animals the angle between the ischium and the pubis is reduced by 44% compared to *Tbx4*$^{+/-}$ controls making the pelvic bone take on a "squished" appearance (Figure 2.8, Table 2.1). With the exception of the ischiopubic ramus width, all other measurements of *Tbx4*$^{\Delta HLEB/-}$ pelvic bones are significantly reduced compared to controls (Table 2.1) consistent with our initial observations. In striking contrast to the severe pelvic malformations observed in *Tbx4*$^{\Delta HLEB/-}$ mice, the pelvic morphology of *Tbx4*$^{\Delta HLEA/-}$ adult mice appeared normal.

Abnormal pelvic phenotypes can be seen as early as E14.5 when the ischium and the pubic bone, normally not fused at this time, are almost entirely connected in *Tbx4*$^{\Delta HLEB/-}$ individuals (Fig 2.9, A,B). At this time point there also seems to be a smaller angle between these two structures that is more prominent at E15.5 (compare D to C) and even more so at E16.5 (compare F to E).

The expression patterns of Alx4 and Bmp4 are disrupted in Tbx4$^{\Delta HLEB/-}$ *mutant embryos*

We went on to try to identify potential genes downstream of Tbx4 that may be involved in the severe pelvic phenotypes we observed in *Tbx4*$^{\Delta HLEB/-}$ mice. We performed quantitative RT-PCR on E10.5 hindlimb buds as well as the hindlimb somatopleure of

Tbx4$^{\Delta HLEA/-}$, *Tbx4*$^{\Delta HLEB/-}$, *Tbx4*$^{+/-}$ and WT embryos. We found that the expression of *Alx4* and *Alx3* in the somatopleure was shown to be statistically significantly different in *Tbx4*$^{\Delta HLEB/-}$ embryos compared to wild type, while levels detected from *Tbx4*$^{\Delta HLEA/-}$ embryos were not significantly different from control. We then performed mRNA *in situ* hybridization using probes against *Alx4, Sox9, Hoxc10* and *Bmp4*. Although by Q-RT-PCR *Hoxc10* did not show a difference between mutant and control that was statistically significant, the nature of the pelvic phenotype of *Hoxc10* knockout mutants compelled us to add it to our *in situ* assay.

Although not statistically significant, *Bmp4* was added as well due to the lowered expression seen in all three *Tbx4*$^{\Delta HLEB/-}$ biological replicates and does show a pontentially reduced level of expression in the *Tbx4*$^{\Delta HLEB/-}$ embryo compared to control. *In situ* hybridization using probes against *Sox9* and *Hoxc10* on E10.5 *Tbx4*$^{\Delta HLEA/-}$ and *Tbx4*$^{\Delta HLEB/-}$ mutant embryos yielded a pattern of gene expression that was identical to that of controls. In contrast, *Tbx4*$^{\Delta HLEA/-}$ and control embryos that were used with a probe against *Alx4*, showed a different pattern of expression when compared to *Tbx4*$^{\Delta HLEB/-}$. In figure 2.10, panels A and B show control and *Tbx4*$^{\Delta HLEA/-}$ hindlimbs of E10.25 embryos that display an anteriorly restricted domain of *Alx4* expression. However, *Tbx4*$^{\Delta HLEB/-}$ embryos of the same stage show an expanded domain of expression, well into the posterior margin of the hindlimb, a trend which is maintained at E10.5 as well.

Discussion

Tbx4 is modulated by two hindlimb regulatory elements with non overlapping functions

We wanted to compare function of the two hindlimb enhancers of *Tbx4*, A and B. To do so we generated enhancer knockout mice that lacked the endogenous B enhancer (*ΔHLEB/ΔHLEB* mice). We then compared the observable and measureable adult phenotypes of these mice to those of $Tbx4^{\Delta HLEA/\Delta HLEA}$ mice, which were previously reported (Menke et al., 2008). Our measurements of the hindlimb bones of the $Tbx4^{\Delta HLEA/\Delta HLEA}$ adult mice compared to controls where consistent with those published by Menke et al. (Menke et al., 2008). The most proximal long bone of the hindlimb, the femur, is also impacted in $Tbx4^{\Delta HLEB/\Delta HLEB}$ mutant mice, being reduced by 2% (Table 2.3). The more distal patella as well as the tibia width, although the most impacted hindlimb bones by HLEA deletion, are not significantly altered in ΔHLEB homozygotes. It seems that both enhancers are involved in the proper development of the more proximal femur, while HLEA plays a greater role in the proper development of the more distal parts of the hindlimb. These observations are supported by the different impacts each of these enhancer deletions has when put on a sensitive $Tbx4^{+/-}$ background. Hindlimbs of $Tbx4^{\Delta HLEA/-}$ are mainly impacted distally, where the tibia is grossly abnormal and fails to fuse properly with the fibula near the ankle (Fig 2.8,F).

In the homozygous mutants, the differential phenotypes caused by the deletion of either enhancer A or B within the posterior part of the pelvic girdle are approximately equal in magnitude but opposite in effect. While deleting enhancer A increased the posterior pelvic girdle area by about 6%, ΔHLEB homozygous pelves have a reduction of the same magnitude. It is not yet clear why this opposing effect is occurring, however, not all the bones of the posterior

pelvic girdle are affected in the same way in these mutants. Upon a closer look, we observed that the area of the ischium in animals homozygous for either enhancer deletion is not significantly different that of wild type controls as some linear measurements of the width initially lead us to believe. For example, although the anterior width of the ischium in ΔHLEB homozygous mutants is significantly reduced (7.2%), the length is also increased by over 5% (Table 2.4) suggesting that the effect on this bone may be due to patterning rather than cellular proliferation defects. The only structure with an affected area in ΔHLEA homozygous mutants is the IPR (Fig2.5), which shows an increase of 17.2%. In contrast, in *Tbx4*$^{\Delta HLEB/\Delta HLEB}$ mutants, not only is the IPR decreased by a significant 12.2% amount but the pubic bone also shows a reduction of 5.9%. Taken together, the data makes it clear that hindlimb enhancers A and B have different roles to play in modulating *Tbx4* expression with respect to hindlimb skeletal development.

Clues on how this differential regulation may be achieved at the molecular level came from expression analyses, both *in situ* hybridization and allele specific expression (Fig 2.6, Fig2.7) which clearly show that the relative important of each enhancer in driving *Tbx4* expression differs within different compartments of the growing hindlimb bud and across different development stages of hindlimb development. HLEA seems to drive strong gene expression in the anterior part of the limb at multiple time points, while HLEB seems to drive expression of *Tbx4* in the proximal part of the limb at E10.5. It is unclear exactly how these spatio-temporal differences interact to give rise to the observed phenotypes. There is no complete overlap in spatial pattern of expression from these two enhancers, suggesting either the existence of at least another hind limb enhancer, or synergistic interaction as suggested by

deletion of both enhancers in the BAC transgenic work reported in Menke et al. (Menke et al., 2008).

An interesting study would be a comparison between the phenotypes of each individual enhancer deletion versus phenotypes associated with the deletion of both enhancers simultaneously. Because the deletion of both enhancers from a *Tbx4* BAC transgene resulted in no detectable expression at E12.5, indicates that these enhancers may work in synergy to modulate gene expression (Menke et al., 2008). Based on this result alone, one cannot infer how the *Tbx4* limb expression of such a double mutant may behave at earlier time points, therefore exact phenotypic consequences are difficult to predict. However due to synergistic interactions, we can speculate that the morphology of such mutants may be more severe than what is observed from either $Tbx4^{\Delta HLEA/\Delta HLEA}$ or $Tbx4^{\Delta HLEB/\Delta HLEB}$ mutants.

Xiong and colleagues (Xiong et al., 2002) used the known cis-regulatory elements of the TCR Cry1 gene cluster to specifically address enhancer redundancy. They knocked out each copy of the two known endogenous enhancers for this locus and noticed some minor alterations compared to controls. Just like us, they noticed that some of these minor alterations produced by either enhancer deletion, overlapped with each other only to a small degree. For example, the total observed number of a particular cell type ($V\gamma2^{+}$) was reduced by different magnitudes in each enhancer deletion (40% compared to 25%). In our study we also noticed some overlapping phenotypes in terms of tissues affected as well as magnitude, but we also observe striking differences between the two enhancer deletion knockouts. Interestingly, Xiong and colleagues also deleted both enhancers simultaneously and noticed significant defects in Tcell development, something not previously observed in either enhancer knockout.

Taken together, this data suggests a new outlook on the term "redundant". Data gathered from transgene constructs and reporter assays limits the definition of redundancy to mean just overlapping activity. However, as we have shown, overlapping activity of enhancers does not necessarily allow us to infer that the enhancers have the same function in development. Indeed, when we delete either HLEA or HLEB, we notice that few of the phenotypes we measured are exactly the same in terms of tissue affected or the magnitude of the effect. We suggest that the term "redundant" be used more selectively to refer to quantifiable functional effects rather than visual observations based on intrinsically variable transgenic constructs.

Tbx4 may modulate ectodermal signals to the underlying mesenchyme, in part, through Alx4

Possible clues in the mechanism that *Tbx4* has on proper pelvic girdle development came from the analysis of $Tbx4^{\Delta HLEB/-}$ mutants. Their adult pelvic girdle is grossly abnormal in the posterior part just as seen in the homozygous mutants (Fig 2.8). We show that these phenotypes occur prenatally, with the first observable defects observed as early as E14.5 (Fig2.9). At this stage the pubic bone seems to form a more acute angle with the ischium to which it is joined prematurely. It was shown though 3-D reconstruction of serial histological sections that these two bones are in fact, separate at this stage and fuse later (Pomikal and Streicher, 2010). We know that the posterior part of the pelvis develops under different regulatory signals that the anterior portion that becomes the ilium. Malashichev and colleagues (Malashichev et al., 2005) showed that in chicken, if the ectoderm of the developing hind limb bud is removed before AER formation, the resulting pelvic girdle forms an ilium but ischium and posterior pelvis development is massively disrupted. This data suggests that the pelvic

phenotype observed in *Tbx4*$^{\Delta HLEB/-}$ mutants may be due to an ectodermal signal early in development that is indirectly disrupted by altered *Tbx4* expression in the underlying mesenchyme. In this case, enhancer B seems to play a crucial role because pelvic girdles of *Tbx4*$^{\Delta HLEA/-}$ mutants, although slightly reduced in size, do not display gross phenotypic abnormalities (Fig2.8). The different phenotypes observed in our homozygous mutants still may occur later in development, after AER formation, as these phenotypes are very subtle and could not have been uncovered without careful measurements. Therefore it is possible that *Tbx4* has multiple roles in pelvic development.

To elucidate a possible mechanism of how *Tbx4* may modulate development of the posterior part of the pelvic girdle we performed quantitative RT-PCR on genes known to have pelvic phenotypes when their expression is disrupted (Table 2.3). Knowing that the pelvic primordia is formed from the mesodermal part of the somatopleure (Malashichev et al., 2008; Pomikal and Streicher, 2010) we tried to carefully dissect the hindlimb somatopleure and tested this tissue as well as the limb bud at E10.5 for expression differences. Amongst the genes tested, *Alx4* shows a statistically significant increase in expression in the somatopleure tissue from *Tbx4*$^{\Delta HLEB/-}$ embryos. It was previously shown that *Alx4* null mutants display an abnormally truncated pubic bone, while *Alx4/Alx3* double mutants lack this bone entirely (Kuijper et al., 2005a). By *in situ* hybridization, we show a clear posterior expansion of the *Alx4* expression domain not seen in either control or *Tbx4*$^{\Delta HLEA/-}$ embryos (Fig 2.10). Naiche and Papaioannou (2007) showed that when *Tbx4* is conditionally knocked out, *Alx4* domain is expanded posteriorly. Normal proximal anterior *Alx4* (and *Pax1*) expression were also shown by Malaschiev et al. (2008) to be entirely down-regulated after the removal of the ectoderm and

concluded that ectodermal signals mediate expression of this gene. Given that the ectoderm is involved in the proper development of the posterior part of the pelvis, it is possible that *Tbx4* may mediate its influence over pelvic development, at least in part, through *Alx4*.

We also show that *Bmp4* expression is disrupted in the hindlimbs of E10.5 $Tbx4^{\Delta HLEB/-}$ embryos compared to controls. By *in situ* hybridization we see a posterior expansion of expression domain as well lowered levels of anterior expression (Fig2.10). However, Naiche and Papaioannou (2007) reported that *Bmp4* expression is not disrupted in their *Tbx4* conditional mutants, but they do state that there was significant medial tissue loss that may have altered these results indirectly.

Materials and methods

Knockout and transgenic mice

The transgenic mice used for this study as well as the HLEA knockout mice where previously described by Menke et al. (2008).

Skeletal preparations and measurements

The adult mouse skeletons as well as E16.5 embryos were prepared using Alcian Blue and Alizarin Red as previously described (Lufkin et al., 1992). Embryos of E15.5 and younger stages were stained using only Alcian Blue. Adult mice, as well as embryos of E16.5 and E15.5, were eviscerated and skinned before staining. Adult alcian/alizarin stained bones were carefully dissected and cleaned, placed flat on a Petri dish containing 100% glycerol and photographed next to a ruler. Images were uploaded to GIMP2 (www.gimp.org), an image analysis program that allowed us to measure the exact number of pixels between two bone features. The ruler gave us the pixel/mm ratio that was used to convert the measurements made from GIMP2 to

mm. The angle between the ischium and the pubic bone was measured in two steps using the same program. The distance measuring tool also gives the angle between the bone and the perceived horizontal of the image. This was used to calculate the angle between the ischium and the horizontal as well as the angle between the horizontal and the pubis, the two measurements giving the total angle between these bones. The area of the bones was measured using ImageJ (for an online tutorial http://rsb.info.nih.gov/ij/docs/pdfs/examples.pdf). P values were calculated using a two-tailed t-test assuming unequal variation.

Wanting to make sure that deleting HLEB from the *Tbx4* locus does not cause other effects that may skew a comprehensive comparison between the roles of HLEA and HLEB in the hindlimb. To do so, we made careful measurements of the humerus of adult mouse forelimbs that is not expected to be affected by any alterations in *Tbx4* expression. The measurement of each humerus (mm) was normalized to the respective body length (mm) and the resulting normalized values of mutant humeri were compared to those of their respective controls (Fig 2.2).

In situ hybridization

In situ hybridization was performed as previously described (Gall and Pardue, 1969) *Tbx4* riboprobe was generated as described (Menke et al., 2008), digested with NotI, and transcribed with T3 RNA polymerase. *Alx4* riboprobe was digested with EcoRI, transcribed with T7 RNA polymerase and hydrolyzed (Menke et al., 2008). *Bmp4* riboprobe was graciously donated by the lab of James Lauderdale (Kim and Lauderdale, 2008). *Sox9* and *Hoxc10* riboprobes were generated using primers already described (Peterson et al., 1992; Wright et al.,

1995), cloned into a pBS KS(+) vector, digested with BamHI and transcribed using T7 RNA polymerase.

Primers:

Sox9_F_BamHI ATATGGATCCGGGCGAGCACTCTGGGCAAT

Sox9_R_NotI ATATGCGGCCGCGCTGGGGCTCAGCTGCTCCGT

MusHoxC10_F_BamHI ATATGGATCCGAGCGCTATAACCGTAACGC

MusHoxC10_R_NotI ATATGCGGCCGCCTGAGGCGATTCCAGATGTT

Staining with BMpurple was performed for 2 hours for *Tbx4* riboprobes, 2 hours and 20 minutes for *Alx4* and 2 hours and 40 minutes for *Bmp4*.

Allele specific expression

Allele specific expression was performed as previously described (Jeong et al., 2007) Briefly, we made use of a naturally occurring SNP in the *Tbx4* transcript between C57BL/6J and DBA/2J (C->T). We bred C57BL/6J females with the enhancer deletion allele to DBA/2J males to obtain either ΔHLEA or ΔHLEB heterozygous embryos on mixed background. These embryos would have the enhancer deleted allele from one background harboring a C within the *Tbx4* transcript while the wild type allele containing the enhancer drives expression of *Tbx4* that harbors a T. We designed a FRET probe complementary to the C57BL/6J Tbx4 transcript that would bind the DBA/2J transcript with a different melting temperature due to the base pair mismatch. If both transcripts are present that would give a measureable difference in melting curves and thus we could then quantify the relative ratio of C containing transcript (driven by the mutant allele) versus the T (driven by the wild type allele) from each individual embryo. A

ratio of 1 means that both alleles are equally efficient at driving *Tbx4* expression, while a C/T ratio of less than one means the mutant allele is not as efficient.

Quantitative RT-PCR

E10.5 hindlimb somatopleure and the hindlimb buds were dissected and stored in RNA later. RNA extraction was performed using the Trizol method (Verhofstede et al., 1996). Synthesis of cDNA was performed using Thermoscientific Revertaid kit using the protocol provided. All RNA samples tested, that where meant to be compared to each other, where synthesized simultaneously using the same kit and where run on the same plate for quantitative analysis. Qualitative RT-PCR was performed using Roche Universal Probe assay. Primers used are listed in table 2.6. We performed quantitative RT-PCR on E10.5 hindlimb buds as well as the hindlimb somatopleure of the same embryos. We selected to continue our assays with the genes that showed signals which deviated most from control (WT) but that also had good CT values. A total of 14 genes were tested for gene expression in the hindlimb somatopleure (Table 2.3) and 9 for the hindlimb (Table 2.4). We continued with 6 genes that showed promising results when tested for somatopleure expression (Table 2.5). P values were calculated using a two-tailed t-test assuming equal variation.

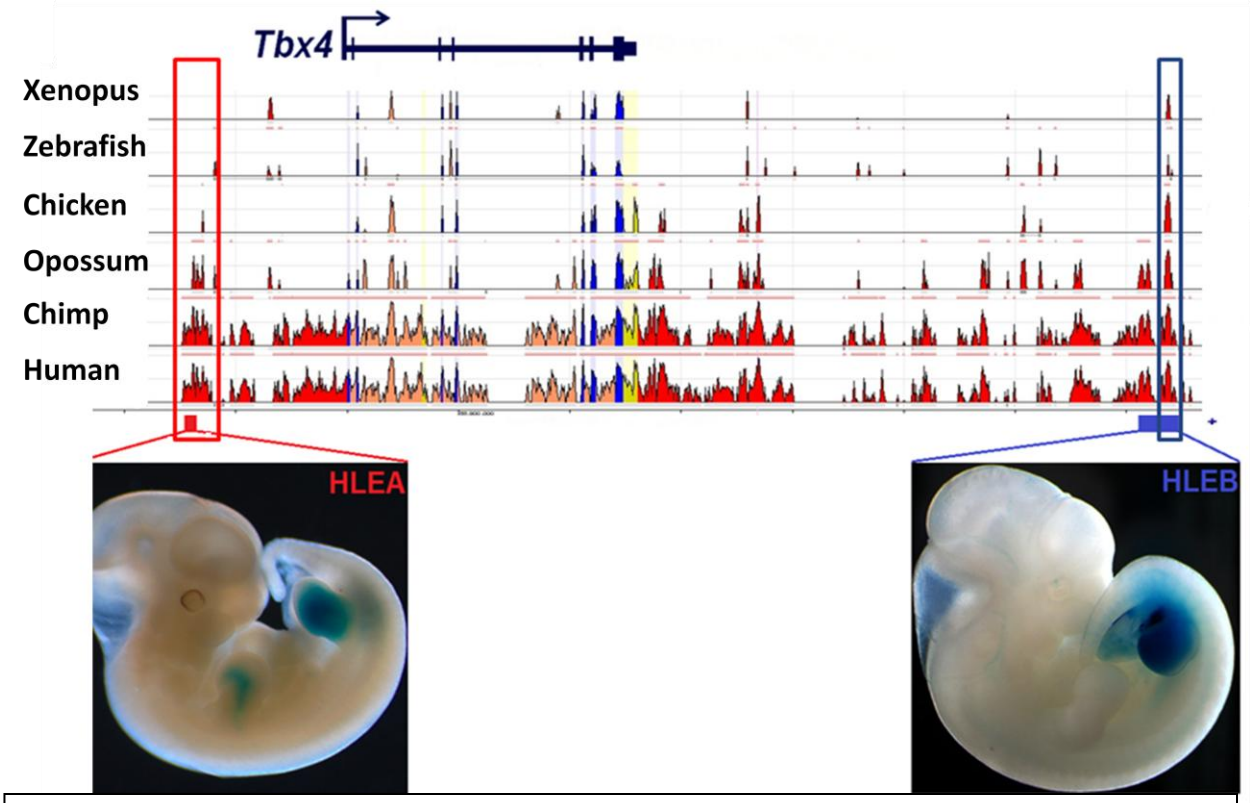


Figure 2.1 *Tbx4* limb expression is regulated by two distinct enhancers, hindlimb enhancer A (HLEA) and B (HLEB). A conservation track from VISTA (A) shows the extent of conservation of HLEA (red block) and HLEB (blue block) among vertebrates. Whole-mount *LacZ* stained E11.5 mouse embryos carrying HLEA-hsp68*LacZ* (B) and HLEA-hsp68*LacZ* (C) transgenes, a schematic of the HLEA and HLEB transgenes is represented in panel D.

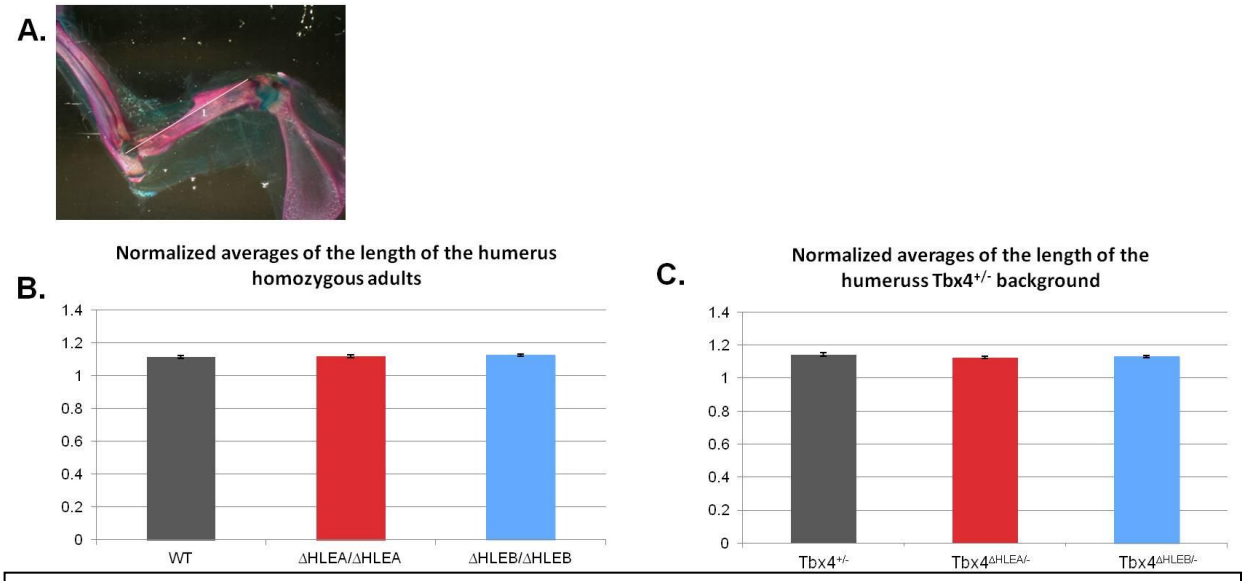
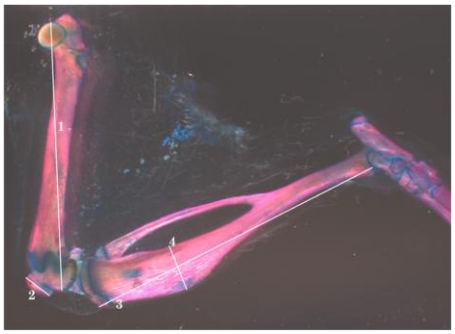


Figure 2.2. The genotypes used in this study do not show any significant alterations in the length of their respective humeri as compared to controls. A schematic (A) shows how the length of the humerus was measured. Bar graphs (B,C) showing the average length of the humerus for each genotype, normalized to body length and compared to their respective control. Error bars indicate standard error of the mean. WT (N=8); *ΔHLEA/ΔHLEA* (N=13); *ΔHLEB/ΔHLEB* (N=8); *Tbx4*$^{-/+}$ (N=11); *Tbx4*$^{\Delta HLEA/-}$ (N=13); *Tbx4*$^{\Delta HLEB/-}$ (N=14).

A.

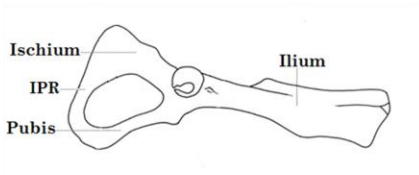


B.

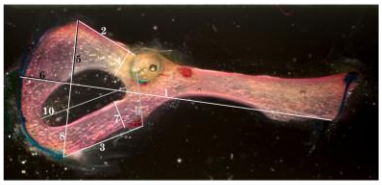
Genotype	Percent Reduction of Femur Length Compared to WT (1)	Percent Reduction of Patella Compared to WT (2)	Percent Reduction of Tibia Length Compared to WT (3)	Percent Reduction of Tibia Width Compared to WT (4)
ΔHLEA/ΔHLEA	3.2% ($p=2x10^{-4}$)	16.1% ($p=5.6x10^{-6}$)	1.7% (p=0.022)	18.3% ($p=1.2x10^{-7}$)
ΔHLEB/ΔHLEB	2% ($p=8.9x10^{-4}$)	3.3% (N.S.)	+1.6% (p=0.011)	2.8% (N.S.)

Figure 2.3. Hindlimb bone measurements of ΔHLEA and ΔHLEB homozygous mutant mice. Schematic (A) shows how the measurements were made. The table shows the values in percent, of the comparisons performed between the hindlimbs of the two transgenic adult mice, *ΔHLEB/ΔHLEB and ΔHLEA/ΔHLEA.* These values have been normalized to the length of the humerus. Next to them, are their respective p-values. N.S. stands for "not significant" meaning the two mutant mice do not significantly differ for that particular measurement. "+" indicates that the measurement was greater than control. Only males were used for this study.

A.



B.

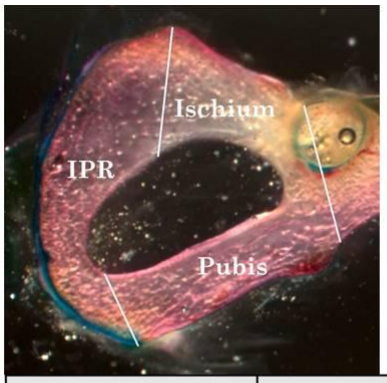


B.

Genotype	Percent Reduction of Pelvis Length Compared to WT (1)	Percent Reduction of Ischium Length Compared to WT (2)	Percent Reduction of Pubis Length Compared to WT (3)	Percent Reduction of Anterior Ischium Width Compared to WT (4)	Percent Reduction of Posterior Ischium Width Compared to WT (5)	Percent Reduction of Ischiopubic Ramus Width Compared to WT (6)	Percent Reduction of Pubis Width Compared to WT (7)	Percent Reduction of Posterior Pelvic Width Compared to WT (8)	Percent Reduction of Anterior Pelvic Width Compared to WT (9)	Percent Reduction of Foramen Obturatum Length Compared to WT (10)	Percent Reduction of the Angle Between Ischium and Pubis Compared to WT
ΔHLEA/ΔHLEA	+0.81% (N.S.)	+5 %(N.S.)	2.5%(N.S.)	+4.3%(p=0.0096)	+7.2%(p=0.057)	+15.9%(p=0.00085)	+3.35%(N.S.)	+1.3%(N.S.)	5.7%(p=0.0025)	7.9%(p=0.00061)	+7.1%(p=0.025)
ΔHLEB/ΔHLEB	0.7%(N.S.)	+5.4%(p=0.094)	7.7%(p=0.028)	+1.3%(N.S.)	10.5%(p=0.03)	22.7%(p=3.5X10^{-4})	4.6%(p=0.06)	4.4%(p=0.08)	0.2%(N.S.)	3.2%(N.S)	3.5%(N.S.)

Figure 2.4. Pelvic measurements of ΔHLEA and ΔHLEB homozygous mutant mice. Schematic of the pubic bone (A) shows the ischium, pubis, ilium as well as the ischiopubic ramus (IPR). Schematic (B) shows how each measurement was made. The table shows the values in percent, of the comparisons performed between the pelvic bones of the two transgenic adult mice, *ΔHLEB/ΔHLEB and ΔHLEA/ΔHLEA.* These values have been normalized to the length of the humerus. Next to them, are their respective p-values which are shown as N.S. if the respective p value is greater than 0.1. "+" indicates that the measurement was greater than control. Only adult males were used for the preparations and measurements of pelvic bones.

A.



B.

Genotype	Percent Decrease of Whole Posterior Pelvis as Compared to WT	Percent Decrease of Ischiopubic Ramus (IPR) as Compared to WT	Percent Decrease of Ischium as Compared to WT	Percent Decrease of Pubis as Compared to WT
ΔHLEA/ΔHLEA	+6.1%(p=0.01)	+17.2%($p=4x10^{-5}$)	4%(N.S.)	0.81%(N.S)
ΔHLEB/ΔHLEB	6.3%(p=0.006)	12.2%(p=0.003)	+3.9%(N.S.)	5.9%(p=0.006)

Figure 2.5. Area measurements of the posterior part of the pelvis. Schematic (A) shows how the posterior pelvis was delineated in order to make the area measurements. The table (B) shows the values in percent, of the comparisons performed between the posterior part of the pelvis of the two transgenic adult mice, *ΔHLEB/ΔHLEB and ΔHLEA/ΔHLEA.* These values have been normalized to the length of the humerus. Next to them, are their respective p-values. N.S. stands for "not significant" meaning the two mutant mice do not significantly differ for that particular measurement. "+" indicates that the measurement was greater than control (WT).

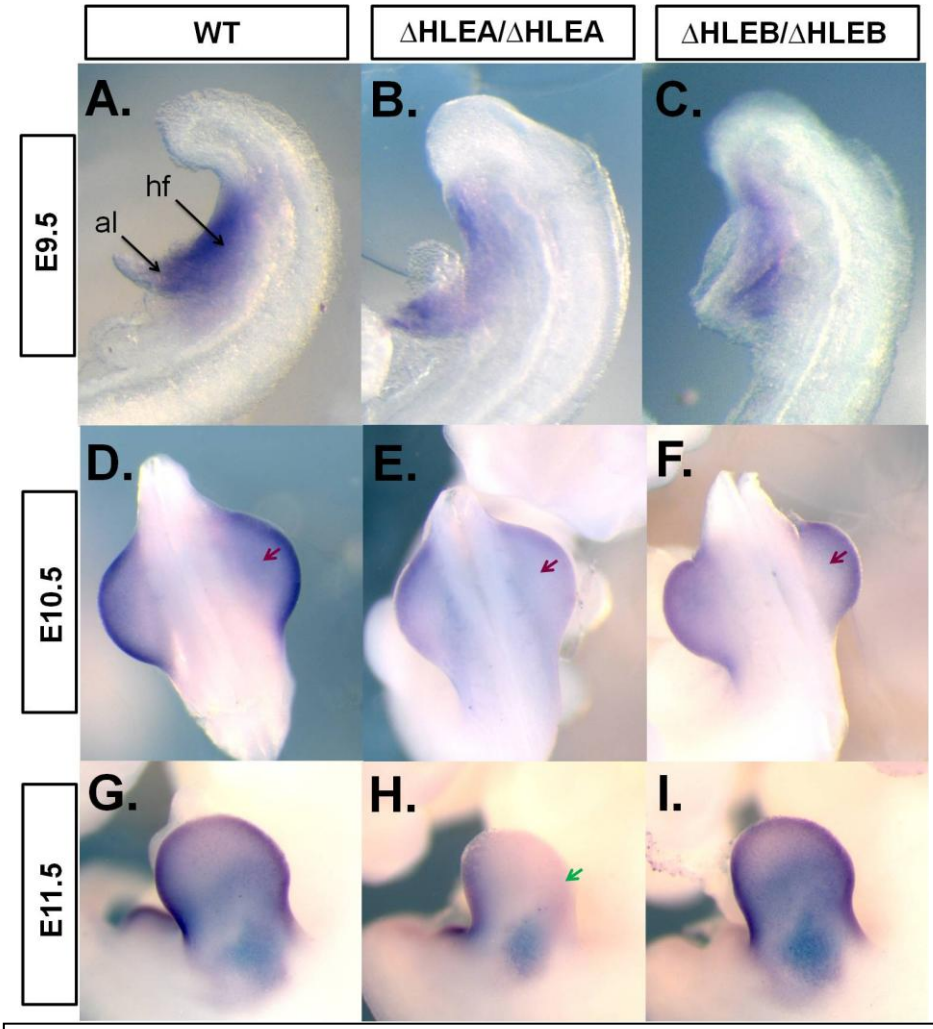
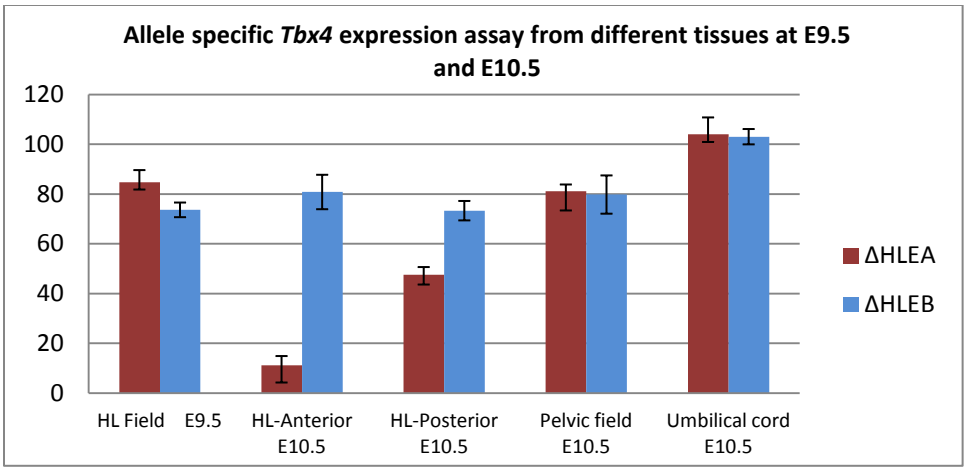
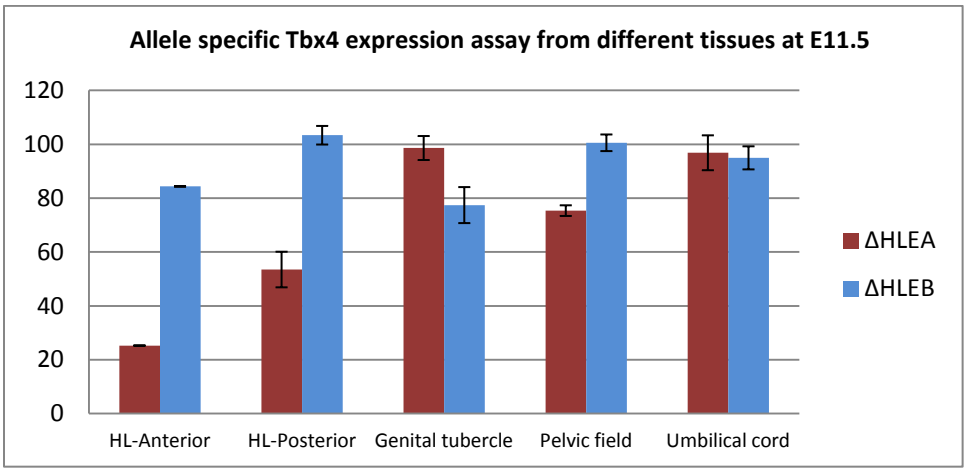


Figure 2.6. HLEB and HLEA differentially regulate the spatio-temporal expression of *Tbx4*. At E9.5 expression of *Tbx4* is downregulated in the hindlimb field (hl) of both *ΔHLEA/ΔHLEA (*B) and *ΔHLEB/ΔHLEB (*C) mutant embryos compared to wild type (A). At E10.5 the evident reduction of *Tbx4* expression in the hindlimbs of homozygous mutant embryos is no longer uniform throughout the hindlimb, different compartments of lowered expression now becoming evident (compare red arrows panels D,E and F). At E11.5 the anterior part of the hindlimb of HLEA homozygous mutant embryos (H) shows little to no expression of *Tbx4* (green arrow panel H) while expression of this gene is maintained in the posterior end. In contrast, the hindlimbs of both WT and *ΔHLEB/ΔHLEB* display a relative uniform level of expression between anterior and posterior parts of the hindlimb (G,I). al (allantois), hf (hindlimb field)


Allele specific *Tbx4* expression assay from different tissues at E9.5 and E10.5
120
100
80
60
40
20
0
HL Field E9.5
HL-Anterior E10.5
HL-Posterior E10.5
Pelvic field E10.5
Umbilical cord E10.5
ΔHLEA
ΔHLEB


Allele specific Tbx4 expression assay from different tissues at E11.5
120
100
80
60
40
20
0
HL-Anterior
HL-Posterior
Genital tubercle
Pelvic field
Umbilical cord
ΔHLEA
ΔHLEB

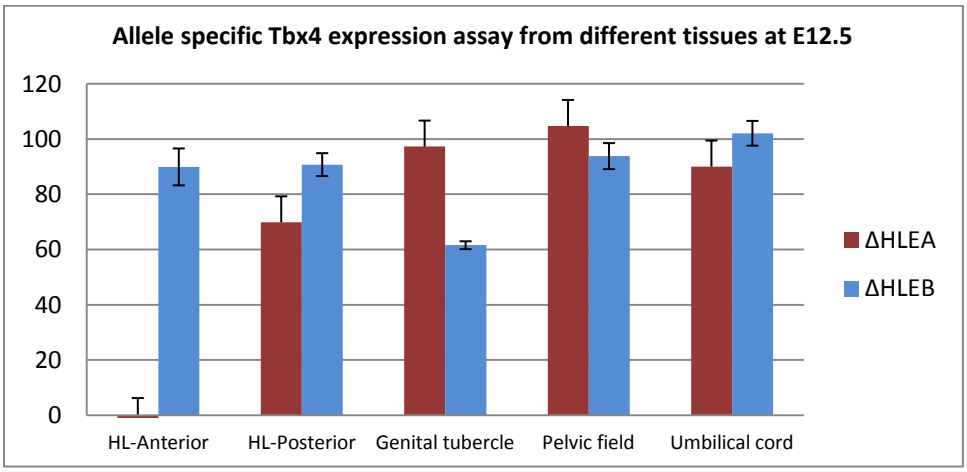

Allele specific Tbx4 expression assay from different tissues at E12.5
120
100
80
60
40
20
0
HL-Anterior
HL-Posterior
Genital tubercle
Pelvic field
Umbilical cord
ΔHLEA
ΔHLEB

Figure 2.7. Allele specific *Tbx4* expression assay from different tissues shows that HLEA and HLEB have different roles in the spatial regulation of gene expression. Making use of the different melting temperatures of DNA with a single base pair mismatch, in our case a naturally occurring SNP within the Tbx4 locus C->T, we were able to determine the relative levels of Tbx4 expression driven by either the ΔHLEA or ΔHLEB locus compared to the WT locus. Pure bred *ΔHLEA/ΔHLEA* or *ΔHLEB/ΔHLEB* mice on a C57BL/6J background were bred to pure bred DBA/2J mice to generate heterozygous embryos with the deleted enhancer on one background and the wild type on the other. Using a FRET probe homologous to the Tbx4 transcript from the C57BL/6J sequence, we were able to generate a melting curve that gave us the relative ratio of Tbx4 transcripts containing a C versus *Tbx4* transcripts containing a T allowing us to determine how efficiently each deletion was able to modulate gene expression. Panel A shows the results of this assay on tissues taken from embryos of stages E9.5 and E10.5. In these figures the relative expression levels of *Tbx4* as driven by either the *ΔHLEA* or the *ΔHLEB* allele were compared to the levels driven by the wild type allele, meaning that 100% represents equal expression between mutant and wild type alleles. Umbilical cord was used as negative control, since neither HLEA nor HLEB are known to drive expression of *Tbx4* in this tissue, therefore both alleles were expected to drive 100% levels of expression as the *WT* allele. Tissues at E11.5 (B) and E12.5 (C) were also assayed. N=4 for each measurement

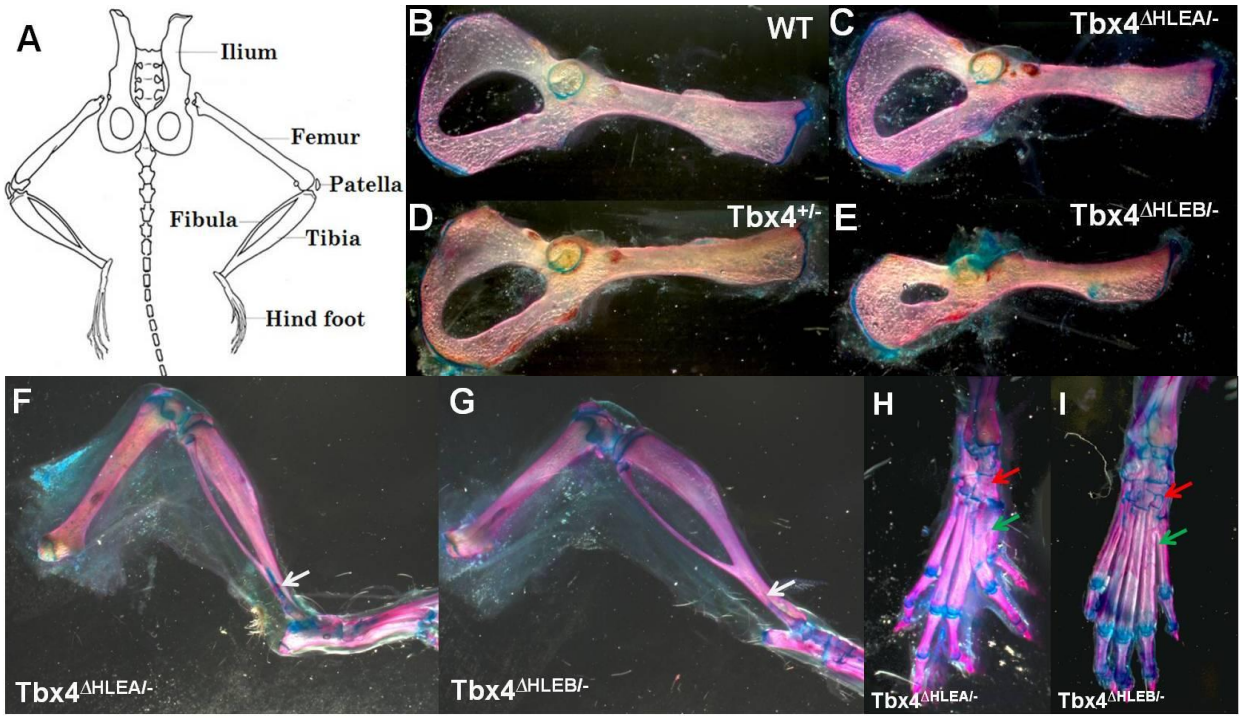


Figure 2.8. Compared to *Tbx4*$^{\Delta HLEA/-}$ mice (A), *Tbx4*$^{\Delta HLEB/-}$ mice display more severe phenotypes in proximal parts of the limb. Panel A is an illustration of a mouse pelvis and hindlimb bones for reference. *WT* disarticulated pelvic bone (B) and *Tbx4*$^{-/+}$ pelvic bones (D) have been used as controls for this study. *Tbx4*$^{\Delta HLEB/-}$ (E) hip bone displays significant abnormalities compared to *Tbx4*$^{\Delta HLEA/-}$ (C) and controls. The tibia and fibula of *Tbx4*$^{\Delta HLEA/-}$ mice are not entirely fused, the calcaneous bone and overall ankle structure is abnormal (compare white arrow from panel F to that from panel G). A closer look at the ankle of *Tbx4*$^{\Delta HLEA/-}$ mice shows a significant number of fused ankle bones as well as an abnormal structure and fused digits while the ankle and digits of *Tbx4*$^{\Delta HLEB/-}$ mice appear normal (compare panels H and I).

Table 2.1. Pelvic measurements of *Tbx4*$^{\Delta HLEA/-}$ and *Tbx4*$^{\Delta HLEB/-}$ adult mutant mice.

Genotype	Percent Reduction of Pelvis Length Compared to Tbx4$^{-/+}$ (1)	Percent Reduction of Ischium Length Compared to Tbx4$^{-/+}$ (2)	Percent Reduction of Pubis Length Compared to Tbx4$^{-/+}$ (3)	Percent Reduction of Anterior Ischium Width Compared to Tbx4$^{-/+}$ (4)	Percent Reduction of Posterior Ischium Width Compared to Tbx4$^{-/+}$ (5)	Percent Reduction of Ischiopubic Ramus Width Compared to Tbx4$^{-/+}$ (6)	Percent Reduction of Pubis Width Compared to Tbx4$^{-/+}$ (7)	Percent Reduction of Posterior Pelvic Width Compared to Tbx4$^{-/+}$ (8)	Percent Reduction of the Angle Between Ischium and Pubis Compared to Tbx4$^{-/+}$
Tbx4$^{\Delta HLEA/-}$	0.2% (N.S.)	+4.7 %(N.S.)	+6.7%(p=0.004)	6.5%(p=0.002)	1.8%(N.S.)	3.7%(N.S.)	7.7%(p=0.001)	+1.9%(N.S.)	0.6%(N.S.)
Tbx4$^{\Delta HLEB/-}$	7.9%(p=1.1x10^{-9})	8.5%(p=0.019)	7.4%(p=0.06)	13.3%(p=3.8x10^{-6})	26.6%(p=1.4x10^{-10})	+26.4%(p=8X10^{-5})	20%(p=4.8x10^{-8})	32.9%(p=1.7x10^{-18})	44%(p=3.5x10^{-12})

The table shows the values in percent, of the comparisons performed between the pelvic bones of the two transgenic adult mice, *Tbx4*$^{\Delta HLEA/-}$ and *Tbx4*$^{\Delta HLEB/-}$. These values have been normalized to the length of the humerus as well. Next to them, are their respective p-values. A respective p value that is greater than 0.1 is represented by "N.S." (not significant). "+" indicates that the measurement was greater than control. Only adult males were used for the preparations and measurements of these pelvic bones.

Table 2.2. Measurements of the hindlimb bones of *Tbx4*$^{\Delta HLEA/-}$ and *Tbx4*$^{\Delta HLEB/-}$ adult mutant mice.

Genotype	Percent Reduction of Femur Length Compared to Tbx4$^{-/+}$ (1)	Percent Reduction of Patella Compared to Tbx4$^{-/+}$ (2)	Percent Reduction of Tibia Length Compared to Tbx4$^{-/+}$ (3)	Rercent Reduction of Tibia Width Compared to Tbx4$^{-/+}$ (4)
Tbx4$^{\Delta HLEA/-}$	3.5% ($p=6\times10^{-4}$)	9.7% (p=0.004)	1.5% (p=0.06)	16.7% ($p=2.9\times10^{-5}$)
Tbx4$^{\Delta HLEB/-}$	4.8% ($p=4.3\times10^{-5}$)	5.8% ($p=4.9\times10^{-5}$)	0.4% (N.S)	3.5% (N.S.)

The table shows the values in percent, of the comparisons performed between the pelvic bones of the two transgenic adult mice, *Tbx4*$^{\Delta HLEA/-}$ and *Tbx4*$^{\Delta HLEB/-}$. These values have been normalized to the length of the humerus as well. Next to them, are their respective p-values. A respective p value that is greater than 0.1 is represented by "N.S." (not significant). "+" indicates that the measurement was greater than control.

Table 2.3. Preliminary quantitative RT-PCR on the somatopleure of the E10.5 hindlimb.

Gene	N (number of biological replicates)	Average fold enrichment of $Tbx4^{\Delta HLEB/-}$ compared to wild type
Sox9	3	0.56
Bmp4	3	0.75
Alx4	3	1.41*
Alx3	3	1.51
Alx1	1	2.51
Scx	2	0.28
Hoxc10	3	1.1
Pbx1	3	1.08
Pbx2	3	1.85
Wnt4	2	1.65
Msx1	2	1.18
Pitx1	2	1.04
Fgf10	2	0.48
Prx1	1	0.66

Table shows quantitative RT-PCR results expressed in fold enrichment as compared to wild type littermates of E10.5 *Tbx4*$^{\Delta HLEB/-}$. The tissue used for this preliminary study was the hindlimb somatopleure, known to be the tissue responsible for the development of the pelvic primordia. Genes were selected based on their pelvic phenotypes associated with the disruption of gene expression. Here "*" signifies significance, $p<0.05$

Table 2.4. Quantitative RT-PCR on the hindlimb of E10.5 embryos

Gene	N (number of biological replicates)	Average fold enrichment of $Tbx4^{\Delta HLEB/-}$ compared to wild type	N (number of biological replicates)	Average fold enrichment of $Tbx4^{-/+}$ compared to wild type
Fgf10	3	1.14	3	1.09
Pitx1	3	1.3	3	0.79
Sox9	3	0.87	3	0.46
Twist1	3	1.63	3	1.12
Isl1	3	1.6	2	1.24
Alx4	3	3.9	3	1.44
Prx1	3	1.67	3	1.15
Prx2	3	2.55	3	1.09
Msx1	2	2.14	2	0.63

Table shows quantitative RT-PCR results expressed in fold enrichment as compared to wild type littermates of E10.5 *Tbx4*$^{\Delta HLEB/-}$ and *Tbx4*$^{-/+}$ of hindlimb tissue. P values were not calculated at this step.

Table 2.5. Quantitative RT-PCR on the hindlimb somatopleure of E10.5 embryos

Gene	Average fold enrichment of $Tbx4^{\Delta HLEA/+}$ compared to wild type	Average fold enrichment of $Tbx4^{\Delta HLEB/-}$ compared to wild type	Average fold enrichment of $Tbx4^{-/+}$ compared to wild type
Alx4	1.01 (N.S.)	1.91 (p=0.017)	1.21 (N.S.)
Alx3	1.12 (N.S.)	1.64 (p=0.0025)	1.22 (N.S.)
Alx1	2.24 (N.S.)	2.1 (N.S.)	1.77 (N.S.)
Hoxc10	0.88 (N.S.)	1.15 (N.S.)	0.97 (N.S.)
Sox9	0.62 (N.S.)	0.84 (N.S.)	0.52 (N.S.)
Prx1	0.64 (N.S.)	0.79 (N.S.)	0.65 (N.S.)

Table shows quantitative RT-PCR results expressed in fold enrichment as compared to wild type E10.5 $Tbx4^{\Delta HLEA/-}$, $Tbx4^{\Delta HLEB/-}$ and $Tbx4^{-/+}$. P values are noted to the left. The tissue used for this study was the hindlimb somatopleure, known to be the tissue responsible for the development of the pelvic primordia. Genes were selected based on the results of the preliminary study (Table 2.3).

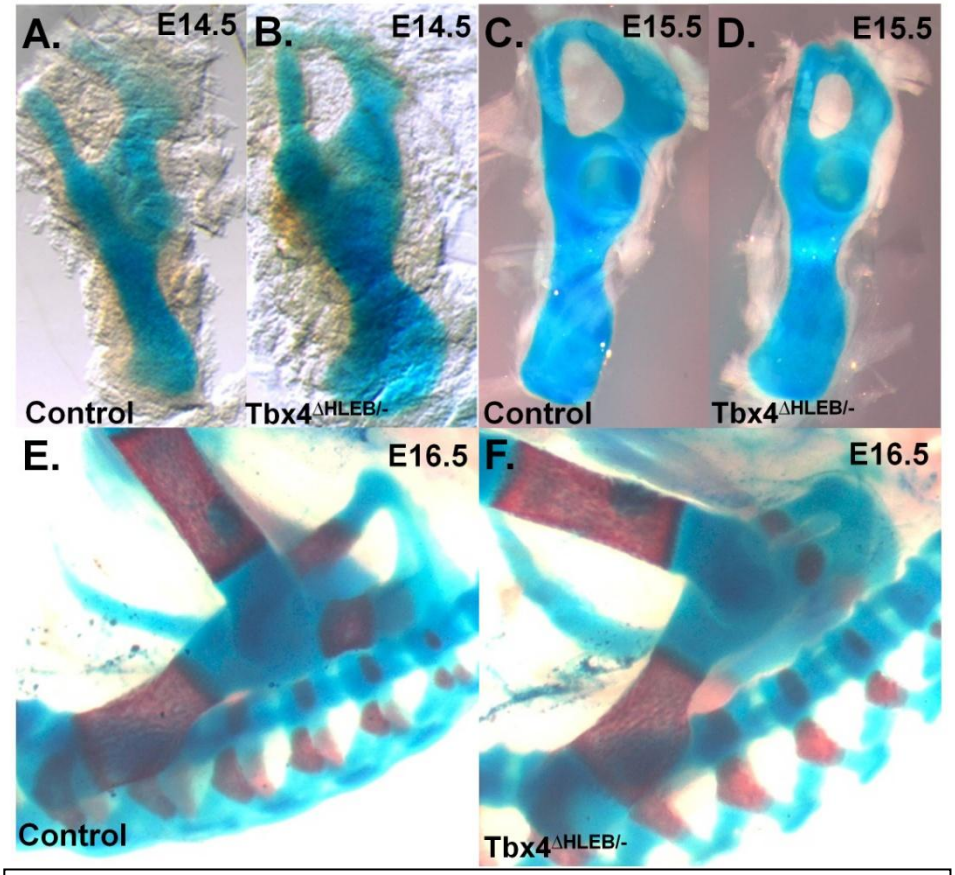


Figure 2.9 Pelvic abnormalities observed in adult *Tbx4*$^{\Delta HLEB/-}$ mice occur prenatally. *Tbx4*$^{\Delta HLEB/-}$ mutant embryos at E14.5, E15.5 and E16.5 were stained with alcian blue for cartilage (blue color) and alizarin red for bone (red color). Right pelvises of E14.5 (A,B) and E15.5 (C,D) were dissected away from the main body, cleaned and photographed. Right pelvises of E16.5 were photographed while still attached to the main body (E,F).

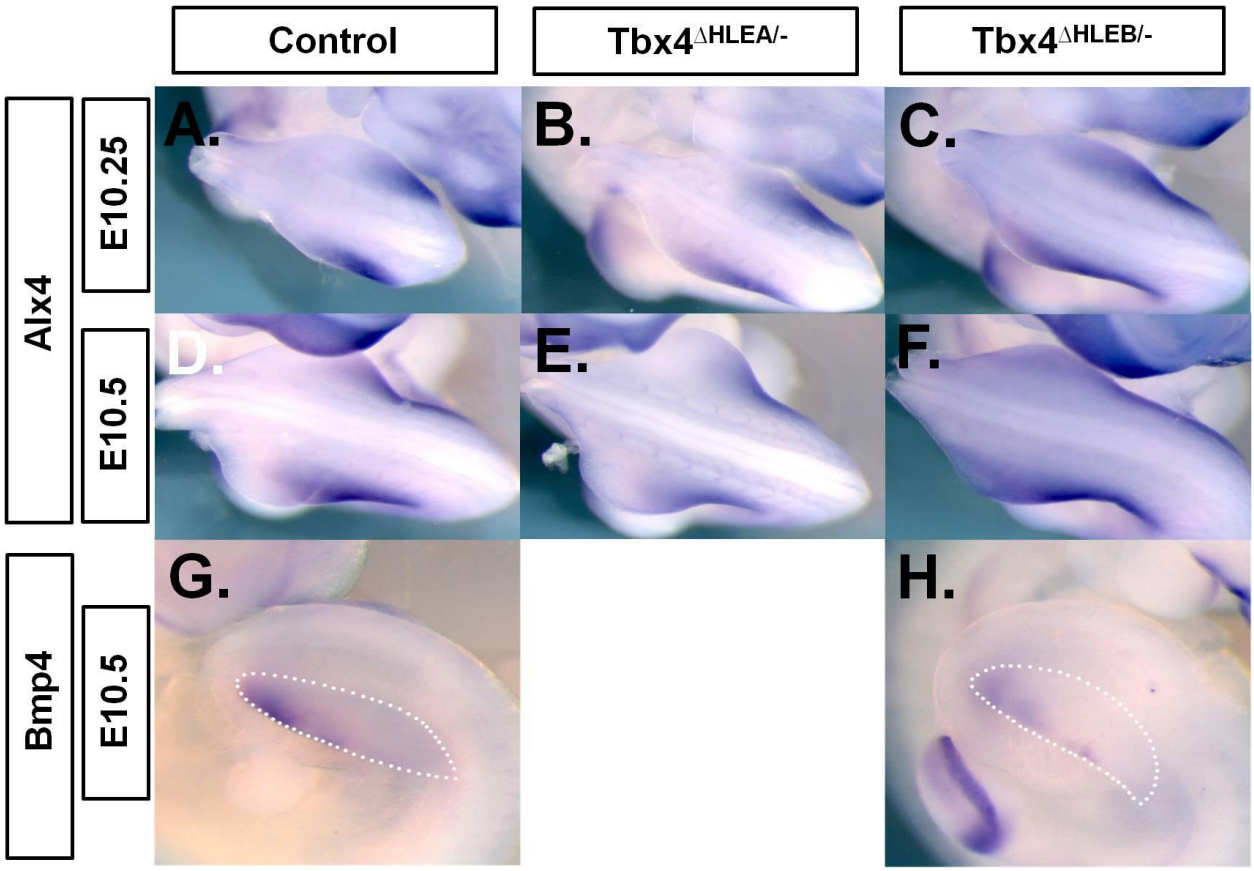


Figure 2.10. At E10.5, the activities of *Alx4* and *BMP4* are disrupted only in the hindlimbs of *Tbx4*$^{\Delta HLEB/-}$ mutant embryos as shown by *in situ* hybridization. At E10.75 and E10.5, *Alx4* shows a pattern of expression that is restricted to the anterior part of the hindlimb in control (A and D respectively) as well as in *Tbx4*$^{\Delta HLEA/-}$ mutant embryos of the same stage (B and E respectively). Quantitative real-time PCR using cDNA from the somatopleure of the hindlimb shows that *Alx4* is upregulated in *Tbx4*$^{\Delta HLEB/-}$ mutant embryos at E10.5 by almost a factor of two compared to wild type controls (p=0.017, N=4). Conversely, there is no statistical difference in *Alx4* expression from *Tbx4*$^{\Delta HLEA/-}$ nor *Tbx4*$^{-/+}$ mutant mice of comparable as compared to wild type, suggesting that the disruptions in *Tbx4* gene expression associated with the deletion of enhancer HLEB alone is necessary for the observed alterations in *Alx4* expression. *Bmp4* also shows altered expression within the hindlimb of E10.5 *Tbx4*$^{\Delta HLEB/-}$ mutant embryos (H) compared to control (G). In situ hybridization using a *Bmp4* probe on *Tbx4*$^{\Delta HLEA/-}$ embryos could not have been preformed due to an insufficient number. However, preliminary quantitative RT-PCR showed that *Bmp4* transcripts in the somatopleure of E10.5 hindlimbs, are reduced by 25% in *Tbx4*$^{\Delta HLEB/-}$ mutants as compared to controls (N=3).

Table 2.6. Primers used for quantitative RT-PCR

Gene	Name	Sequence
Hoxc10	F_qHoxc10_Mmus_80	tcaatatgtatttgacgcgagag
	R_qHoxc10_Mmus_80	tgtctgtaaggttaatggtcttgc
PBX1	F_qPBX1_Mmus_69	tcccagcacttgcaggat
	R_qPBX1_Mmus_69	gcagtttaaagcatgttttctgg
PBX2	F_qPBX2_Mmus_93	ttctgcaacagataatgaccatc
	R_qPBX2_Mmus_93	gtgacagtttagggcatgtttct
EMX2	F_qEMX2_Mmus_33	ctgggtcatcgcttccaa
	R_qEMX2_Mmus_33	ctcaaaagcgtgctctagcc
Sox9	F_qSox9_Mmus_66	gtacccgcatctgcacaac
	R_qSox9_Mmus_66	ctcctccacgaagggtctct
Pitx1	F_qPitx1_Mmus_60	atcgtccgacgctgatct
	R_qPitx1_Mmus_60	cttagctgggtcctctgcac
Tbx15	F_qTbx15_Mmus_10	taccatgcaaaagcagcaag
	R_qTbx15_Mmus_10	gagatgagaagaagccgaagg
Scx	F_qScx_Mmus_29	acacccagcccaaacagat
	R_qScx_Mmus_29	tctgtcacggtctttgctca
Alx1	F_qAlx1_Mmus_78	agaggagacaccgaactaccttt
	R_qAlx1_Mmus_78	ctgggtaatgggttttctgg
Alx3	F_qAlx3_Mmus_73	acagcctatgacatctccgtact
	R_qAlx3_Mmus_73	actgggccacagggagtt
Alx4	F_qAlx4_Mmus_106	acggtagcctcaagctcca
	R_qAlx4_Mmus_106	cacgggttcagagtcagga
Prrx1	F_qPrrx1_Mmus_18	agtcaccgggactgacca
	R_qPrrx1_Mmus_18	tcttctcctcagagttcaactgg
Prrx2	F_qPrrx2_Mmus_13	agtgaggcacgtgtccaag
	R_qPrrx2_Mmus_13	cctggccataagacttgagc
Twist1	F_qTwist1_Mmus_58	agctacgccttctccgtct
	R_qTwist1_Mmus_58	tccttctctggaaacaatgaca
Isl1	F_qIslet1_Mmus_83	gcaacccaacgacaaaactaa
	R_qIslet1_Mmus_83	ccatcatgtctctccggact
Wnt4	F_qWnt4_Mmus_25	gcgtagccttctcacagtcc
	R_qWnt4_Mmus_25	cgcatgtgtgtcaagatgg
Cv2	F_qCv2_Mmus_16	tcagccagaaggaaacaaatg
	R_qCv2_Mmus_16	acagacttctctcacgcactgt
Msx1	F_qMsx1_Mmus_7	gccccgagaaactagatcg
	R_qMsx1_Mmus_7	ttggtcttgtgcttgcgtag
Msx2	F_qMsx2_Mmus_92	aattccgaagacggagcac
	R_qMsx2_Mmus_92	gtggtggggctcatatgtct
Fgf10	F_qFgf10_Mmus_80	cgggaccaagaatgaagact
	R_qFgf10_Mmus_80	gcaacaactccgatttccac
Pax1	F_qPax1_Mmus_105	ctccgcacattcagtcagc
	R_qPax1_Mmus_105	tcttccatcttgggggagta
Bmp4	F_qBmp4_Mmus_63	gatctttaccggctccagtct
	R_qBmp4_Mmus_63	tgggatgttctccagatgttc

Table showing the primers used for qualitative RT-PCR assays. The number at the end of the primer's name represents the number of the universal probe used for that particular assay.

References

Abbasi, A.A., Paparidis, Z., Malik, S., Bangs, F., Schmidt, A., Koch, S., Lopez-Rios, J., Grzeschik, K.H., 2010. Human intronic enhancers control distinct sub-domains of Gli3 expression during mouse CNS and limb development. BMC developmental biology 10, 44.

Ahituv, N., Zhu, Y., Visel, A., Holt, A., Afzal, V., Pennacchio, L.A., Rubin, E.M., 2007. Deletion of ultraconserved elements yields viable mice. PLoS biology 5, e234.

Barolo, S., 2012. Shadow enhancers: frequently asked questions about distributed cis-regulatory information and enhancer redundancy. Bioessays 34, 135-141.

Cande, J., Goltsev, Y., Levine, M.S., 2009. Conservation of enhancer location in divergent insects. Proceedings of the National Academy of Sciences of the United States of America 106, 14414-14419.

Cretekos, C.J., Wang, Y., Green, E.D., Martin, J.F., Rasweiler, J.J.t., Behringer, R.R., 2008. Regulatory divergence modifies limb length between mammals. Genes & development 22, 141-151.

Degenhardt, K.R., Milewski, R.C., Padmanabhan, A., Miller, M., Singh, M.K., Lang, D., Engleka, K.A., Wu, M., Li, J., Zhou, D., Antonucci, N., Li, L., Epstein, J.A., 2010. Distinct enhancers at the Pax3 locus can function redundantly to regulate neural tube and neural crest expressions. Developmental biology 339, 519-527.

DiLeone, R.J., Russell, L.B., Kingsley, D.M., 1998. An extensive 3' regulatory region controls expression of Bmp5 in specific anatomical structures of the mouse embryo. Genetics 148, 401-408.

Epner, E., Reik, A., Cimbora, D., Telling, A., Bender, M.A., Fiering, S., Enver, T., Martin, D.I.K., Kennedy, M., Keller, G., Groudine, M., 1998. The Globin LCR Is Not Necessary for an Open Chromatin

Structure or Developmentally Regulated Transcription of the Native Mouse Globin Locus. Molecular cell 2, 447-455.

Frankel, N., Davis, G.K., Vargas, D., Wang, S., Payre, F., Stern, D.L., 2010. Phenotypic robustness conferred by apparently redundant transcriptional enhancers. Nature 466, 490-493.

Frankel, N., Erezyilmaz, D.F., McGregor, A.P., Wang, S., Payre, F., Stern, D.L., 2011. Morphological evolution caused by many subtle-effect substitutions in regulatory DNA. Nature 474, 598-603.

Hasson, P., DeLaurier, A., Bennett, M., Grigorieva, E., Naiche, L.A., Papaioannou, V.E., Mohun, T.J., Logan, M.P., 2010. Tbx4 and tbx5 acting in connective tissue are required for limb muscle and tendon patterning. Developmental cell 18, 148-156.

Hobert, O., 2010. Gene regulation: enhancers stepping out of the shadow. Current biology : CB 20, R697-699.

Hong, J.W., Hendrix, D.A., Levine, M.S., 2008. Shadow enhancers as a source of evolutionary novelty. Science 321, 1314-1314.

Jeong, S., Hahn, Y., Rong, Q., Pfeifer, K., 2007. Accurate quantitation of allele-specific expression patterns by analysis of DNA melting. Genome research 17, 1093-1100.

Jeong, Y., El-Jaick, K., Roessler, E., Muenke, M., Epstein, D.J., 2006. A functional screen for sonic hedgehog regulatory elements across a 1 Mb interval identifies long-range ventral forebrain enhancers. Development 133, 761-772.

Juan, A.H., Ruddle, F.H., 2003. Enhancer timing of Hox gene expression: deletion of the endogenous Hoxc8 early enhancer. Development 130, 4823-4834.

Kuijper, S., Beverdam, A., Kroon, C., Brouwer, A., Candille, S., Barsh, G., Meijlink, F., 2005. Genetics of shoulder girdle formation: roles of Tbx15 and aristaless-like genes. Development 132, 1601-1610.

Levine, M., 2010. Transcriptional enhancers in animal development and evolution. Current biology : CB 20, R754-763.

Lufkin, T., Mark, M., Hart, C.P., Dolle, P., LeMeur, M., Chambon, P., 1992. Homeotic transformation of the occipital bones of the skull by ectopic expression of a homeobox gene. Nature 359, 835-841.

Malashichev, Y., Borkhvardt, V., Christ, B., Scaal, M., 2005. Differential regulation of avian pelvic girdle development by the limb field ectoderm. Anatomy and embryology 210, 187-197.

Malashichev, Y., Christ, B., Prols, F., 2008. Avian pelvis originates from lateral plate mesoderm and its development requires signals from both ectoderm and paraxial mesoderm. Cell and tissue research 331, 595-604.

Menke, D.B., Guenther, C., Kingsley, D.M., 2008. Dual hindlimb control elements in the Tbx4 gene and region-specific control of bone size in vertebrate limbs. Development 135, 2543-2553.

Naiche, L.A., 2003. Loss of Tbx4 blocks hindlimb development and affects vascularization and fusion of the allantois. Development 130, 2681-2693.

Naiche, L.A., Papaioannou, V.E., 2007. Tbx4 is not required for hindlimb identity or post-bud hindlimb outgrowth. Development 134, 93-103.

Nakada, Y., Parab, P., Simmons, A., Omer-Abdalla, A., Johnson, J.E., 2004. Separable enhancer sequences regulate the expression of the neural bHLH transcription factor neurogenin 1. Developmental biology 271, 479-487.

Nobrega, M.A., Zhu, Y., Plajzer-Frick, I., Afzal, V., Rubin, E.M., 2004. Megabase deletions of gene deserts result in viable mice. Nature 431, 988-993.

Pappu, K.S., Ostrin, E.J., Middlebrooks, B.W., Sili, B.T., Chen, R., Atkins, M.R., Gibbs, R., Mardon, G., 2005. Dual regulation and redundant function of two eye-specific enhancers of the Drosophila retinal determination gene dachshund. Development 132, 2895-2905.

Perry, M.W., Boettiger, A.N., Bothma, J.P., Levine, M., 2010. Shadow enhancers foster robustness of Drosophila gastrulation. Current biology : CB 20, 1562-1567.

Peterson, R.L., Jacobs, D.F., Awgulewitsch, A., 1992. Hox-3.6: isolation and characterization of a new murine homeobox gene located in the 5' region of the Hox-3 cluster. Mechanisms of development 37, 151-166.

Pomikal, C., Streicher, J., 2010. 4D-analysis of early pelvic girdle development in the mouse (Mus musculus). Journal of morphology 271, 116-126.

Schaffner, G., Schirm, S., Mullerbaden, B., Weber, F., Schaffner, W., 1988. Redundancy of Information in Enhancers as a Principle of Mammalian Transcription Control. J Mol Biol 201, 81-90.

Verhofstede, C., Fransen, K., Marissens, D., Verhelst, R., van der Groen, G., Lauwers, S., Zissis, G., Plum, J., 1996. Isolation of HIV-1 RNA from plasma: evaluation of eight different extraction methods. Journal of virological methods 60, 155-159.

Wright, E., Hargrave, M.R., Christiansen, J., Cooper, L., Kun, J., Evans, T., Gangadharan, U., Greenfield, A., Koopman, P., 1995. The Sry-related gene Sox9 is expressed during chondrogenesis in mouse embryos. Nat Genet 9, 15-20.

Xiong, N., Kang, C., Raulet, D.H., 2002. Redundant and unique roles of two enhancer elements in the TCRgamma locus in gene regulation and gammadelta T cell development. Immunity 16, 453-463.

Zhou, X., Sigmund, C.D., 2008. Chorionic enhancer is dispensable for regulated expression of the human renin gene. American journal of physiology. Regulatory, integrative and comparative physiology 294, R279-287.

CHAPTER 3

CIS-REGULATORY ELEMENTS WITH SHARED ACTIVITY IN BOTH LIMBS AND GENITAL TUBERCLE ARE FUNCTIONALLY IMPORTANT IN BOTH STRUCTURES[2]

[2] Alexandra G. Mihala, Carlos Infante, Sungdae Park, Jim Lauderdale and Douglas Menke. To be submitted to *Developmental Cell*.

Abstract

The development of limb and genital tubercle derived tissues are known to have many shared transcription factors and signaling pathways. However, little is known about how the network of *cis*-regulatory elements is used by these appendages to modulate such shared factors into very different morphological outputs. To investigate the active *cis*-regulome of limbs and genital tubercle we performed ChIP-seq on these tissues against H3K27ac, a marker of active enhancers, and discovered a remarkable overlap between the set of enhancers activated during embryonic development of these different appendage types. We functionally tested one such enhancer, HLEB, from the *Tbx4* locus by knocking out this *cis*-regulatory element from the mouse genome. Deletion of this enhancer resulted in reduced *Tbx4* expression in embryonic mouse hindlimbs and the genital tubercle. Loss of the HLEB element was associated with morphological defects in the both the pelvis and external genitalia of mutant mice. Surprisingly, we also report the discovery a novel role for *Tbx4* in the proper development of kidneys. Together, our results demonstrate pervasive sharing of enhancer elements between the limbs and genital appendages and shows that some of these shared elements are functionally important for the development of these different appendage types.

Introduction

The proper development of limbs and external genitalia involve very similar developmental processes that are orchestrated by many of the same molecular mechanisms. Both appendages emerge as mesenchymal swellings from the lateral plate mesoderm that must undergo growth and patterning in three dimensions away from the body axis. Limb outgrowth is promoted by a signaling center in the overlying ectoderm called the AER that expresses Fgf

transcription factors. Just as in limbs, Fgfs have been shown to be indispensable for genital tubercle outgrowth (Lin et al., 2013). Furthermore, successive removal of ectoderm and endoderm from cultured genital tubercles show similar stage-dependent loss of distal structures (Murakami and Mizuno, 1986) as seen in limbs. In particular, Fgf8 has recently been shown to play an important role in the proximo-distal outgrowth of both appendages through a conserved pathway involving canonical Wnt signaling (Lin et al., 2013).

Conserved functions of signaling molecules such as BMPs, have also been implicated in the proper development of both the genital tubercle (Suzuki et al., 2003) and limbs (Dunn et al., 1997). Shh, a signaling molecule vital for the proper patterning of both appendages (Chiang et al., 1996; Seifert et al., 2010) is expressed from distinct appendage regions, the urethral plate (UP) in genital tubercle and the zone of polarizing activity (ZPA) in limbs. Transplantation experiments in chick have shown that the limb ZPA can cause anterior cells to be posteriorized causing ectopic expression of Shh, which results in mirror-image duplication of digits (Lopez-Martinez et al., 1995; Tickle et al., 1975). Similar transplantation experiments using mouse genital tubercle tissue including the UP also resulted in digital duplication (Izpisua-Belmonte et al., 1992) suggesting a shared patterning role via Shh between these two structures (Lin et al., 2009; Miyagawa et al., 2009; Perriton et al., 2002).

Hox genes, mainly *Hoxa13* and *Hoxd13*, have been shown to be very important in the proper patterning of both appendages (Dollé et al., 1993; Morgan, 2003; Warot et al., 1997; Zakany et al., 1997). *Hoxa13* mutants display significant genital tubercle phenotypes, many due to proliferation defects that can be rescued by the addition of Fgf8 soaked beads (Morgan, 2003). Furthermore, human mutations in *HOXA13* were shown to be responsible for many of

the observed limb and urogenital phenotypes in Hand-Foot-Genital Syndrome (Goodman et al., 2000). Using microarrays to identify genes disrupted by the deletion of the entire Hoxd cluster, Cobb and Duboule have observed that the vast majority of the same genes were disrupted in both limb and genital tissues (Cobb and Duboule, 2005). This data suggests that a significant portion of genes downstream of HoxD genes are shared between appendages raising the possibility that parts of their regulatory networks may also be shared. Indeed, the expression of *Hoxd13* in both limbs and the genital tubercle has been linked to the same cis-regulatory element (Spitz et al., 2003). However not all shared transcription factors provide the same function in both appendages. For example, Fgf10 expressed from the AER is involved in limb outgrowth, however in the genital tubercle it functions in the closure of the urethral tube (Haraguchi et al., 2000).

The shared expression of many developmentally important genes between limb buds and the genital tubercle poses an interesting question: what are the molecular mechanisms by which shared patterning genes drive the development of appendages towards significantly distinct morphological outcomes? To begin answering this question it is important to uncover the make up and function of the active appendage regulome. However, no one has yet determined the extent of overlap between active limb and genital tubercle enhancers. To answer this question we performed chromatin immunoprecipitation followed by massive parallel sequencing (ChIP-seq) on several mouse tissues using an antibody against H3K27ac, a histone modification known to associate strongly with active enhancers and promoters (Creyghton et al., 2010; Ernst et al., 2011). Although ChIP-seq using this marker has previously been performed on forelimbs and hindlimbs of E11.5 mice (Cotney et al., 2012), we wanted all

the datasets to be fully comparable to those obtained from other tissues, thus we generated all of our own data from limbs, genital tubercle, and control tissues using standardized conditions.

We found that out of the 906 total putative active limb enhancers, 284 (31%) are also active in the genital tubercle showing a high degree of overlap between the active regulomes of these structures. Among the shared putative *cis*-regulatory elements is hindlimb enhancer B (HLEB), previously shown to drive robust appendage expression of *Tbx4* (Menke et al., 2008), a transcription factor necessary for hind-limb development and outgrowth (Naiche and Papaioannou, 2007). The role of HLEB in the development of the hindlimbs has been established (see Chapter 2). However, we wanted to further investigate the functional role of HLEB in the development of the genital tubercle derived tissues. We observed that the baculum of male $Tbx4^{\Delta HLEB/-}$ mutants is significantly smaller than controls displaying a 14.6 % reduction in length and a 25.9% reduction in width. Furthermore we discover a novel role for Tbx4 in the proper development of the urogenital system.

Results

Activity of limb-specific enhancers in the genital tubercle

Many of the signaling pathways and transcription factors that are involved in the development of the limb are also required for the proper development of the genital tubercle (Lin et al., 2013; Lin et al., 2009; Miyagawa et al., 2009; Seifert et al., 2010). We wanted to understand the active regulome of these developmentally important tissues and to uncover the extent of overlap between them. To do so we performed ChIP-Seq against H3K27ac, a histone modification known to be strongly associated with active enhancers and promoters, on E11.5 forelimb, E11.5 hindlimb, E12.5 genital tubercle, E11.5 flank and eye tissues. Due to the later

development of the genital tubercle relative to the limbs, genital tubercles were collected at E12.5.

After removal of promoter regions and exons from our generated datasets, we identified a total of 16,350 putative *cis*-regulatory elements enriched for H3K27ac in at least one of the tissues analyzed. We clustered these elements from each tissue type and formed a heatmap based on signal intensity (Figure 3.1, A). Some of the newly identified putative enhancers are marked in all tissue types with the same signature, most likely due to them being ubiquitously active (clusters 5 thought 10).

As we were interested in identifying the active regulome of limbs and genital tubercle, we concentrated on putative enhancers that do not cluster with the control tissues flank and eye (clusters 1 and 2). We noticed that many of the regions represented in the limb dataset were also represented in the dataset derived from genital tubercle. To further explore this relationships we looked at how well 171 previously reported limb enhancers are represented in each of our datasets. These functionally validated limb regulatory elements were identified using other methods, and thus only about half of them (81) were represented in our limb H3K27ac dataset (Visel et al., 2009). This observation is consistent with previous reports that H3K27ac and P300 associate with most but not all active enhancers (Cotney et al., 2012). Of these 81 previously validated limb enhancers, over half (63%) are marked by significant H3K27ac signal in our E12.5 genital tubercle derived data set (Figure 3.1, B). Taken together this data suggests that there is a significant overlap in not only the genes that are expressed in these appendage types but also between the cis-regulatory elements that are active in these different tissues.

HLEB is functionally important in the development of the hindlimb and the genital tubercle

In order to determine if enhancers with shared activity in both limbs and genital tubercle are functionally important in both of these structures, we decided to focus on the *Tbx4* locus. Tbx4 is a developmentally crucial transcription factor that is expressed in the hindlimbs and the genital tubercle (Fig 3.2, A). Furthermore, this gene is under the regulation of at least two distinct limb enhancers, HLEA and HLEB, but out of the two only HLEB drives robust expression in both hindlimbs and genital tubercle (Fig 3.3 C) (Menke et al., 2008). Investigating where H3K27ac is enriched across the *Tbx4* locus revealed that the histone mark associates strongly with HLEA only in limbs, however this mark associates strongly with HLEB in both limbs and genital tubercle (Fig 3.2, B).

We wanted to see whether or not HLEB drives *in vivo* expression of *Tbx4* in the genital tubercle. To do so we used previously generated knockout mutant mice with a deletion of the endogenous HLEB (ΔHLEB/ΔHLEB) (see Chapter 2). Upon performing mRNA *in situ* hybridization on these mutants it was apparent that deleting HLEB and not HLEA significantly reduces the expression of *Tbx4* in the genital tubercle (Fig 3.4, A). This observation was confirmed via allele specific expression, a method of quantifying levels of *Tbx4* transcript driven by a specific allele. Using this method we observed that at E11.5 the expression level of *Tbx4* from the ΔHLEB allele is reduced by 23% compared to wild type, and that a day later reduction in expression is more significant (39%) (Figure 3.4, B and C). In contrast, *Tbx4* expression from the HLEA knockout allele is not statistically different from wild type within the genital tubercle at any time point studied. HLEB has been established as an enhancer with a very important function in driving

correct *Tbx4* expression in various domains or compartments of the developing limb and if disrupted the resulting mutant mice display hindlimb skeletal abnormalities (see Chapter 2).

Given that *Tbx4* is also expressed in the genital tubercle, we wanted to determine if there are any genital phenotypes. 11% of the HLEB heterozygous mutant females exhibit vaginal septum, while homozygous animals have a higher incidence of nearly 50% (Fig 3.5). This abnormality separates the vagina into two separate compartments. To ensure that the deletion of the enhancer was in fact responsible for this phenotype, we crossed HLEB homozygous mutant males to wildtype C57/BL6J females purchased from the Jackson Laboratory. Interestingly, the incidence of vaginal septum in resulting HLEB heterozygous mutant females increased almost three fold (Fig 3.5, B). This confirms that the vaginal abnormality is in fact due to disruption of HLEB.

We bred ΔHLEB/ΔHLEB females to heterozygous $Tbx4^{+/-}$ null males to generate $Tbx4^{\Delta HLEB/-}$ mice and noticed that the overall size of the baculum is smaller in these animals (Fig. 3.6 compare C to A). The length is reduced by 14.6% while the width at the base of the baculum is reduced by 25.9% (Fig. 3.6, D). In contrast, the baculi of $Tbx4^{\Delta HLEA/-}$ mice are not statistically different from wild type further confirming that HLEA does not contribute to *Tbx4* expression in the genital tubercle. The disruption of HLEB affects both the hindlimbs and the tissues that arise from the genital tubercle, which supports our initial observations regarding the association of H3K27ac with this enhancer in both tissues examined.

Interestingly, a further analysis of 12 $Tbx4^{\Delta HLEB/-}$ mice revealed that half harbored significant renal abnormalities (Fig. 3.7). Three individuals displayed kidney agenesis, two showed polycystic kidneys and one individual showed grossly abnormal renal tissue (Fig 3.7,

Table 3.1). All the male mutants had abnormally shaped seminal vesicles and many displayed extended bulbourethral glands (Fig 3.8, Table 3.2). Similarly, 50% of the *Tbx4*$^{\Delta HLEB/-}$ females suffered from a high transverse septum resulting in imperforate vagina which caused, by improper drainage, an accumulation of mucosa resulting in significantly distended uterine horns (Fig., 3.8, Table 3.3). We also noticed that one of the uterine horns of several mutant females was significantly underdeveloped (Fig 3.8, H). Taken together this data shows that *Tbx4*, under the regulation of HLEB, is responsible for the proper development of both hindlimbs as well as the urogenital system in mice. Due to the activity of HLEB in multiple tissues during development, it is understandable why it is strongly associated with H3K27ac in multiple tissues. Given that a significant number of other putative enhancers display the same histone profiles it is possible that they provide a similar multi-tissue function.

Discussion

Shared enhancers are important for the development of both hindlimb and external genitalia

We know a lot about what genes and signaling pathways are involved in the early development of the limb but we know virtually nothing about how these genes interact to give rise to such a complex structure. Given the large extent to which transcription factors and signaling cascades are shared between forelimbs, hindlimbs and the genital tubercle, this raised the possibility that there may be subtle differences in the spatio-temporal distribution of these factors. Such differences would be under the control of *cis*-regulatory elements, and thus we sought to elucidate the active regulomes of limbs and genital tubercle in the hopes of understanding how shared elements can interact to give rise to multiple appendages with distinct morphologies.

Microarray analyses between forelimb and hindlimb autopods of E12.5 mouse embryos showed a remarkable similarities in gene expression between the two (Shou et al., 2005), suggesting that the morphological differences between appendages arises from differential use of commonly expressed genes. These differences could arise from distinct regulatory elements active in each appendage. A global analysis using ChIP-seq on E10.5 and E11.5 mouse forelimb and hindlimb buds uncovered a very high degree of shared regulatory element usage between the two appendages (Cotney et al., 2012) which mirrors our findings exactly.

More remarkable however, is the degree of shared active enhancers that we uncovered between forelimbs, hindlimbs and the genital tubercle. The genital tubercle gives rise to the external genitalia, including the penis in males and clitoris in females. The mineralized bones of these appendages are relatively simple in structure compared to the complexity of both forelimbs and hindlimbs such as the lack of articulation. Why then, would genital tubercles contain such a remarkably complex regulatory network that is shared with the limbs? Some speculate that the genital tubercle arose as a fin derived appendage (Minelli, 2002; Rosa-Molinar and Burke, 2002) and thus may have co-opted both the gene networks as well as the regulome of fins. This however does not explain the morphological differences underlined by the functional divergence between appendages. We speculate that minor differences in the use of shared enhancers may occur, such as the availability of only a subset of transcription factor binding sites in each appendage.

Indeed, given the nature of histone marks, we cannot precisely tell whether the entire sequence of the enhancer is available or not for transcription binding within a specific tissue. Also, putative appendage enhancers do display subtle differences in their histone signatures

that could allow different sequences to be exposed for transcription binding in different appendages. It is also possible that different subsets of shared enhancers are active in different domains of each appendage, thus performing distinct spatio-temporal functions. We have previously reported the case of the *Tbx4* locus, where two distinct limb enhancers drive gene expression in different compartments of the limb bud across different developmental stages, and when independently disrupted result in distinct phenotypes (See Chapter2). Yet another explanation may be from subtle differences in levels of gene expression. Microarray analyses have determined that although forelimbs, hindlimbs and genital tubercles share much of their genetic networks, there are subtle differences in terms of levels of gene expression (Cobb and Duboule, 2005; Shou et al., 2005). Nonetheless all of these mechanisms may work together to use the core conserved appendage "toolkit" to generate morphologically and functionally distinct structures.

Tbx4 contributes to the proper development of external genitalia and urogenital system

Unexpectedly, we found a previously unreported role for *Tbx4*. When we took a close look at the urogenital tissues of adult $Tbx4^{\Delta HLEB/-}$ mutant mice we noticed severe abnormalities of the kidneys and reproductive organs, including the seminal vesicles in males and uterine tubes in females. In contrast, the complex role of *Tbx4* during limb development has been previously described (Hasson et al., 2010; Menke et al., 2008; Naiche et al., 2011; Naiche and Papaioannou, 2007). The function of this gene in the development of external genitalia, however, has reported here for the first time.

Using a hypomorphic allele on a sensitive background we noticed a significant decrease in the overall size of the male baculum which correlates with lowered gene expression in the

early stages of genital development. These results indicate that the proper expression of *Tbx4* via a genital tubercle enhancer, HLEB, is necessary for the correct development of external genitalia. Although *Tbx4* has been shown to be expressed in both developing limbs and genital tubercles (Chapman et al., 1996; Menke et al., 2008), reporter assays have failed to detect gene expression in tissues that give rise to the urogenital system. However, cell lineage tracing experiments have shown that by E14.5 most of the tissues that give rise to some of the urogenital tissues are derived from cells that expressed Tbx4 (Naiche et al., 2011). This leaves a few questions that will need to be answered in the future, such as when and by what mechanism does Tbx4 mediate development of urogenital system?

Materials and methods

Chromatin immunoprecipitation

Timed matings were performed with outbred ICR mice (Harlan Laboratories) and embryonic hindlimb and forelimb buds (from 32 and 27 embryos respectively per each biological replicate) as well as flank (16 embryos per each biological replicate) were collected at E11.5 while genital tubercles (from 39 and 34 embryos per each biological replicate) were collected at E12.5. After cross-linking in 1% formaldehyde in PBS for 20 min, dissected tissues were rinsed and treated with trypsin for 5 min to generate a single cell suspension. Samples were then sheared by sonication to generate a chromatin size range of 200–600 bp. PureProteome^{TM} Protein G Magnetic Beads (Millipore) were pre-incubated with H3K27ac antibody (Milipore NG-1918613) before incubating overnight with 500µg of chromatin from limb and flank tissue and 400µg from genital tubercle. After washing, immune complexes were eluted from the beads, and protein-DNA crosslinks were reversed by incubating at 65 °C

overnight. After treatment with RNase followed by proteinase K, samples were purified with the GeneJET™ PCR Purification Kit (Fermentas). Two independent biological replicates were used to generate two Illumina ChIP-Seq and two control libraries for each tissue type. All ChIP and input chromatin control libraries were produced using the NEBNext ChIP-Seq Library Prep kit using Hudson Alpha provided adapters. Single-end libraries were sequenced on an Illumina GA IIx, producing 36 bp reads (replicate 1), or a HiSeq 2000, producing 50 bp reads (replicate 2), at the HudsonAlpha Institute for Biotechnology.

Quantitative PCR was used to evaluate the relative abundance of four distinct enhancers known to be active in limbs, in both input and H3K27ac-ChIP DNA as compared to the intron of the *Stra8* gene known to be inactive in these tissues.. qPCR assays were performed in triplicate using Maxima^{TM} SYBR Green ROX mix (Fermentas) on an ABI7500 (Applied Biosystems). To control for normalization we used the promoter region of Stra8, a gene known to be expressed exclusively in premeiotic germ cells. The $2^{-\Delta\Delta t}$ method was used for calculating enrichments for each target in ChIP DNA relative to input. The primers used in qPCR were as follows: hs1430F: 5′-TTCTCCCAGGCTCAGGCAGCA -3′; hs1430R: 5′-TCCAGTCAAGGCCACGGGACT -3′; hs1432F: 5′-CGACTGCACAAATGAGACACG -3′; hs1432R: 5′-TGAAGTTGGCAGGAGCTGAGC -3′; hs1452_mouse_F1: 5′- AGCTCTGCCTGTTATAAGTCAG-3′; hs1452_mouse_R1: 5′-GACATGTGGTGGAGCAGTTA -3′; hs1473_mouse_F1: 5′- AACCTGAAGTCAGGAGCGAG-3′; hs1452_mouse_R1: 5′-GTCATCAGGCTATAGGGTGAG -3′; Mouse_GAPDHEx2Ex3_F1: 5′-AAGGTCGGTGTGAACGGATTTG -3′; Mouse_GAPDHEx3_R1: 5′-GTCGTTGATGGCAACAATCTCC -3′; mStra8_intron1_ChIPcontrol_F3: 5′-ATGGCTGGCTAAATGAGCTAGC -3′; mStra8_intron1_ChIPcontrol_R3: 5′-GAGATGATACAACACATGGTCATGC -3′.

Transgenic and mutant mice

Both transgenic and mutant mice were previously described ; also see Chapter 2).

Skeletal preparations and measurements

The adult mouse skeletons as were prepared using Alcian Blue and Alizarin Red as previously described (Lufkin et al., 1992). Adult stained bones were carefully dissected and cleaned, placed flat on a Petri dish containing 100% glycerol and photographed next to a ruler. Images were uploaded to GIMP2 (www.gimp.org), an image manipulation program that allowed us to measure the exact number of pixels between two bone features. The ruler gave us the pixel/mm ratio that was used to convert the measurements made from GIMP2 to mm. The angle between the ischium and the pubic bone was measured in two steps using the same program. The distance measuring tool also gives the angle between the bone and the perceived horizontal of the image. This was used to calculate the angle between the ischium and the horizontal as well as the angle between the horizontal and the pubis, the two measurements giving the total angle between these bones. The area of the bones was measured using ImageJ (for an online tutorial http://rsb.info.nih.gov/ij/docs/pdfs/examples.pdf). P values were calculated using a two-tailed t-test assuming unequal variation.

Allele specific expression

The allele specific expression assay was designed following the protocol outlined by Jeong and colleagues (Jeong et al., 2007). Briefly, we made use of a naturally occurring SNP in the *Tbx4* transcript between C57BL/6J and DBA/2J (G->A). We bred C57BL/6J females with the enhancer deletion allele to DBA/2J males to obtain either ΔHLEA or ΔHLEB heterozygous embryos on mixed background. All embryos would have a wild type DBA/2J allele containing

Tbx4 that harbors an “A” and either a wild type C57BL/6J allele or a C57BL/6J allele harboring a “G” within the *Tbx4* transcript. We designed a FRET probe complementary to the C57BL/6J Tbx4 transcript that would bind the DBA/2J transcript with a different melting temperature due to the base pair mismatch. If both transcripts are present that would give a measureable difference in melting curves and thus we could then quantify the relative ratio of G containing transcript (driven by the mutant allele) versus the A (driven by the wild type allele) from each individual embryo. A ratio of 1 means that both alleles are equally efficient at driving *Tbx4* expression, while a G/A ratio of less than one means the mutant allele is not as efficient.

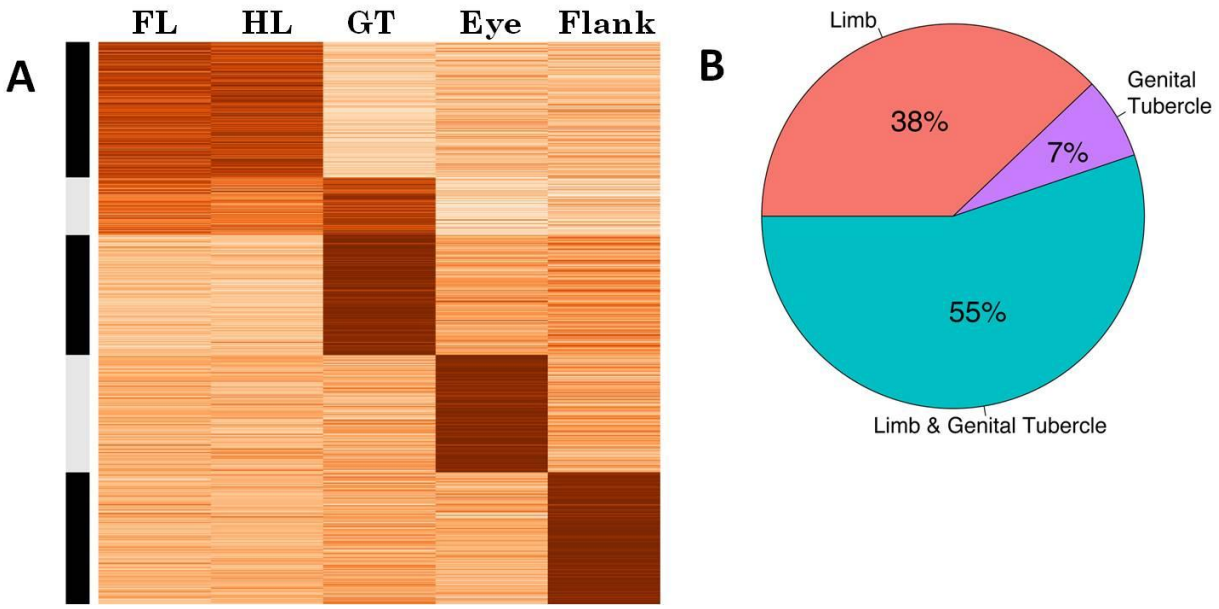


Figure 3.1 Over half of the putative enhancers identified by H3K27ac mark are shared between limb and genital tubercle. Clustering of H3K27ac signals reveals groups of putative *cis*-regulatory elements with similar patterns of activity across tissue types (A). Pie chart shows the extent to which the H3K27ac signal of 171 functionally validated limb enhancers overlap between limbs and genital tubercle (B).

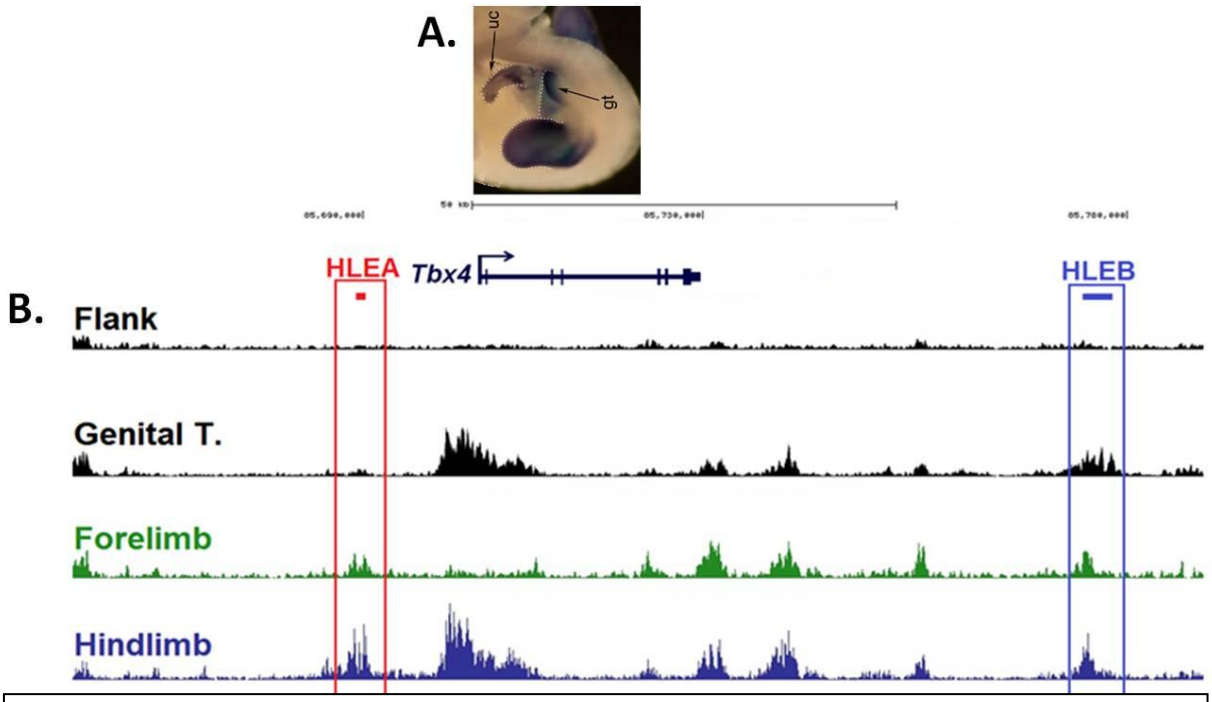


Figure 3.2 *Tbx4* limb and genital expression is regulated by hindlimb enhancer B (HLEB). Tbx4 is expressed in both hindlimbs and genital tubercle (A) and is under the regulation of two distinct enhancers, hindlimb enhancer A (HLEA) and B (HLEB) (B). Panel B shows how H3K27ac mark is distributed cross the Tbx4 locus in four distinct tissues.

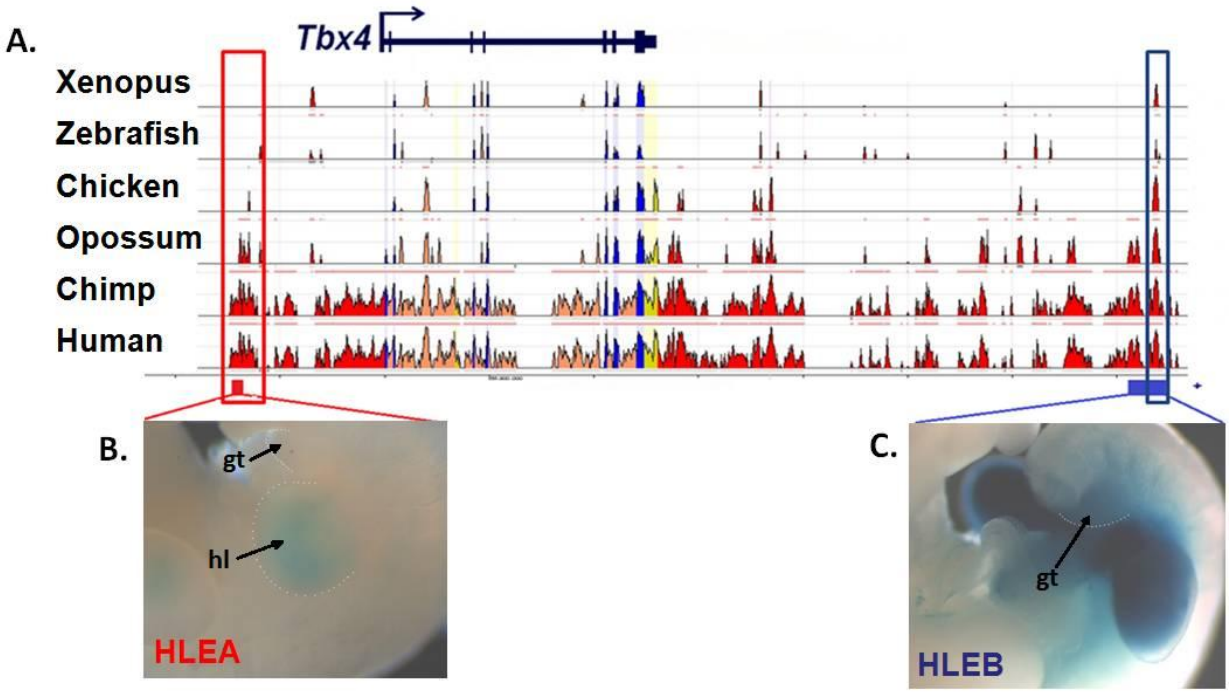


Figure 3.3. *Tbx4* limb expression is regulated by two distinct enhancers, hindlimb enhancer A (HLEA) and B (HLEB). A conservation track from VISTA (A) shows the extent of conservation of HLEA (red block) and HLEB (blue block) among vertebrates. Whole-mount *LacZ* stained E11.5 mouse embryos carrying HLEA-hsp68*LacZ* (B) and HLEA-hsp68*LacZ* (C) transgenes. Gt stands for genital tubercle, hl stands for hind limb

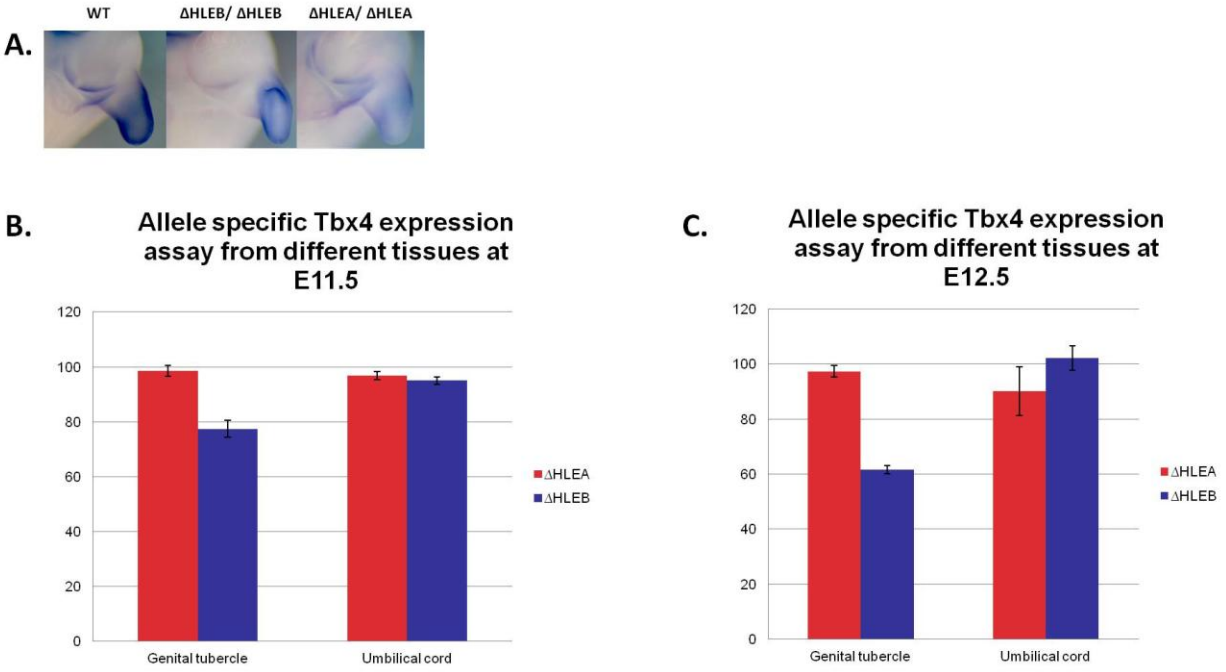
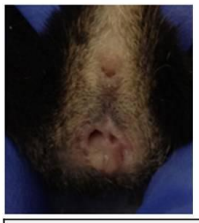


Figure 3.4 Expression data supports that HLEB is important for the proper expression of Tbx4 in the genital tubercle. In situ hybridization using a Tbx4 probe was performed on E11.5 homozygous mutant embryos. Allele specific expression data which quantifies how efficiently each of the two alleles, HLEA and HLEB, are at driving expression of Tbx4 compared to wild type (set at 100%) (B).

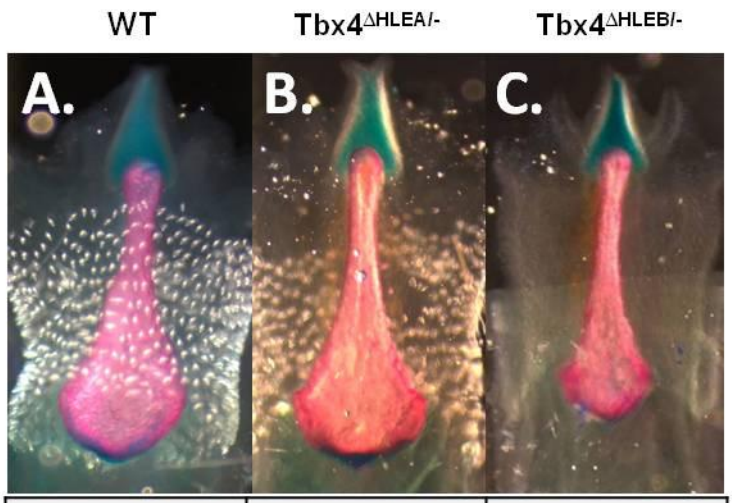
A. ΔHLEB/ ΔHLEB



B.

Home Strain	Number of Females with Vaginal Septum	Percent Affected	Home Strain Crossed to C57/*BL6*	Number of Females with Vaginal Septum	Percent Affected
WT	0/7	0%	WT	0/9	0%
ΔHLEB/ +	2/19	11%	ΔHLEB/ +	5/16	31%
ΔHLEB/ ΔHLEB	7/15	47%			

Figure 3.5 Heterozygous HLEB females display a high frequency of vaginal septum. A homozygous HLEB female displays vaginal septum representative of both homozygous and heterozygous genotypes (A). Table (B) shows the frequency at which this phenotype is seen within our home strain colony and within females from a cross between home strain males and C57/BL6 females.



D.

Genotype Compared to WT	Length of Bachulum	Width of Bachulum
Tbx4$^{\Delta HLEA/-}$	1.6%	+0.5%
	N.S.	N.S.
Tbx4$^{\Delta HLEB/-}$	14.6%	25.9%
	P=1.5E-05	P=3.3E-08

Figure 3.6 Adult HLEB males on a hypomorphic allele display significantly smaller baculi. The baculum length and width of *Tbx4*$^{\Delta HLEB/-}$ is significantly smaller than wild type (compare C and A). In contrast the baculi of adult *Tbx4*$^{\Delta HLEA/-}$ are indistinguishable from wild type (compare B to A). Table (D) displays percent reduction of baculi as compared to wild type controls.

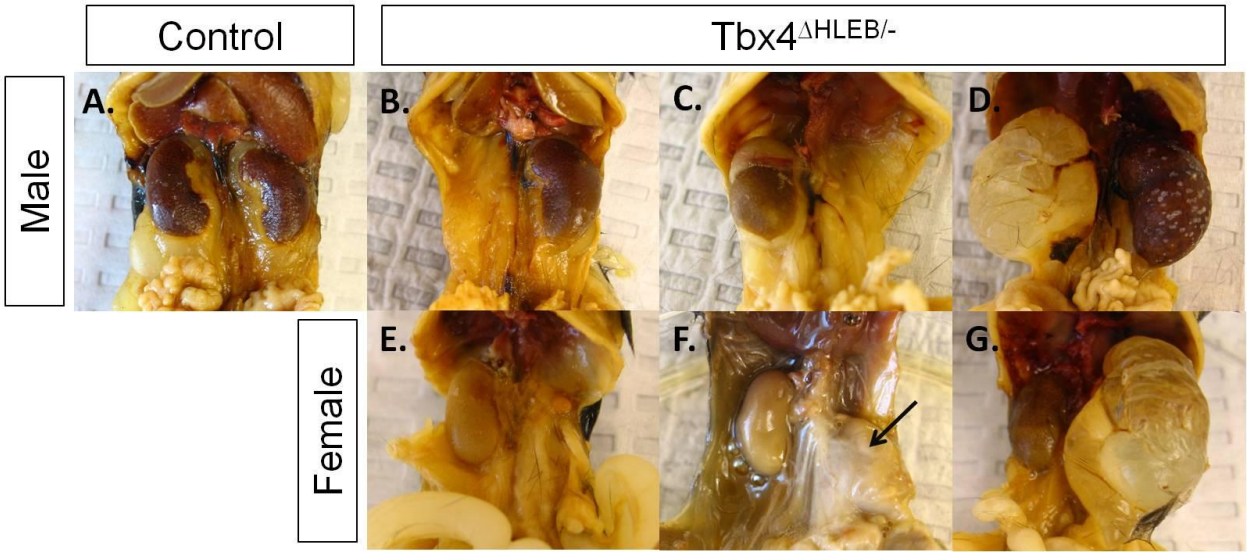


Figure 3.7 *Tbx4* is involved in proper kidney development. Both male and female *Tbx4*$^{\Delta HLEB/-}$ adult mice display renal agenesis (B, C, E), multicystic (D and G) or grossly deformed kidneys (F, black arrow).

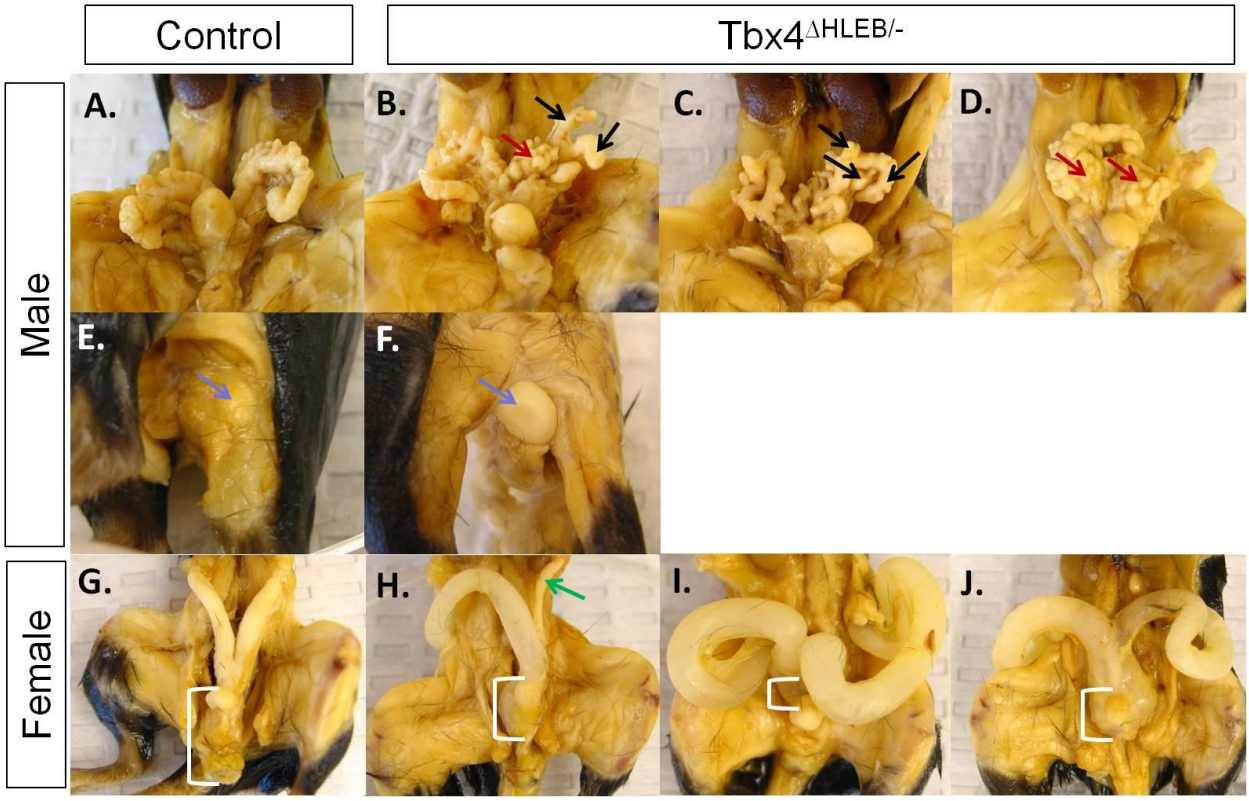


Figure 3.8 *Tbx4* is involved in the proper development of the internal reproductive tissues. Male *Tbx4*$^{\Delta HLEB/-}$ adult mice display mispatterned seminal vesicles (B, C and D) and enlarged bulbourethral glands (F, blue arrow). The uteri of females can be underdeveloped (H, green arrow) or massively distended (H,I and J). The vagina of *Tbx4*$^{\Delta HLEB/-}$ female mice does not fully extend posteriorly and in such cases, no vaginal opening is observed (H,I and J white brackets).

Table 3.1 *Tbx4*$^{\Delta HLEB/-}$ adult mice display a high incidence of kidney abnormalities

Kidney Agenesis	Malformed Kidney	Polycystic Kidney	Total percent of individuals with a kidney defect
3/24	1/24	2/24	50%

Six out of twelve male and female individuals display at least one abnormal kidney phenotype such as kidney agenesis. None of the observed abnormalities seem to be biased to either the left or right side of the animals.

Table 3.2 Incidence of reproductive abnormalities in *Tbx4*$^{\Delta HLEB/-}$ adult males

Seminal Vesicle Overgrowth	Seminal Vesicle Multiple Horns	Distended Bulbourethral Gland	Absent Bulbourethral Gland
6/12	8/12	8/12	1/12

Six total male *Tbx4*$^{\Delta HLEB/-}$ individuals display grossly abnormal seminal vesicle as well as enlarged bulbourethral glands. None of the observed abnormalities seem to be biased to either the left or right side of the animals.

Table 3.3 Incidence of reproductive abnormalities in Tbx4$^{\Delta HLEB/-}$ adult females

Absent Vaginal Opening	Incomplete Vaginal Canal	Distended Uterine Horns	Underdeveloped Uterine Horns
2/4	2/4	4/10	2/10

Five total female *Tbx4*$^{\Delta HLEB/-}$ individuals display abnormalities of the uterine horns and vaginal canals. The vaginal canal of one female could not be assessed. The ovaries of these mice seem normal, though more careful observations need to be made. None of the observed abnormalities seem to be biased to either the left or right side of the animals.

References

Chiang, C., Litingtung, Y., Lee, E., Young, K.E., Corden, J.L., Westphal, H., Beachy, P.A., 1996. Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. Nature 383, 407-413.

Cobb, J., Duboule, D., 2005. Comparative analysis of genes downstream of the Hoxd cluster in developing digits and external genitalia. Development 132, 3055-3067.

Cotney, J., Leng, J., Oh, S., DeMare, L.E., Reilly, S.K., Gerstein, M.B., Noonan, J.P., 2012. Chromatin state signatures associated with tissue-specific gene expression and enhancer activity in the embryonic limb. Genome research 22, 1069-1080.

Creyghton, M.P., Cheng, A.W., Welstead, G.G., Kooistra, T., Carey, B.W., Steine, E.J., Hanna, J., Lodato, M.A., Frampton, G.M., Sharp, P.A., Boyer, L.A., Young, R.A., Jaenisch, R., 2010. Histone H3K27ac separates active from poised enhancers and predicts developmental state. Proceedings of the National Academy of Sciences of the United States of America 107, 21931-21936.

Dollé, P., Dierich, A., LeMeur, M., Schimmang, T., Schuhbaur, B., Chambon, P., Duboule, D., 1993. Disruption of the Hoxd-13 gene induces localized heterochrony leading to mice with neotenic limbs. Cell 75, 431-441.

Dunn, N.R., Winnier, G.E., Hargett, L.K., Schrick, J.J., Fogo, A.B., Hogan, B.L., 1997. Haploinsufficient phenotypes in Bmp4 heterozygous null mice and modification by mutations in Gli3 and Alx4. Developmental biology 188, 235-247.

Ernst, J., Kheradpour, P., Mikkelsen, T.S., Shoresh, N., Ward, L.D., Epstein, C.B., Zhang, X., Wang, L., Issner, R., Coyne, M., Ku, M., Durham, T., Kellis, M., Bernstein, B.E., 2011. Mapping and analysis of chromatin state dynamics in nine human cell types. Nature 473, 43-49.

Goodman, F.R., Bacchelli, C., Brady, A.F., Brueton, L.A., Fryns, J.P., Mortlock, D.P., Innis, J.W., Holmes, L.B., Donnenfeld, A.E., Feingold, M., Beemer, F.A., Hennekam, R.C., Scambler, P.J., 2000. Novel HOXA13 mutations and the phenotypic spectrum of hand-foot-genital syndrome. Am J Hum Genet 67, 197-202.

Haraguchi, R., Suzuki, K., Murakami, R., Sakai, M., Kamikawa, M., Kengaku, M., Sekine, K., Kawano, H., Kato, S., Ueno, N., Yamada, G., 2000. Molecular analysis of external genitalia formation: the role of fibroblast growth factor (Fgf) genes during genital tubercle formation. Development 127, 2471-2479.

Hasson, P., DeLaurier, A., Bennett, M., Grigorieva, E., Naiche, L.A., Papaioannou, V.E., Mohun, T.J., Logan, M.P., 2010. Tbx4 and tbx5 acting in connective tissue are required for limb muscle and tendon patterning. Developmental cell 18, 148-156.

Izpisua-Belmonte, J.C., Brown, J.M., Crawley, A., Duboule, D., Tickle, C., 1992. Hox-4 gene expression in mouse/chicken heterospecific grafts of signalling regions to limb buds reveals similarities in patterning mechanisms. Development 115, 553-560.

Jeong, S., Hahn, Y., Rong, Q., Pfeifer, K., 2007. Accurate quantitation of allele-specific expression patterns by analysis of DNA melting. Genome research 17, 1093-1100.

Lin, C., Yin, Y., Bell, S.M., Veith, G.M., Chen, H., Huh, S.H., Ornitz, D.M., Ma, L., 2013. Delineating a conserved genetic cassette promoting outgrowth of body appendages. PLoS genetics 9, e1003231.

Lin, C., Yin, Y., Veith, G.M., Fisher, A.V., Long, F., Ma, L., 2009. Temporal and spatial dissection of Shh signaling in genital tubercle development. Development 136, 3959-3967.

Lopez-Martinez, A., Chang, D.T., Chiang, C., Porter, J.A., Ros, M.A., Simandl, B.K., Beachy, P.A., Fallon, J.F., 1995. Limb-patterning activity and restricted posterior localization of the amino-terminal product of Sonic hedgehog cleavage. Current biology : CB 5, 791-796.

Lufkin, T., Mark, M., Hart, C.P., Dolle, P., LeMeur, M., Chambon, P., 1992. Homeotic transformation of the occipital bones of the skull by ectopic expression of a homeobox gene. Nature 359, 835-841.

Menke, D.B., Guenther, C., Kingsley, D.M., 2008. Dual hindlimb control elements in the Tbx4 gene and region-specific control of bone size in vertebrate limbs. Development 135, 2543-2553.

Minelli, A., 2002. Homology, limbs, and genitalia. Evolution & development 4, 127-132.

Miyagawa, S., Moon, A., Haraguchi, R., Inoue, C., Harada, M., Nakahara, C., Suzuki, K., Matsumaru, D., Kaneko, T., Matsuo, I., Yang, L., Taketo, M.M., Iguchi, T., Evans, S.M., Yamada, G., 2009. Dosage-dependent hedgehog signals integrated with Wnt/beta-catenin signaling regulate external genitalia formation as an appendicular program. Development 136, 3969-3978.

Morgan, E.A., 2003. Loss of Bmp7 and Fgf8 signaling in Hoxa13-mutant mice causes hypospadia. Development 130, 3095-3109.

Murakami, R., Mizuno, T., 1986. Proximal-distal sequence of development of the skeletal tissues in the penis of rat and the inductive effect of epithelium. Journal of embryology and experimental morphology 92, 133-143.

Naiche, L.A., Arora, R., Kania, A., Lewandoski, M., Papaioannou, V.E., 2011. Identity and fate of Tbx4-expressing cells reveal developmental cell fate decisions in the allantois, limb, and external genitalia. Developmental dynamics : an official publication of the American Association of Anatomists 240, 2290-2300.

Naiche, L.A., Papaioannou, V.E., 2007. Tbx4 is not required for hindlimb identity or post-bud hindlimb outgrowth. Development 134, 93-103.

Perriton, C.L., Powles, N., Chiang, C., Maconochie, M.K., Cohn, M.J., 2002. Sonic hedgehog signaling from the urethral epithelium controls external genital development. Developmental biology 247, 26-46.

Rosa-Molinar, E., Burke, A.C., 2002. Starting from fins: parallelism in the evolution of limbs and genitalia: the fin-to-genitalia transition. Evolution & development 4, 124-126.

Seifert, A.W., Zheng, Z., Ormerod, B.K., Cohn, M.J., 2010. Sonic hedgehog controls growth of external genitalia by regulating cell cycle kinetics. Nature communications 1, 23.

Shou, S., Scott, V., Reed, C., Hitzemann, R., Stadler, H.S., 2005. Transcriptome analysis of the murine forelimb and hindlimb autopod. Developmental dynamics : an official publication of the American Association of Anatomists 234, 74-89.

Spitz, F., Gonzalez, F., Duboule, D., 2003. A global control region defines a chromosomal regulatory landscape containing the HoxD cluster. Cell 113, 405-417.

Suzuki, K., Bachiller, D., Chen, Y.P., Kamikawa, M., Ogi, H., Haraguchi, R., Ogino, Y., Minami, Y., Mishina, Y., Ahn, K., Crenshaw, E.B., 3rd, Yamada, G., 2003. Regulation of outgrowth and apoptosis for the terminal appendage: external genitalia development by concerted actions of BMP signaling [corrected]. Development 130, 6209-6220.

Tickle, C., Summerbell, D., Wolpert, L., 1975. Positional signalling and specification of digits in chick limb morphogenesis. Nature 254, 199-202.

Visel, A., Blow, M.J., Li, Z., Zhang, T., Akiyama, J.A., Holt, A., Plajzer-Frick, I., Shoukry, M., Wright, C., Chen, F., Afzal, V., Ren, B., Rubin, E.M., Pennacchio, L.A., 2009. ChIP-seq accurately predicts tissue-specific activity of enhancers. Nature 457, 854-858.

Warot, X., Fromental-Ramain, C., Fraulob, V., Chambon, P., Dolle, P., 1997. Gene dosage-dependent effects of the Hoxa-13 and Hoxd-13 mutations on morphogenesis of the terminal parts of the digestive and urogenital tracts. Development 124, 4781-4791.

Zakany, J., Fromental-Ramain, C., Warot, X., Duboule, D., 1997. Regulation of number and size of digits by posterior Hox genes: a dose-dependent mechanism with potential evolutionary implications.

Proceedings of the National Academy of Sciences of the United States of America 94, 13695-13700.

CHAPTER 4

FUTURE DIRECTIONS

Identification of Other Hindlimb and Genital Tubercle Enhancers

Thus far only two enhancers that drive *Tbx4* expression in the hindlimbs, HLEA and HLEB, have been identified (Menke et al., 2008). Our data shows that HLEB drives robust expression in the genital tubercle as well and thus can be considered an enhancer with multiple functions in mice. However, when HLEB is knocked out, the resulting *Tbx4* expression in the genital tubercle is not entirely reduced at E12.5, suggesting the existence of at least one other enhancer that drives the remaining gene expression in this tissue. Similarly, the hindlimb buds of $Tbx4^{\Delta HLEA/\Delta HLEA}$ and $Tbx4^{\Delta HLEB/\Delta HLEB}$ mutant embryos display only partial loss of gene expression that together do not recapitulate what would be expected from a homozygous null mutant. These observations indicate that the *Tbx4* locus is more complex that previously considered, with more than two enhancers driving tissue specific expression in both hindlimbs and genital tubercle. Such enhancers should be identified if we are to have a better understanding of how *Tbx4* is regulating during the development of the hindlimbs and the external genitalia.

By comparing the relative signal strength of H3K27ac around the *Tbx4* locus from different tissues we identified three regions harboring putative new appendage regulatory elements (Fig 4.1). Regions I and III show areas with a significant and strong association of this histone mark only in the genital tubercle and not any of the other tissue examined. In contrast,

region III harbors sequences more strongly associated with H3K27ac in the hindlimbs as compared to the forelimbs and therefore could be novel hindlimb *cis*-regulatory elements of *Tbx4*. The exact function of these putative appendage enhancer regions should be investigated using transgenic reporter assays. Depending on the initial results, each region can be further divided into distinct sequences with strong H3K27ac association that can be tested individually.

Because H3k27ac only marks a subset of active enhancers, there is a distinct possibility that some *Tbx4* enhancers active in hindlimbs or genital tubercle may not be identified using this approach. For further identification of other possible appendage regulatory elements not represented in our data we would need to use other markers such as P300.

Identifying the Potential Transcriptome Diversity of Hindlimb Compartments Delineated by *Tbx4* Expression

Charting the expression patterns of *Tbx4* in the developing hindlimb buds of $Tbx4^{\Delta HLEA/\Delta HLEA}$ and $Tbx4^{\Delta HLEB/\Delta HLEB}$ mutant embryos, we have determined that each enhancer drives gene expression in distinct areas of the developing limb. HLEA function is important for driving expression in the anterior part of the growing limb from E10.5 to E12.5, while HLEB is most critical for driving *Tbx4* expression in a discrete proximo-medial domain of the E10.5 hindlimb. Nonetheless, Tbx4 is a transcription factor that is expressed strongly throughout the mesenchyme of the early hindlimb bud, from E9.5 to E11.5. During these early stages *Tbx4* is involved in the patterning of hindlimb bones (Naiche and Papaioannou, 2007). Later in development, *Tbx4* expression becomes dynamically restricted and has been shown to be involved in muscle and tendon patterning (Hasson et al., 2010).

Very little is known about the molecular map of the limb bud mesenchyme in terms of developmental potential. The ZPA for example, is made up of mesenchymal cells located posteriorly within the limb and it expresses Shh, a morphogen involved in the patterning of the antero-posterior axis (Harfe, 2011). When the ZPA compartment is transplanted to the anterior part of the limb, some mesenchymal cells become repatterned resulting in mirror-like duplication of digits (Suzuki, 2013). Gli3, is an important limb transcriptional mediator shown to be under the regulation of at least two distinct *cis*-regulatory elements with a very dynamic pattern of limb expression (Abbasi et al., 2010). Just like *Tbx4*, *Gli3* has multiple functions within the developing limb, an early role in proximo-distal patterning of limb bones and a later role in antero-posterior patterning of the autopod (Barna et al., 2005). These examples highlight the complexity of the developing limb bud and the need to analyze this tissue as such in future experiments. Thus far most genome-wide analyses, such as our own, have focused heavily on the whole limb tissue and are therefore averaged outcomes where important signals from a small population of cells may be lost. For example, enhancers known to be active in the AER (a small population of ectodermal cells located at the distal tip of the limb bud) are not represented in our data suggesting the possibility that other important limb regulatory elements can not be identified using such approaches on the entire limb bud.

However, using the *Tbx4* locus, we can tackle this problem by analyzing the transcriptome and the corresponding active regulome of delineated *Tbx4* expressing limb compartments. The E10.5 hindlimb looks like a good choice due the stronger impact on gene expression when either HLEA or HLEB is knocked out at this stage (Fig 4.2). Performing RNA-seq and ChIP-seq using markers of active enhancers such as H3K27ac on subdomains of the limb

from wild type E10.5 embryos will allow us to determine how similar the transcriptome and regulome of each compartment are from each other.

We would also be able to identify putative limb enhancers previously missed due to noise. Comparing such a dataset with a similar one generated from either *Tbx4*$^{\Delta HLEA/\Delta HLEA}$ or *Tbx4*$^{\Delta HLEB/\Delta HLEB}$ mutant embryos would allow us to determine possible downstream gene targets of *Tbx4*, which have remained for the most part illusive. From such a comparison we would also understand how these compartments interact. For example, if a gene X is downregulated in the anterior compartment of *Tbx4*$^{\Delta HLEB/\Delta HLEB}$ mutant hindlimbs (compartment shown to be under the regulation of HLEA) but the expression of this gene is fairly constant in *Tbx4*$^{\Delta HLEA/\Delta HLEA}$ mutants, would indicate a long range effect from one compartment to the other. Nevertheless, the future of limb development studies will surely migrate from studying the limb as a population study of all the cells of the limb and focus more on the complex differences and distinct developmental potentials already present in the growing limb buds.

Exploring the Relationship between *Tbx4* and *Shh* via *Alx4*

We have shown that disruption of *Tbx4* expression in the hindlimbs of *Tbx4*$^{\Delta HLEB/-}$ mutants, leads to a posterior expansion of the *Alx4* expression domain. *Alx4* null mutants have skeletal phenotypes including polydactility which was shown to be associated with an ectopic anterior expression of *Shh* in both mice (Qu et al., 1997) and chick (Takahashi et al., 1998). In *Alx4* null mutant mice, the ectopic expression of *Shh* is observed at E11.0 (Qu et al., 1997). Other important limb factors such as Hoxd13 and Hoxd11 are also seen ectopically expressed in the anterior portion of *Alx4* null limbs (Kuijper et al., 2005b; Panman et al., 2005). Interestingly, we noticed polydactyly in a mouse knock-in mutant established by our lab which the native

mouse HLEB as been replaced with the orthologous enhancer of *Anolis lineatopus* (data not shown). Preliminary data showed that this particular lizard HLEB drives higher levels of *Tbx4* expression than the mouse HLEB, which could potentially act to disrupt *Alx4* expression anteriorly. Such a disruption in gene expression could cause ectopic expression of Shh causing polydactyly. Nonetheless, the possible indirect relationship between *Tbx4*, *Shh*, *Hoxd11* and *Hoxd13* should be explored.

Using *in situ* hybridization and quantitative RT-PCR, we can determine whether the expression of *Shh* or *Hox* genes are indeed altered in the hindlimbs of $Tbx4^{\Delta HLEB/\Delta HLEB}$ and $Tbx4^{\Delta HLEB/-}$ mice. It is interesting to note that altered *Alx4* expression is observed only in $Tbx4^{\Delta HLEB/-}$ and not $Tbx4^{\Delta HLEA/-}$ mutants which indicates that this alteration is caused by disrupted *Tbx4* expression in a spatio-temporal way associated with HLEB control. This observation is quite interesting due to the more severe impact in *Tbx4* gene expression seen when HLEA is knocked out at later developmental time points. As seen from allele specific expression, the *ΔHLEA* allele drives less *Tbx4* expression than the *ΔHLEB* allele in both the anterior and posterior domains of hindlimb. It is possible that *Tbx4* expression from the medial compartment of the E10.5 hindlimb, which is under the control of HLEB, is responsible for inhibiting *Alx4* expression in that domain thus restricting its expression anteriorly. Alternatively, HLEB could have a greater impact than HLEA at early stages when Alx4 expression domain is being established.

Comprehensive Histology of Urogenital Phenotypes and Charting *Tbx4* Expression in these Tissues

We have discovered that *Tbx4* is important in the proper development of urogenital system, something which has never been reported before. However, this opens up a new set of questions to be answered, such as when in development is *Tbx4* needed and how does this gene effect development in these tissues? In order to fully understand what key role or roles *Tbx4* is playing in urogenital development, we must have a full understanding of the phenotypes associated with gene disruption.

So far we have characterized phenotypes by visual observations which may have missed subtle but nonetheless important abnormalities. For example, some females seem to be incontinent which may underline abnormalities in bladder or urethral development. The urogenital sinus gives rise to both the urethra and the bladder but also to the bulbourethral glands and the lower two-thirds of the vaginal canal, tissues shown to be greatly affected in *Tbx4* mutant mice. The presence of bladder or urethral abnormalities in these mutants would suggest that the effects of gene disruption may happen early in development with the specification of these tissues within the developing urogenital sinus.

Other tissues that need to be investigated for potential phenotypes include the prostate gland, ovaries, efferent ducts, rete testis and vas deferens. Once a comprehensive understanding of all adult urogenital phenotypes is made in these mutants, we should investigate how and when these abnormalities arise by looking at earlier time points in development. This would give us a good understanding of how *Tbx4* may affect the development of these tissues and help us create hypotheses to be tested.

It would also be important to determine the extent of *Tbx4* expression in the tissues known to give rise to the urogenital organs observed to be affected by disruption of gene expression. Cell lineage tracing experiments in mouse have determined that by as early as E13.5 the urogenital sinus is entirely made up of *Tbx4* lineage cells (Naiche et al., 2011), pointing to an earlier expression of *Tbx4* in this tissue.

We should not limit our investigation of *Tbx4* expression in the urogenital system to E13.5 or earlier. Given that *Tbx4* has a dual role in limb development, an early role in limb bud outgrowth and a later role in limb patterning (Naiche, 2003; Naiche and Papaioannou, 2007), it is possible that *Tbx4* expression and function in the urogenital system are dynamic. The urethra, which like the bladder is also derived from the urogenital sinus, expresses *Tbx4* at E15.5 (Harding et al., 2011). Therefore, *Tbx4* expression is dynamic in the tissues that give rise to a fully developed urethra and suggests that this gene may have multiple roles in the development of the bladder and possibly the whole urogenital system. The best way to address function of *Tbx4* in these tissues is by use of a conditional knockout allele that we would use to inactivate gene expression at different times or in specific tissues of the urogenital system.

Possible Mechanisms of *Tbx4* Function in the Urogenital System

Naiche and colleagues did not report any *Tbx4*-lineage cells contributing to the developing kidneys, either because they have not looked at this tissue or possibly because there is no contribution to be observed (2011). Nevertheless, in $Tbx4^{\Delta HLEB/-}$ mutants we observe kidney agenesis as well as polycystic kidneys. There are a few possible mechanisms for the development of these phenotypes, either *Tbx4* is directly required for kidney development or these phenotypes arise indirectly by interactions with another tissue where *Tbx4* is involved. It

is important therefore to explore whether or not *Tbx4* is expressed at any time during kidney development in this tissue (see above).

Kidney agenesis has also been reported in *Isl1* conditional mutants (Kaku et al., 2013). *Isl1* is a gene crucial for the onset of hindlimb bud outgrowth and is thought to function upstream of *Tbx4* (Kawakami et al., 2011). However, Kaku and colleagues reported that the kidney phenotypes they observed may be indirectly due to ureteric bud malformations (2013). Nevertheless a possible genetic interaction between *Isl1* and *Tbx4* in urogenital development is intriguing.

Tbx4 was shown by our experiments to be upstream of *Alx4* in the developing hindlimb. Interestingly *Alx4* is also expressed at E10.5 in tissues that give rise to the seminal vesicles (mesonephric duct) in males (Kuijper et al., 2005b). The seminal vesicles are abnormal in $Tbx4^{\Delta HLEB/-}$ male mutants and thus *Alx4* is a good candidate gene to lie downstream of *Tbx4* in the development of this tissue.

We have also noticed striking phenotypes in the genital development of females. Unlike males where the mesonephric duct gives rise to Wolffian ducts which further develop into most of the reproductive organs, female reproductive organs develop from Mullerian ducts that have a slightly different origin. Genes shown to affect Mullerian duct development include: *Emx2* (Miyamoto et al., 1997), *Lim1* (Kobayashi et al., 2005; Kobayashi et al., 2004; Pedersen et al., 2005), *Pax2* (Kuschert et al., 2001; Torres et al., 1995) and amongst others *Hoxa10* and *Hoxa13* (Ekici et al., 2013). Interestingly, all of these genes, when disrupted, include other abnormalities of the urogenital system reminiscent of the phenotypes we observed in our *Tbx4* mutant mice. Also, *Hoxa13* has been linked to Hand-Foot-Genital Syndrome, a disease that affects both the

urogenital system and the limbs (Goodman et al., 2000). Therefore these genes make great candidates to test for interactions with *Tbx4* in the development of the urogenital system.

Investigating the Identity of *Tbx4* Expressing Cells in the Hindlimbs and Genital Tubercle at E10.5

At E10.5 in wild type embryos there is a continuous domain of *Tbx4* expression between the hindlimbs and the genital tubercle. Whole mount in situ hybridization of E10.5 embryos followed by paraffin sectioning though this domain showed that these cells indeed express *Tbx4* and are morphologically distinct from the underlying mesenchymal cells that do not express *Tbx4* (data not shown). It would be interesting to determine the identity of these cells and also if they contribute to the development of a particular tissue. Valasek and colleagues have discovered in mice that some mesenchymal cells of the hindlimb bud migrate towards the genital tubercle and form the perineal musculature (Valasek et al., 2005). This experiment clearly shows that the hindlimb buds can directly contribute to other tissues and indicates a possible long range signaling interaction between the genital tubercle and the hindlimbs. Such signaling could potentially occur through the *Tbx4* expressing cells located between these two appendages.

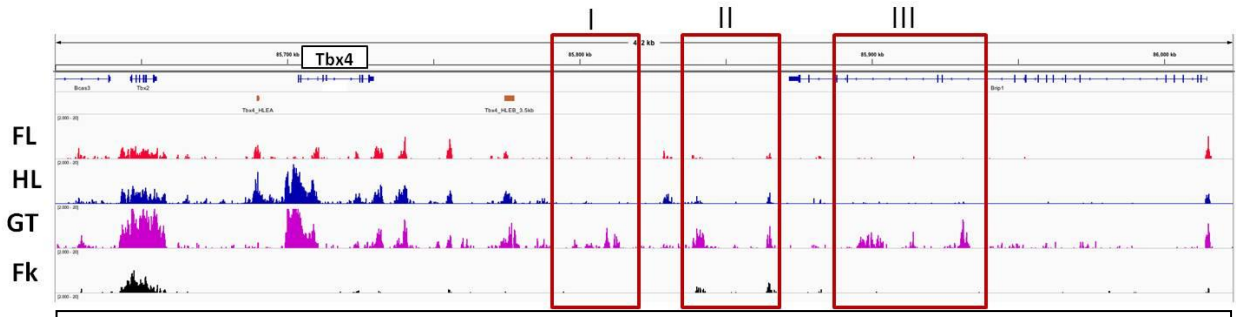


Figure 4.1 Three new regions harboring putative appendage enhancers have been identified. Shown is a 400kb genomic region spanning the *Tbx4* locus. Peaks indicate H3K27ac association with a sequence of DNA from four different tissues: E11.5 forelimb (red, FL), E11.5 hindlimb (blue, HL), E12.5 genital tubercle (pink, GT) and E11.5 flank (black, Fk). Signal intensity is represented by peak height, with regions of DNA more strongly associated with H3K27ac displaying larger peaks. Three red boxes indicate regions harboring putative appendage enhancers of *Tbx4.*

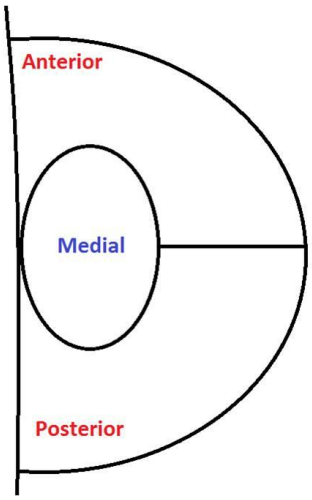


Figure 4.2 A diagram of the hindlimb at E10.5 showing the domains of *Tbx4* expression. The three domains represent distinct compartments differentially affected by knockout of HLEA (anterior compartment most affected as compared to the proximal) or HLEB (medial and proximal compartment).

References

Abbasi, A.A., Paparidis, Z., Malik, S., Bangs, F., Schmidt, A., Koch, S., Lopez-Rios, J., Grzeschik, K.H., 2010. Human intronic enhancers control distinct sub-domains of Gli3 expression during mouse CNS and limb development. BMC developmental biology 10, 44.

Acquaah-Mensah, G.K., Malhotra, D., Vulimiri, M., McDermott, J.E., Biswal, S., 2012. Suppressed expression of T-box transcription factors is involved in senescence in chronic obstructive pulmonary disease. PLoS computational biology 8, e1002597.

Agulnik, S.I., Garvey, N., Hancock, S., Ruvinsky, I., Chapman, D.L., Agulnik, I., Bollag, R., Papaioannou, V., Silver, L.M., 1996. Evolution of mouse T-box genes by tandem duplication and cluster dispersion. Genetics 144, 249-254.

Ahituv, N., Zhu, Y., Visel, A., Holt, A., Afzal, V., Pennacchio, L.A., Rubin, E.M., 2007. Deletion of ultraconserved elements yields viable mice. PLoS biology 5, e234.

Alvarado, D.M., Aferol, H., McCall, K., Huang, J.B., Techy, M., Buchan, J., Cady, J., Gonzales, P.R., Dobbs, M.B., Gurnett, C.A., 2010. Familial isolated clubfoot is associated with recurrent chromosome 17q23.1q23.2 microduplications containing TBX4. American journal of human genetics 87, 154-160.

Amemiya, C.T., Alfoldi, J., Lee, A.P., Fan, S., Philippe, H., Maccallum, I., Braasch, I., Manousaki, T., Schneider, I., Rohner, N., Organ, C., Chalopin, D., Smith, J.J., Robinson, M., Dorrington, R.A., Gerdol, M., Aken, B., Biscotti, M.A., Barucca, M., Baurain, D., Berlin, A.M., Blatch, G.L., Buonocore, F., Burmester, T., Campbell, M.S., Canapa, A., Cannon, J.P., Christoffels, A., De Moro, G., Edkins, A.L., Fan, L., Fausto, A.M., Feiner, N., Forconi, M., Gamieldien, J., Gnerre, S., Gnirke,

A., Goldstone, J.V., Haerty, W., Hahn, M.E., Hesse, U., Hoffmann, S., Johnson, J., Karchner, S.I., Kuraku, S., Lara, M., Levin, J.Z., Litman, G.W., Mauceli, E., Miyake, T., Mueller, M.G., Nelson, D.R., Nitsche, A., Olmo, E., Ota, T., Pallavicini, A., Panji, S., Picone, B., Ponting, C.P., Prohaska, S.J., Przybylski, D., Saha, N.R., Ravi, V., Ribeiro, F.J., Sauka-Spengler, T., Scapigliati, G., Searle, S.M., Sharpe, T., Simakov, O., Stadler, P.F., Stegeman, J.J., Sumiyama, K., Tabbaa, D., Tafer, H., Turner-Maier, J., van Heusden, P., White, S., Williams, L., Yandell, M., Brinkmann, H., Volff, J.N., Tabin, C.J., Shubin, N., Schartl, M., Jaffe, D.B., Postlethwait, J.H., Venkatesh, B., Di Palma, F., Lander, E.S., Meyer, A., Lindblad-Toh, K., 2013. The African coelacanth genome provides insights into tetrapod evolution. Nature 496, 311-316.

Barna, M., Pandolfi, P.P., Niswander, L., 2005. Gli3 and Plzf cooperate in proximal limb patterning at early stages of limb development. Nature 436, 277-281.

Barolo, S., 2012. Shadow enhancers: frequently asked questions about distributed cis-regulatory information and enhancer redundancy. Bioessays 34, 135-141.

Bongers, E.M., Duijf, P.H., van Beersum, S.E., Schoots, J., Van Kampen, A., Burckhardt, A., Hamel, B.C., Losan, F., Hoefsloot, L.H., Yntema, H.G., Knoers, N.V., van Bokhoven, H., 2004. Mutations in the human TBX4 gene cause small patella syndrome. American journal of human genetics 74, 1239-1248.

Cande, J., Goltsev, Y., Levine, M.S., 2009. Conservation of enhancer location in divergent insects. Proceedings of the National Academy of Sciences of the United States of America 106, 14414-14419.

Chapman, D.L., Garvey, N., Hancock, S., Alexiou, M., Agulnik, S.I., Gibson-Brown, J.J., Cebra-Thomas, J., Bollag, R.J., Silver, L.M., Papaioannou, V.E., 1996. Expression of the T-box family genes, Tbx1-Tbx5, during early mouse development. Developmental dynamics : an official publication of the American Association of Anatomists 206, 379-390.

Chen, H., Johnson, R.L., 1999. Dorsoventral patterning of the vertebrate limb: a process governed by multiple events. Cell and tissue research 296, 67-73.

Chiang, C., Litingtung, Y., Lee, E., Young, K.E., Corden, J.L., Westphal, H., Beachy, P.A., 1996. Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. Nature 383, 407-413.

Cobb, J., Duboule, D., 2005. Comparative analysis of genes downstream of the Hoxd cluster in developing digits and external genitalia. Development 132, 3055-3067.

Cotney, J., Leng, J., Oh, S., DeMare, L.E., Reilly, S.K., Gerstein, M.B., Noonan, J.P., 2012. Chromatin state signatures associated with tissue-specific gene expression and enhancer activity in the embryonic limb. Genome research 22, 1069-1080.

Cotney, J., Leng, J., Yin, J., Reilly, S.K., DeMare, L.E., Emera, D., Ayoub, A.E., Rakic, P., Noonan, J.P., 2013. The evolution of lineage-specific regulatory activities in the human embryonic limb. Cell 154, 185-196.

Cretekos, C.J., Wang, Y., Green, E.D., Martin, J.F., Rasweiler, J.J.t., Behringer, R.R., 2008. Regulatory divergence modifies limb length between mammals. Genes & development 22, 141-151.

Creyghton, M.P., Cheng, A.W., Welstead, G.G., Kooistra, T., Carey, B.W., Steine, E.J., Hanna, J., Lodato, M.A., Frampton, G.M., Sharp, P.A., Boyer, L.A., Young, R.A., Jaenisch, R., 2010. Histone H3K27ac separates active from poised enhancers and predicts developmental state. Proceedings of the National Academy of Sciences of the United States of America 107, 21931-21936.

Davis, A.P., Witte, D.P., Hsieh-Li, H.M., Potter, S.S., Capecchi, M.R., 1995. Absence of radius and ulna in mice lacking hoxa-11 and hoxd-11. Nature 375, 791-795.

Degenhardt, K.R., Milewski, R.C., Padmanabhan, A., Miller, M., Singh, M.K., Lang, D., Engleka, K.A., Wu, M., Li, J., Zhou, D., Antonucci, N., Li, L., Epstein, J.A., 2010. Distinct enhancers at the Pax3 locus can function redundantly to regulate neural tube and neural crest expressions. Developmental biology 339, 519-527.

DeLaurier, A., Schweitzer, R., Logan, M., 2006. Pitx1 determines the morphology of muscle, tendon, and bones of the hindlimb. Developmental biology 299, 22-34.

Dollé, P., Dierich, A., LeMeur, M., Schimmang, T., Schuhbaur, B., Chambon, P., Duboule, D., 1993. Disruption of the Hoxd-13 gene induces localized heterochrony leading to mice with neotenic limbs. Cell 75, 431-441.

Dunn, N.R., Winnier, G.E., Hargett, L.K., Schrick, J.J., Fogo, A.B., Hogan, B.L., 1997. Haploinsufficient phenotypes in Bmp4 heterozygous null mice and modification by mutations in Gli3 and Alx4. Developmental biology 188, 235-247.

Ekici, A.B., Strissel, P.L., Oppelt, P.G., Renner, S.P., Brucker, S., Beckmann, M.W., Strick, R., 2013. HOXA10 and HOXA13 sequence variations in human female genital malformations including congenital absence of the uterus and vagina. Gene 518, 267-272.

Epner, E., Reik, A., Cimbora, D., Telling, A., Bender, M.A., Fiering, S., Enver, T., Martin, D.I.K., Kennedy, M., Keller, G., Groudine, M., 1998. The Globin LCR Is Not Necessary for an Open Chromatin Structure or Developmentally Regulated Transcription of the Native Mouse Globin Locus. Molecular cell 2, 447-455.

Ernst, J., Kheradpour, P., Mikkelsen, T.S., Shoresh, N., Ward, L.D., Epstein, C.B., Zhang, X., Wang, L., Issner, R., Coyne, M., Ku, M., Durham, T., Kellis, M., Bernstein, B.E., 2011. Mapping and analysis of chromatin state dynamics in nine human cell types. Nature 473, 43-49.

Frankel, N., Davis, G.K., Vargas, D., Wang, S., Payre, F., Stern, D.L., 2010. Phenotypic robustness conferred by apparently redundant transcriptional enhancers. Nature 466, 490-493.

Frankel, N., Erezyilmaz, D.F., McGregor, A.P., Wang, S., Payre, F., Stern, D.L., 2011. Morphological evolution caused by many subtle-effect substitutions in regulatory DNA. Nature 474, 598-603.

Gall, J.G., Pardue, M.L., 1969. Formation and detection of RNA-DNA hybrid molecules in cytological preparations. Proceedings of the National Academy of Sciences of the United States of America 63, 378-383.

Gibson-Brown, J.J., Agulnik, S.I., Chapman, D.L., Alexiou, M., Garvey, N., Silver, L.M., Papaioannou, V.E., 1996. Evidence of a role for T-box genes in the evolution of limb morphogenesis and the specification of forelimb/hindlimb identity. Mechanisms of development 56, 93-101.

Gibson-Brown, J.J., Agulnik, S.I., Silver, L.M., Niswander, L., Papaioannou, V.E., 1998. Involvement of T-box genes Tbx2-Tbx5 in vertebrate limb specification and development. Development 125, 2499-2509.

Goodman, F.R., Bacchelli, C., Brady, A.F., Brueton, L.A., Fryns, J.P., Mortlock, D.P., Innis, J.W., Holmes, L.B., Donnenfeld, A.E., Feingold, M., Beemer, F.A., Hennekam, R.C., Scambler, P.J., 2000. Novel HOXA13 mutations and the phenotypic spectrum of hand-foot-genital syndrome. Am J Hum Genet 67, 197-202.

Haraguchi, R., Suzuki, K., Murakami, R., Sakai, M., Kamikawa, M., Kengaku, M., Sekine, K., Kawano, H., Kato, S., Ueno, N., Yamada, G., 2000. Molecular analysis of external genitalia formation: the role of fibroblast growth factor (Fgf) genes during genital tubercle formation. Development 127, 2471-2479.

Harding, S.D., Armit, C., Armstrong, J., Brennan, J., Cheng, Y., Haggarty, B., Houghton, D., Lloyd-MacGilp, S., Pi, X., Roochun, Y., Sharghi, M., Tindal, C., McMahon, A.P., Gottesman, B., Little, M.H., Georgas, K., Aronow, B.J., Potter, S.S., Brunskill, E.W., Southard-Smith, E.M., Mendelsohn, C., Baldock, R.A., Davies, J.A., Davidson, D., 2011. The GUDMAP database--an online resource for genitourinary research. Development 138, 2845-2853.

Harfe, B.D., 2011. Keeping up with the zone of polarizing activity: New roles for an old signaling center. Developmental dynamics : an official publication of the American Association of Anatomists 240, 915-919.

Hasson, P., DeLaurier, A., Bennett, M., Grigorieva, E., Naiche, L.A., Papaioannou, V.E., Mohun, T.J., Logan, M.P., 2010. Tbx4 and tbx5 acting in connective tissue are required for limb muscle and tendon patterning. Developmental cell 18, 148-156.

Hobert, O., 2010. Gene regulation: enhancers stepping out of the shadow. Current biology : CB 20, R697-699.

Hong, J.W., Hendrix, D.A., Levine, M.S., 2008. Shadow enhancers as a source of evolutionary novelty. Science 321, 1314-1314.

Horton, A.C., Mahadevan, N.R., Minguillon, C., Osoegawa, K., Rokhsar, D.S., Ruvinsky, I., de Jong, P.J., Logan, M.P., Gibson-Brown, J.J., 2008. Conservation of linkage and evolution of developmental function within the Tbx2/3/4/5 subfamily of T-box genes: implications for the origin of vertebrate limbs. Development genes and evolution 218, 613-628.

Infante, C.R., Park, S., Mihala, A.G., Kingsley, D.M., Menke, D.B., 2013. Pitx1 broadly associates with limb enhancers and is enriched on hindlimb cis-regulatory elements. Developmental biology 374, 234-244.

Izpisua-Belmonte, J.C., Brown, J.M., Crawley, A., Duboule, D., Tickle, C., 1992. Hox-4 gene expression in mouse/chicken heterospecific grafts of signalling regions to limb buds reveals similarities in patterning mechanisms. Development 115, 553-560.

Jeong, S., Hahn, Y., Rong, Q., Pfeifer, K., 2007. Accurate quantitation of allele-specific expression patterns by analysis of DNA melting. Genome research 17, 1093-1100.

Jeong, S., Rebeiz, M., Andolfatto, P., Werner, T., True, J., Carroll, S.B., 2008. The evolution of gene regulation underlies a morphological difference between two Drosophila sister species. Cell 132, 783-793.

Jeong, Y., El-Jaick, K., Roessler, E., Muenke, M., Epstein, D.J., 2006. A functional screen for sonic hedgehog regulatory elements across a 1 Mb interval identifies long-range ventral forebrain enhancers. Development 133, 761-772.

Juan, A.H., Ruddle, F.H., 2003. Enhancer timing of Hox gene expression: deletion of the endogenous Hoxc8 early enhancer. Development 130, 4823-4834.

Kaku, Y., Ohmori, T., Kudo, K., Fujimura, S., Suzuki, K., Evans, S.M., Kawakami, Y., Nishinakamura, R., 2013. Islet1 deletion causes kidney agenesis and hydroureter resembling CAKUT. Journal of the American Society of Nephrology : JASN 24, 1242-1249.

Kawakami, Y., Capdevila, J., Buscher, D., Itoh, T., Rodriguez Esteban, C., Izpisua Belmonte, J.C., 2001. WNT signals control FGF-dependent limb initiation and AER induction in the chick embryo. Cell 104, 891-900.

Kawakami, Y., Marti, M., Kawakami, H., Itou, J., Quach, T., Johnson, A., Sahara, S., O'Leary, D.D., Nakagawa, Y., Lewandoski, M., Pfaff, S., Evans, S.M., Izpisua Belmonte, J.C., 2011. Islet1-mediated activation of the beta-catenin pathway is necessary for hindlimb initiation in mice. Development 138, 4465-4473.

Kim, J., Lauderdale, J.D., 2008. Overexpression of pairedless Pax6 in the retina disrupts corneal development and affects lens cell survival. Developmental biology 313, 434-454.

Kimmel, R.A., Turnbull, D.H., Blanquet, V., Wurst, W., Loomis, C.A., Joyner, A.L., 2000. Two lineage boundaries coordinate vertebrate apical ectodermal ridge formation. Genes Dev 14, 1377-1389.

Kobayashi, A., Kwan, K.M., Carroll, T.J., McMahon, A.P., Mendelsohn, C.L., Behringer, R.R., 2005. Distinct and sequential tissue-specific activities of the LIM-class homeobox gene Lim1 for tubular morphogenesis during kidney development. Development 132, 2809-2823.

Kobayashi, A., Shawlot, W., Kania, A., Behringer, R.R., 2004. Requirement of Lim1 for female reproductive tract development. Development 131, 539-549.

Korzh, V., Grunwald, D., 2001. Nadine Dobrovolskaia-Zavadskaia and the dawn of developmental genetics. Bioessays 23, 365-371.

Kuijper, S., Beverdam, A., Kroon, C., Brouwer, A., Candille, S., Barsh, G., Meijlink, F., 2005a. Genetics of shoulder girdle formation: roles of Tbx15 and aristaless-like genes. Development 132, 1601-1610.

Kuijper, S., Feitsma, H., Sheth, R., Korving, J., Reijnen, M., Meijlink, F., 2005b. Function and regulation of Alx4 in limb development: complex genetic interactions with Gli3 and Shh. Developmental biology 285, 533-544.

Kuschert, S., Rowitch, D.H., Haenig, B., McMahon, A.P., Kispert, A., 2001. Characterization of Pax-2 regulatory sequences that direct transgene expression in the Wolffian duct and its derivatives. Developmental biology 229, 128-140.

Lanctot, C., Moreau, A., Chamberland, M., Tremblay, M.L., Drouin, J., 1999. Hindlimb patterning and mandible development require the Ptx1 gene. Development 126, 1805-1810.

Levine, M., 2010. Transcriptional enhancers in animal development and evolution. Current biology : CB 20, R754-763.

Lin, C., Yin, Y., Bell, S.M., Veith, G.M., Chen, H., Huh, S.H., Ornitz, D.M., Ma, L., 2013. Delineating a conserved genetic cassette promoting outgrowth of body appendages. PLoS genetics 9, e1003231.

Lin, C., Yin, Y., Veith, G.M., Fisher, A.V., Long, F., Ma, L., 2009. Temporal and spatial dissection of Shh signaling in genital tubercle development. Development 136, 3959-3967.

Logan, M., Simon, H.G., Tabin, C., 1998. Differential regulation of T-box and homeobox transcription factors suggests roles in controlling chick limb-type identity. Development 125, 2825-2835.

Loomis, C.A., Harris, E., Michaud, J., Wurst, W., Hanks, M., Joyner, A.L., 1996. The mouse Engrailed-1 gene and ventral limb patterning. Nature 382, 360-363.

Loomis, C.A., Kimmel, R.A., Tong, C.X., Michaud, J., Joyner, A.L., 1998. Analysis of the genetic pathway leading to formation of ectopic apical ectodermal ridges in mouse Engrailed-1 mutant limbs. Development 125, 1137-1148.

Lopez-Martinez, A., Chang, D.T., Chiang, C., Porter, J.A., Ros, M.A., Simandl, B.K., Beachy, P.A., Fallon, J.F., 1995. Limb-patterning activity and restricted posterior localization of the amino-terminal product of Sonic hedgehog cleavage. Current biology : CB 5, 791-796.

Lufkin, T., Mark, M., Hart, C.P., Dolle, P., LeMeur, M., Chambon, P., 1992. Homeotic transformation of the occipital bones of the skull by ectopic expression of a homeobox gene. Nature 359, 835-841.

MacCabe, J.A., Errick, J., Saunders, J.W., Jr., 1974. Ectodermal control of the dorsoventral axis in the leg bud of the chick embryo. Developmental biology 39, 69-82.

Malashichev, Y., Borkhvardt, V., Christ, B., Scaal, M., 2005. Differential regulation of avian pelvic girdle development by the limb field ectoderm. Anatomy and embryology 210, 187-197.

Malashichev, Y., Christ, B., Prols, F., 2008. Avian pelvis originates from lateral plate mesoderm and its development requires signals from both ectoderm and paraxial mesoderm. Cell and tissue research 331, 595-604.

McGregor, A.P., Orgogozo, V., Delon, I., Zanet, J., Srinivasan, D.G., Payre, F., Stern, D.L., 2007. Morphological evolution through multiple cis-regulatory mutations at a single gene. Nature 448, 587-590.

Menke, D.B., Guenther, C., Kingsley, D.M., 2008. Dual hindlimb control elements in the Tbx4 gene and region-specific control of bone size in vertebrate limbs. Development 135, 2543-2553.

Min, H.S., Danilenko, D.M., Scully, S.A., Bolon, B., Ring, B.D., Tarpley, J.E., DeRose, M., Simonet, W.S., 1998. Fgf-10 is required for both limb and lung development and exhibits striking functional similarity to Drosophila branchless. Gene Dev 12, 3156-3161.

Minelli, A., 2002. Homology, limbs, and genitalia. Evolution & development 4, 127-132.

Minguillon, C., Del Buono, J., Logan, M.P., 2005. Tbx5 and Tbx4 are not sufficient to determine limb-specific morphologies but have common roles in initiating limb outgrowth. Developmental cell 8, 75-84.

Minguillon, C., Gibson-Brown, J.J., Logan, M.P., 2009. Tbx4/5 gene duplication and the origin of vertebrate paired appendages. Proceedings of the National Academy of Sciences of the United States of America 106, 21726-21730.

Minguillon, C., Nishimoto, S., Wood, S., Vendrell, E., Gibson-Brown, J.J., Logan, M.P., 2012. Hox genes regulate the onset of Tbx5 expression in the forelimb. Development 139, 3180-3188.

Miyagawa, S., Moon, A., Haraguchi, R., Inoue, C., Harada, M., Nakahara, C., Suzuki, K., Matsumaru, D., Kaneko, T., Matsuo, I., Yang, L., Taketo, M.M., Iguchi, T., Evans, S.M., Yamada, G., 2009. Dosage-dependent hedgehog signals integrated with Wnt/beta-catenin signaling regulate external genitalia formation as an appendicular program. Development 136, 3969-3978.

Miyamoto, N., Yoshida, M., Kuratani, S., Matsuo, I., Aizawa, S., 1997. Defects of urogenital development in mice lacking Emx2. Development 124, 1653-1664.

Morgan, E.A., 2003. Loss of Bmp7 and Fgf8 signaling in Hoxa13-mutant mice causes hypospadia. Development 130, 3095-3109.

Murakami, R., Mizuno, T., 1986. Proximal-distal sequence of development of the skeletal tissues in the penis of rat and the inductive effect of epithelium. Journal of embryology and experimental morphology 92, 133-143.

Naiche, L.A., 2003. Loss of Tbx4 blocks hindlimb development and affects vascularization and fusion of the allantois. Development 130, 2681-2693.

Naiche, L.A., Arora, R., Kania, A., Lewandoski, M., Papaioannou, V.E., 2011. Identity and fate of Tbx4-expressing cells reveal developmental cell fate decisions in the allantois, limb, and external genitalia. Developmental dynamics : an official publication of the American Association of Anatomists 240, 2290-2300.

Naiche, L.A., Papaioannou, V.E., 2007. Tbx4 is not required for hindlimb identity or post-bud hindlimb outgrowth. Development 134, 93-103.

Nakada, Y., Parab, P., Simmons, A., Omer-Abdalla, A., Johnson, J.E., 2004. Separable enhancer sequences regulate the expression of the neural bHLH transcription factor neurogenin 1. Developmental biology 271, 479-487.

Nelson, C.E., Morgan, B.A., Burke, A.C., Laufer, E., DiMambro, E., Murtaugh, L.C., Gonzales, E., Tessarollo, L., Parada, L.F., Tabin, C., 1996. Analysis of Hox gene expression in the chick limb bud. Development 122, 1449-1466.

Nissim, S., Hasso, S.M., Fallon, J.F., Tabin, C.J., 2006. Regulation of Gremlin expression in the posterior limb bud. Developmental biology 299, 12-21.

Niswander, L., 2003. Pattern formation: old models out on a limb. Nature reviews. Genetics 4, 133-143.

Niswander, L., Jeffrey, S., Martin, G.R., Tickle, C., 1994. A positive feedback loop coordinates growth and patterning in the vertebrate limb. Nature 371, 609-612.

Niswander, L., Tickle, C., Vogel, A., Booth, I., Martin, G.R., 1993. FGF-4 replaces the apical ectodermal ridge and directs outgrowth and patterning of the limb. Cell 75, 579-587.

Nobrega, M.A., Zhu, Y., Plajzer-Frick, I., Afzal, V., Rubin, E.M., 2004. Megabase deletions of gene deserts result in viable mice. Nature 431, 988-993.

Ohuchi, H., Nakagawa, T., Yamauchi, M., Ohata, T., Yoshioka, H., Kuwana, T., Mima, T., Mikawa, T., Nohno, T., Noji, S., 1995. An Additional Limb Can Be Induced from the Flank of the Chick-Embryo by Fgf4. Biochem Bioph Res Co 209, 809-816.

Ohuchi, H., Takeuchi, J., Yoshioka, H., Ishimaru, Y., Ogura, K., Takahashi, N., Ogura, T., Noji, S., 1998. Correlation of wing-leg identity in ectopic FGF-induced chimeric limbs with the differential expression of chick Tbx5 and Tbx4. Development 125, 51-60.

Panman, L., Drenth, T., Tewelscher, P., Zuniga, A., Zeller, R., 2005. Genetic interaction of Gli3 and Alx4 during limb development. The International journal of developmental biology 49, 443-448.

Papaioannou, V.E., 2001. T-box genes in development: from hydra to humans. International review of cytology 207, 1-70.

Pappu, K.S., Ostrin, E.J., Middlebrooks, B.W., Sili, B.T., Chen, R., Atkins, M.R., Gibbs, R., Mardon, G., 2005. Dual regulation and redundant function of two eye-specific enhancers of the Drosophila retinal determination gene dachshund. Development 132, 2895-2905.

Parr, B.A., McMahon, A.P., 1995. Dorsalizing signal Wnt-7a required for normal polarity of D-V and A-P axes of mouse limb. Nature 374, 350-353.

Pedersen, A., Skjong, C., Shawlot, W., 2005. Lim 1 is required for nephric duct extension and ureteric bud morphogenesis. Developmental biology 288, 571-581.

Perriton, C.L., Powles, N., Chiang, C., Maconochie, M.K., Cohn, M.J., 2002. Sonic hedgehog signaling from the urethral epithelium controls external genital development. Developmental biology 247, 26-46.

Perry, M.W., Boettiger, A.N., Bothma, J.P., Levine, M., 2010. Shadow enhancers foster robustness of Drosophila gastrulation. Current biology : CB 20, 1562-1567.

Peterson, R.L., Jacobs, D.F., Awgulewitsch, A., 1992. Hox-3.6: isolation and characterization of a new murine homeobox gene located in the 5' region of the Hox-3 cluster. Mechanisms of development 37, 151-166.

Plageman, T.F., Jr., Yutzey, K.E., 2005. T-box genes and heart development: putting the "T" in heart. Developmental dynamics : an official publication of the American Association of Anatomists 232, 11-20.

Pomikal, C., Streicher, J., 2010. 4D-analysis of early pelvic girdle development in the mouse (Mus musculus). Journal of morphology 271, 116-126.

Qu, S., Niswender, K.D., Ji, Q., van der Meer, R., Keeney, D., Magnuson, M.A., Wisdom, R., 1997. Polydactyly and ectopic ZPA formation in Alx-4 mutant mice. Development 124, 3999-4008.

Rebeiz, M., Pool, J.E., Kassner, V.A., Aquadro, C.F., Carroll, S.B., 2009. Stepwise modification of a modular enhancer underlies adaptation in a Drosophila population. Science 326, 1663-1667.

Riddle, R.D., Johnson, R.L., Laufer, E., Tabin, C., 1993. Sonic hedgehog mediates the polarizing activity of the ZPA. Cell 75, 1401-1416.

Riddle, R.D., Nelson, C., Tsuchida, T., Jessell, T.M., Tabin, C., 1995. Induction of the LIM Homeobox Gene Lmx7 by WNT7a Establishes Dorsoventral Pattern in the Vertebrate Limb. Cell 83, 631-640.

Rodriguez-Esteban, C., Schwabe, J.W., De La Pena, J., Foys, B., Eshelman, B., Izpisua Belmonte, J.C., 1997. Radical fringe positions the apical ectodermal ridge at the dorsoventral boundary of the vertebrate limb. Nature 386, 360-366.

Rodriguez-Esteban, C., Tsukui, T., Yonei, S., Magallon, J., Tamura, K., Izpisua Belmonte, J.C., 1999. The T-box genes Tbx4 and Tbx5 regulate limb outgrowth and identity. Nature 398, 814-818.

Rosa-Molinar, E., Burke, A.C., 2002. Starting from fins: parallelism in the evolution of limbs and genitalia: the fin-to-genitalia transition. Evolution & development 4, 124-126.

Ruvinsky, I., Oates, A.C., Silver, L.M., Ho, R.K., 2000. The evolution of paired appendages in vertebrates: T-box genes in the zebrafish. Development genes and evolution 210, 82-91.

Saunders, J.W., Jr., 1948. The proximo-distal sequence of origin of the parts of the chick wing and the role of the ectoderm. The Journal of experimental zoology 108, 363-403.

Saunders, J.W.a.G., M. T. Saunders, 1968. Ectodermal-mesenchymal interactions in the origin of limb symmetry. Mesenchymal-Epithelial Interactions (ed. R. Fleichmeyer and R. E. Billingham), pp. 78-97.

Schaffner, G., Schirm, S., Mullerbaden, B., Weber, F., Schaffner, W., 1988. Redundancy of Information in Enhancers as a Principle of Mammalian Transcription Control. J Mol Biol 201, 81-90.

Seifert, A.W., Zheng, Z., Ormerod, B.K., Cohn, M.J., 2010. Sonic hedgehog controls growth of external genitalia by regulating cell cycle kinetics. Nature communications 1, 23.

Shapiro, M.D., Marks, M.E., Peichel, C.L., Blackman, B.K., Nereng, K.S., Jonsson, B., Schluter, D., Kingsley, D.M., 2004. Genetic and developmental basis of evolutionary pelvic reduction in threespine sticklebacks. Nature 428, 717-723.

Shou, S., Scott, V., Reed, C., Hitzemann, R., Stadler, H.S., 2005. Transcriptome analysis of the murine forelimb and hindlimb autopod. Developmental dynamics : an official publication of the American Association of Anatomists 234, 74-89.

Spitz, F., Gonzalez, F., Duboule, D., 2003. A global control region defines a chromosomal regulatory landscape containing the HoxD cluster. Cell 113, 405-417.

Stopper, G.F., Wagner, G.P., 2005. Of chicken wings and frog legs: a smorgasbord of evolutionary variation in mechanisms of tetrapod limb development. Developmental biology 288, 21-39.

Sun, X., Lewandoski, M., Meyers, E.N., Liu, Y.H., Maxson, R.E., Jr., Martin, G.R., 2000. Conditional inactivation of Fgf4 reveals complexity of signalling during limb bud development. Nat Genet 25, 83-86.

Sun, X., Mariani, F.V., Martin, G.R., 2002. Functions of FGF signalling from the apical ectodermal ridge in limb development. Nature 418, 501-508.

Suzuki, K., Bachiller, D., Chen, Y.P., Kamikawa, M., Ogi, H., Haraguchi, R., Ogino, Y., Minami, Y., Mishina, Y., Ahn, K., Crenshaw, E.B., 3rd, Yamada, G., 2003. Regulation of outgrowth and apoptosis for the terminal appendage: external genitalia development by concerted actions of BMP signaling [corrected]. Development 130, 6209-6220.

Suzuki, T., 2013. How is digit identity determined during limb development? Development, growth & differentiation 55, 130-138.

Suzuki, T., Takeuchi, J., Koshiba-Takeuchi, K., Ogura, T., 2004. Tbx genes specify posterior digit identity through Shh and BMP signaling. Developmental cell 6, 43-53.

Szeto, D.P., Rodriguez-Esteban, C., Ryan, A.K., O'Connell, S.M., Liu, F., Kioussi, C., Gleiberman, A.S., Izpisua-Belmonte, J.C., Rosenfeld, M.G., 1999. Role of the Bicoid-related homeodomain factor Pitx1 in specifying hindlimb morphogenesis and pituitary development. Genes Dev 13, 484-494.

Takabatake, Y., Takabatake, T., Takeshima, K., 2000. Conserved and divergent expression of T-box genes Tbx2-Tbx5 in Xenopus. Mechanisms of development 91, 433-437.

Takahashi, M., Tamura, K., Buscher, D., Masuya, H., Yonei-Tamura, S., Matsumoto, K., Naitoh-Matsuo, M., Takeuchi, J., Ogura, K., Shiroishi, T., Ogura, T., Izpisua Belmonte, J.C., 1998. The role of Alx-4 in the establishment of anteroposterior polarity during vertebrate limb development. Development 125, 4417-4425.

Takeuchi, J.K., 2003. Tbx5 and Tbx4 trigger limb initiation through activation of the Wnt/Fgf signaling cascade. Development 130, 2729-2739.

Takeuchi, J.K., Koshiba-Takeuchi, K., Matsumoto, K., Vogel-Hopker, A., Naitoh-Matsuo, M., Ogura, K., Takahashi, N., Yasuda, K., Ogura, T., 1999. Tbx5 and Tbx4 genes determine the wing/leg identity of limb buds. Nature 398, 810-814.

Tanaka, M., Munsterberg, A., Anderson, W.G., Prescott, A.R., Hazon, N., Tickle, C., 2002. Fin development in a cartilaginous fish and the origin of vertebrate limbs. Nature 416, 527-531.

Tanaka, M., Tamura, K., Noji, S., Nohno, T., Ide, H., 1997. Induction of additional limb at the dorsal-ventral boundary of a chick embryo. Developmental biology 182, 191-203.

Tanaka, M., Tickle, C., 2004. Tbx18 and boundary formation in chick somite and wing development. Developmental biology 268, 470-480.

Tarchini, B., Duboule, D., 2006. Control of Hoxd genes' collinearity during early limb development. Developmental cell 10, 93-103.

Tickle, C., Summerbell, D., Wolpert, L., 1975. Positional signalling and specification of digits in chick limb morphogenesis. Nature 254, 199-202.

Torres, M., Gomez-Pardo, E., Dressler, G.R., Gruss, P., 1995. Pax-2 controls multiple steps of urogenital development. Development 121, 4057-4065.

Towers, M., Mahood, R., Yin, Y., Tickle, C., 2008. Integration of growth and specification in chick wing digit-patterning. Nature 452, 882-886.

Towers, M., Tickle, C., 2009. Growing models of vertebrate limb development. Development 136, 179-190.

Valasek, P., Evans, D.J., Maina, F., Grim, M., Patel, K., 2005. A dual fate of the hindlimb muscle mass: cloacal/perineal musculature develops from leg muscle cells. Development 132, 447-458.

Verhofstede, C., Fransen, K., Marissens, D., Verhelst, R., van der Groen, G., Lauwers, S., Zissis, G., Plum, J., 1996. Isolation of HIV-1 RNA from plasma: evaluation of eight different extraction methods. Journal of virological methods 60, 155-159.

Visel, A., Blow, M.J., Li, Z., Zhang, T., Akiyama, J.A., Holt, A., Plajzer-Frick, I., Shoukry, M., Wright, C., Chen, F., Afzal, V., Ren, B., Rubin, E.M., Pennacchio, L.A., 2009. ChIP-seq accurately predicts tissue-specific activity of enhancers. Nature 457, 854-858.

Vokes, S.A., Ji, H., Wong, W.H., McMahon, A.P., 2008. A genome-scale analysis of the cis-regulatory circuitry underlying sonic hedgehog-mediated patterning of the mammalian limb. Genes & development 22, 2651-2663.

Warot, X., Fromental-Ramain, C., Fraulob, V., Chambon, P., Dolle, P., 1997. Gene dosage-dependent effects of the Hoxa-13 and Hoxd-13 mutations on morphogenesis of the terminal parts of the digestive and urogenital tracts. Development 124, 4781-4791.

Wright, E., Hargrave, M.R., Christiansen, J., Cooper, L., Kun, J., Evans, T., Gangadharan, U., Greenfield, A., Koopman, P., 1995. The Sry-related gene Sox9 is expressed during chondrogenesis in mouse embryos. Nat Genet 9, 15-20.

Xiong, N., Kang, C., Raulet, D.H., 2002. Redundant and unique roles of two enhancer elements in the TCRgamma locus in gene regulation and gammadelta T cell development. Immunity 16, 453-463.

Zakany, J., Duboule, D., 2007. The role of Hox genes during vertebrate limb development. Current opinion in genetics & development 17, 359-366.

Zakany, J., Fromental-Ramain, C., Warot, X., Duboule, D., 1997. Regulation of number and size of digits by posterior Hox genes: a dose-dependent mechanism with potential evolutionary implications. Proceedings of the National Academy of Sciences of the United States of America 94, 13695-13700.

Zakany, J., Kmita, M., Duboule, D., 2004. A dual role for Hox genes in limb anterior-posterior asymmetry. Science 304, 1669-1672.

Zhou, X., Sigmund, C.D., 2008. Chorionic enhancer is dispensable for regulated expression of the human renin gene. American journal of physiology. Regulatory, integrative and comparative physiology 294, R279-287.

Zhu, J., Nakamura, E., Nguyen, M.T., Bao, X., Akiyama, H., Mackem, S., 2008. Uncoupling Sonic hedgehog control of pattern and expansion of the developing limb bud. Developmental cell 14, 624-632.

Zuniga, A., Haramis, A.P., McMahon, A.P., Zeller, R., 1999. Signal relay by BMP antagonism controls the SHH/FGF4 feedback loop in vertebrate limb buds. Nature 401, 598-602.