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MOLECULAR ANALYSIS OF SPLIT HAND/FOOT MALFORMATION (SHFM) AT THE SHFM3 AND SHFM5 LOCI

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ABSTRACT

Split hand/foot malformation (SHFM) is a congenital limb malformation observed in humans characterized by a reduction or loss of the central digits of the hands and/or feet. The condition affects one in every 8,500 to 25,000 births, accounting for 8-17% of all limb reduction defects. A tandem duplication of approximately 500 Kb has been determined to be the causative mutation at the SHFM3 locus. Patients that are heterozygous for this duplication have three copies of the genes BTRC, POLL, and DPCD as well as an extra copy of exons 6-9 of FBXW4. The SHFM3 critical region also contains the FGF8 and SUFU genes. The aim of this study was to determine if and where these genes are expressed during normal limb development in the chicken. All the genes except poll at embryonic day 6 (E6) were detected by RT-PCR of cDNA from the limbs of E3-E13 embryos. In situ hybridization of paraffin sections from limbs of the chicken at E6 and E8 showed that BTRC, DPCD, FBXW4, FGF8, and SUFU are expressed in the region of the limb where digit formation occurs. Taken together, these data suggest that all of the genes, with the exception of POLL, may play a role in the development and patterning of the limb. The duplication within this region found in patients with SHFM could cause the phenotype by altering the expression of these genes, either through an increase in expression of the duplicated genes, the removal of a gene from its regulatory element, or a combination of the two.

This study also screened a cohort of patients with SHFM with an unknown molecular cause for mutations in two enhancer elements, one located within intron 4-5 of
BTRC in the SHFM3 locus, and the other located within the SHFM5 locus. Sequencing of these elements found no damaging mutations in any patient.
DEDICATION

I dedicate this work to my husband Brian Ladd and our families, whose love and support made this thesis possible.
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TERMS

AUTOPOD  The distal most region of the limb, hand or foot

CLEFT LIP/PALATE  Condition where tissue that form the lip or roof of the mouth fail to fuse before birth

CUTANEOUS  Pertaining to the skin

ECTODERMAL DYSPLASIA  Genetic condition characterized by failure to form ectodermal derivatives

ECTRODACTYLY  Absence of digits

EXPRESSIVITY  Extent to which a genotype is observed in an individual

MONODACTYLY  Having only one digit

OSSEOUS  Pertaining to bone

POLYDACTYLY  Having more than the normal number of digits

PENETRANCE  Frequency that a genotype manifests into a phenotype

STYLOPOD  The long bone region of the limb near the body wall, humerus/femur

SYNDACTYLY  Fusion of the digits

SYNTENIC  Conserved gene order between species

ZEUGOPOD  The middle region of the limb, forearm or shin
CHAPTER ONE
INTRODUCTION

Development of the Limb

The vertebrate skeleton is generated from three different lineages: the somites which give rise to the axial skeleton, the lateral plate mesoderm which generates the limb skeleton, and the cranial neural crest which gives rise to the pharyngeal arches as well as the craniofacial bones and cartilage [1]. In the majority of the skeleton, osteogenesis is achieved through endochondral ossification, where a cartilage intermediate is formed from mesodermal tissue, then replaced by a calcified matrix to form bone [1, 2].

In chicken, the limb first emerges as a bulge on the side of the embryo at embryonic day three (E3). As the bud elongates, distinct stylopod, zeugopod, and autopod elements become visible. The autopod goes from a paddle like structure at E4 to visible digital demarcations at E6. The digits continue to develop with webbing in between. Individual digits form when apoptosis removes the webbing, a process that is complete by E10. In human, this occurs during the 4th-8th weeks of pregnancy.

The limb initially forms when lateral plate mesoderm and somatic mesodermal cells proliferate and accumulate in a bulge under the ectoderm [1]. The positions of these limb buds are related to the level of Hox gene expression along the body’s cranial to caudal (head to tail) axis [1]. The cranial-most expression boundary of Hoxc5 and Hoxc6 corresponds to the level at which the forelimb bud emerges in both mouse and chicken [3]. In the earliest stages of limb formation Fgf10 protein, which is produced throughout
the lateral plate mesoderm, becomes restricted to the regions where the limbs will form, due to the presence of Wnt proteins [4, 5]. Secretion of Fgf10 by these mesoderm cells initiates limb bud formation by Wnt3a mediated induction of Fgf8 expression, and initiates the interactions between the ectoderm and mesoderm [4, 5]. Expression of fgf8 leads to the formation of the major signaling center of the developing limb, the apical ectodermal ridge (AER). The AER is a thickened band of ectoderm along the distal margin of the limb bud and a major signaling center for the limb (Figure 1.1) [4]. The AER expression of fgf8 in turn induces continued expression of fgf10 in the mesoderm under the AER, as well as Shh expression in the posterior mesenchyme [4]. After the limb bud is established, expression of fgf10 and fgf8 become dependent on each other. Ectopic expression of one can rescue the loss of the other [4]. This interaction is hypothesized to be the molecular basis for the interactions between the AER and the underlying mesoderm [4]. Another feedback loop is established between fgf10 and shh. Shh induces further expression of fgf10, which in turn maintains shh expression [4].

Determination of forelimb and hindlimb occurs early, before emergence of the limb buds [6]. Tbx genes, a family of transcription factors, show expression in the flank mesoderm; Tbx5 in the region of the forelimb, Tbx4 in the hindlimb [6]. Members of the WNT family also play a role in determining forelimb and hindlimb; Wnt2b and Wnt8c restrict fgf10 expression to only the level of the forelimb and hindlimb, respectively.

Proper development of the limb involves growth and patterning along three axes: proximal-distal (shoulder-fingertip), anterior-posterior (thumb-little finger), and dorsal-ventral (knuckle-palm). Each axis has its own signaling systems responsible for
patterning (Figure 1.1) and coordination of these three signaling systems results in the correct limb pattern.

**Proximal-Distal (PD) Patterning**

The prevailing model for PD patterning is the progress zone model, which states that the identity of a cell is determined by the amount of time it spends dividing in a region of undifferentiated mesoderm directly behind the AER, called the progress zone (Figure 1.1) [7, 8]. Cells that spend more time in the progress zone will form more distal structures in the autopod [9]. This model does not explain, however, the results obtained by Sun and coworkers in Fgf4 and Fgf8 double knockout mice where proximal elements are lost while normal distal elements develop [10]. The alternate model, the early specification model, has also been proposed, stating that the cells are differentiated early to establish limb pattern. Subsequent growth of the limb, under the influence of the AER, expands these cell populations, leading to the sequential differentiation of structures that is observed [11]. Research to examine these hypotheses is ongoing, and the data thus far does not exclude either model [12]. A temporal element has also been proposed, in which autopod elements along the PD axis form in correlation with the cyclic expression of hairy2 in the mesoderm [13]. This does not favor one model over another, it merely suggests a mechanism of control for either [13].
Figure 1.1: Major Signaling Centers of the Developing Human Limb

Schematic of developing limb bud showing the major signaling centers the apical ectodermal ridge (AER) comprised of ectodermal tissue, the progress zone (PZ), and the zone of polarizing activity (ZPA), both composed of mesoderm. Numbers represent the future digits 1 (thumb)-5 (little finger). Orientation of each developmental axis is also shown.
Dorsal-Ventral (DV) Patterning

DV patterning is the result of signals from the overlying ectoderm acting on the mesoderm below (Figure 1.1) [14]. Early in limb development, \textit{Wnt5a} is expressed in the ventral ectoderm and \textit{Wnt7a} in the dorsal ectodermal half [15]. \textit{Wnt7a} induces the expression of \textit{Lmx1} in the dorsal mesoderm, an isoform of which, \textit{Lmx1b}, appears to be responsible for dorsal compartmentalization of the limb [16, 17]. This compartmentalization of the mesenchyme does not contain all cells, nor does it correlate to any anatomical structures [17]. Instead of being a result of signaling centers, DV compartmentalization may be a result of the movement of mesenchymal cells through a three-dimensional space necessary for proper limb formation [17].

Anterior-Posterior (AP) Patterning

As the limb bud grows along the PD axis there is a widening of the distal region to prepare for the development of the digits [18]. At the posterior margin of the distal limb bud is a region of mesenchymal cells known as the zone of polarizing activity (ZPA, Figure 1.1) [8]. This region secretes Sonic Hedgehog (Shh), which acts as a diffusible morphogen to regulate AP patterning [8, 19]. This diffusion sets up a concentration gradient across the autopod [8]. Posterior digit identity is specified by both the concentration of Shh and the amount of time that mesenchymal cells are exposed to Shh [20]. The pattern is further refined through interactions of Shh and its target gene \textit{Gli3},
where the presence of Shh promotes the Gli3 activator form (Gli3A) and prevents the repressor form of Gli3 (Gli3R) from forming [21]. Thus, a Gli3R counter-gradient is set up against the Shh gradient. Digit identity is further specified by the ratio of Gli3A:Gli3R that cells are exposed to [22].

It is further hypothesized that Shh patterns digits in a step wise manner, in which the protein first induces the cells to be competent to form digits, and then induces expression of Bmp2 which acts on this competent mesoderm to form digits [23].

BMP signaling has also been shown to regulate digit identity from the interdigital mesenchyme, downstream of ZPA signaling, before the interdigital mesenchyme regresses to form individual digits [24].

**Coordination of the Three Axes**

Coordinated signaling is not only required for proper limb formation, but signaling along one axis is often dependent on signals from another. One such example is the positive feedback loop established between the AER and ZPA [25]. Shh from the ZPA induces Fgf4 expression in the AER, responsible for mesoderm proliferation [25]. This in turn maintains Shh expression as well as makes the mesoderm directly under the AER receptive to Shh signals [25]. This enables Shh dependent AP patterning to continue, but because Shh induces expression of Hoxd genes responsible for specification of the zeugopod and autopod, PD patterning is also maintained [25, 26]. The dorsal Wnt7a
signal has also been shown to maintain Shh expression, demonstrating an interaction between the DV and AP axes [27].
Split Hand/Foot Malformation (SHFM)

SHFM is a congenital limb malformation characterized by a reduction or loss of the central digits of the hands and/or feet and affects one in every 8,500 to 25,000 births, accounting for 8-17% of all limb reduction defects [28, 29]. The phenotype is highly variable, ranging from mild changes in the digits, to monodactyly; with all of the limbs affected, or as few as one [30]. It is also common to see cutaneous and/or osseous syndactyly of the remaining digits (Figure 1.2) [30]. SHFM is clinically heterogeneous, presenting in both syndromic and non-syndromic forms [28]. The most common syndromic form of SHFM is the ectrodactyly, ectodermal dysplasia, cleft lip/palate (EEC) syndrome [28]. Non-syndromic SHFM can be either isolated or associated with deficiencies of the long bones (SHFLD) [28]. In familial cases, SHFM is often inherited in an autosomal dominant manner, although autosomal recessive and X-linked forms have been reported [28]. Families show incomplete penetrance, variable expressivity, and segregation distortion characterized by excessive transmission from affected males to sons [28].
Figure 1.2: SHFM phenotype, showing variable expressivity between patients, as well as within, the same patient.

The top figure shows a patient with a normal left hand and SHFM of the right hand with syndactyly of the thumb and index finger. The bottom figure shows a different patient, with both feet affected; the left foot more severely than the right. From Everman DB. Hands and Feet. In: Stevenson RE, Hall JG, eds. Human Malformations and Related Anomalies, 2nd Ed. Oxford University Press: New York, 2006.
To date there are five well characterized loci for SHFM: SHFM1 at 7q21.3 [31], SHFM2 at Xq26 [32], SHFM3 at 10q24 [33, 34], SHFM4 at 3q27 [35], and SHFM5 at 2q31 [36, 37]. Recently, a sixth locus for SHFM has been suggested at 8q21.11-q22.3 [38]. For one locus, SHFM4, mutations in the \textit{TP63} gene have been identified as the causative mechanism for SHFM [35, 39].

The SHFM1 Locus

Patients with SHFM1 associated with 7q21 have been shown to have either a deletion or translocation including the genes \textit{DLX5}, \textit{DLX6}, and \textit{DSS1} [31, 40]. Hearing loss has also been associated with this locus [40, 41]. \textit{DLX5} (Gene ID 1749) is a member of a homeobox transcription factor family and is expressed in the neural tube, lens, and surface ectoderm of early stage chick embryos [42]. In later stages of chick development, Dlx5 expression is found in the wing and wing bones, as well as facial mesenchyme [43, 44]. Another homeobox transcription family member, \textit{DLX6} (Gene ID 1750), and the \textit{DSS1} gene (Gene ID 7979) have not been studied in chicken.

Inactivation of both \textit{Dlx5} and \textit{Dlx6} in mouse results in bilateral ectrodactyly of the posterior limbs and craniofacial defects [45]. In this model the \textit{Dss1} gene is unaffected and mice heterozygous for the deletion do not show any phenotypic anomalies [45].
It is hypothesized that haploinsufficiency of the genes from this locus, either through deletion or the physical separation of the genes from a regulatory element(s) by chromosomal rearrangement [46] is the causative mechanism of SHFM1.

The SHFM2 Locus

The SHFM2 locus was identified in a seven-generation family with multiple affected members, and linked to a 5.1 Mb region on Xq26.3 [32]. Faiyaz-Ul-Haque and coworkers studied several functional and positional genes from the critical region and found no mutations [32]. This is an extensive region, and it is possible that more genes have been identified since the original publication, which would require further examination. Regulatory elements either in this region, or for genes within the locus, also cannot be ruled out as the causative mechanism for SHFM.

The SHFM3 Locus

Linkage analysis using several families identified the SHFM3 locus at 10q24 [33, 34]. De Mollerat and coworkers identified a tandem duplication of approximately 500kb in this region that segregated with the phenotype as the causative mutation (Figure 1.3) [28, 29]. The duplication has been refined and the smallest region identified contains the entire BTRC, DPCD, and POLL genes, as well as exons 6-9 of FBXW4 (Figure 1.3)[28, 47, 48]. Other genes of interest at this region are FGF8 and SUFU (Figure 1.3)[49].
While screening of these genes in SHFM cases has not found any causative mutations [49], the presumption is that this duplication causes the SHFM phenotype by altering the expression levels of one or more of the genes of this locus. This could be either by a 1.5 fold increase in expression of the duplicated genes, or by disrupting the regulation of genes near the breakpoints, or by some combination of these mechanisms [48].
Figure 1.3: SHFM3 critical region at chromosome 10q24.

Normal (top) and partially duplicated chromosome 10 (bottom). SHFM3 patients are heterozygous for the duplication and possess three copies of BTRC, POLL, and DPCD, as well as two normal copies of FBXW4 with the addition of exons 6-9. Adapted from de Mollerat et al, 2003.
**BTRC** (Gene ID 8945) is a member of the F-box/WD40 protein family involved in two pathways responsible for AER maintenance, canonical Wnt signaling and NFκB [48]. As part of the ubiquitination process which targets proteins for further processing or degradation, BTRC is responsible for regulating β-catenin levels which have an effect on apoptosis [50, 51]. BTRC also recruits IκBα for degradation, allowing NFκB to translocate into the nucleus and activate its target genes [52]. As mentioned previously, Gli3 processing is a key component of AP patterning. Wang and coworkers demonstrated that BTRC works downstream of protein kinase activity in Gli3 processing [53]. In lymphoblasts of patients with the SHFM3 duplication, BTRC is over expressed [48].

In the chicken model, btrc expression has been detected in neural tube and midbrain of Hamburger Hamilton (HH) stages 13-26 embryos [54, 55]. To date expression of this gene in the limb has not been reported.

**FBXW4** (Gene ID 6468) is also a member of the F-box/WD40 family implicated in the ubiquitination of proteins, but no specific pathway has been identified [77]. The mouse homolog of this gene, Dactylin (Dac), is implicated in the naturally occurring Dactylaplasia mutant that serves as the current model for SHFM3 [49]. It is believed that Dac is responsible for AER maintenance by degrading a suppressor of AER cell proliferation [56]. The expression of this gene has not been reported in the chicken.

**POLL**, (Gene ID 27343) is a DNA directed repair polymerase of the PolX family of polymerases which is conserved in chimpanzee, dog, cow, mouse, rat, chicken, zebrafish, Arabidopsis, and rice [57] Expression of POLL has been found in a variety of
tissues, the highest in testis and fetal liver, but not in limb specific tissue. There are no reports of studies of this gene having been conducted in chicken.

**DPCD** (Gene ID 25911), a gene deleted in the mouse model for primary ciliary dyskinesia, is believed to be involved in the structure or function of cilia [58]. This gene has not been implicated in limb development specifically, but is expressed in human skeletal muscle [58]. At the time of limb bud formation in the chicken embryo (HH19), over half of the mesenchymal cells of the limb are ciliated, relating to the mitotic phase of the cell [59]. If this gene is involved in the formation or structure of these cilia, then misexpression of **DPCD** could impact cell division that is required for proper limb formation. Expression of dpcd has not been reported in the chicken.

**FGF8** (Gene ID 2253), is a member of the FGF family and, as mentioned above, is one of the key signals involved in the maintenance of the AER. *In vitro* **FGF8** has been shown to stimulate osteoblast proliferation [60]. Another study has shown that Fgf8 works with *Indian hedgehog (Ihh)* and through *Sox9* to control the elongation of digits, implicating a role in distal cartilage element development [61]. The same study also indicated a role for **Fgf8** in mesodermal cell differentiation [61]. **Fgf8** has further been shown to work with *Wnt3a* to promote cell proliferation of limb progenitor cells, while maintaining them in an undifferentiated state [62]

Studies in the embryonic chicken have shown that fgf8 expression is present starting from HH1-HH6 in the primitive streak [63, 64]. When the limb bud is present, fgf8 expression is detected in the AER, forebrain, somites, pharyngeal arches, and the eye [65-67]. At embryonic day 9 (E9) chick limbs, fgf8 expression is found in distal wing
muscle, near where the muscle attaches to bone [68], and also the facial mesenchyme [69].

**SUFU** (Gen ID 51684) is a member of the *SHH* pathway, preventing Gli1 from entering the nucleus and activating its target genes [70]. With strong expression detected in human testis, ovary, prostate, spleen, and peripheral blood leukocyte tissue, expression was also detected in skeletal muscle tissue [71]. *BTRC* and *SUFU* are believed to interact with each other, as *SUFU* interacts with the *Drosophila slimb* gene, which is homologous with *BTRC* [71]. Similar to *BTRC*, *SUFU* has been shown to be over expressed in the lymphoblasts of individuals with the SHFM3 duplication [48], and has been implicated in apoptosis by affecting β-catenin activity [72, 73].

In the chicken model, sufu expression is detected in the primitive streak, Hensen’s node, and the neural tube of early embryos and in the midbrain of embryos at the time of limb formation [55]. Expression has not been reported in the chicken limb.

Of the genes from the SHFM3 critical region, only *BTRC*, *FBXW4*, *FGF8*, and *SUFU* have any known ties to limb development, and only *FGF8* expression has been studied in the limb. However, a sequential study of *FGF8* expression through the stages of autopod development has not been reported.

**The SHFM4 Locus**

The SHFM4 locus, located at 3q27, is the only SHFM locus for which the causative gene is clearly known [35, 39]. In this case, missense mutations in *TP63* are
the causative mechanism for SHFM [35, 39]. *TP63* is transcription factor, and these mutations are hypothesized to disrupt the DNA binding surface of the protein, reducing the transactivation activity of TP63.

**The SHFM5 Locus**

A deletion of approximately 5Mb at chromosome 2q31 has been associated with the SHFM5 locus (Figure 1.4) [36, 37]. This critical region is 5’ to the *HOXD* cluster of genes, and contains the genes *DLX1, DLX2*, and *LNP*, all of which have been implicated in limb development [74, 75]. Although the closely related genes, *DLX5* and *DLX6*, are in the SHFM1 critical region, mice lacking *Dlx1, Dlx2*, or both *Dlx1* and *Dlx2* genes do not show limb anomalies [74, 76]. *Lnp* is shown to be expressed in the developing digits of the mouse [75]. Also within the deleted regions are *SP3* and *SP9*, two transcription factors [77]. *SP3* (Gene ID: 6670) can either stimulate or repress transcription of target genes by binding to their consensus GC- and GT-box regulatory elements [77]. Little is known about the specifics of *SP9* (Gene ID: 100131390) activity, except for the conservation of its sequence across several species [77].
Figure 1.4: SHFM5 critical region.

2q31 showing the approximately 5 Mb region that is deleted in some patients with SHFM5. The deletion is upstream of the gene Evx2 and the HOXD cluster and contains the genes DLX1, DLX2, Sp3, Sp9 and LNP, some of which have been implicated in limb development.
The Dactylaplasia Mouse Mutant

In the mouse a spontaneous mutation resulted in the dactylaplasia (Dac) mouse with an SHFM like phenotype [56]. The manifestation of the phenotype is the result of mutations in two unlinked interacting genes, Modifier of dactylaplasia (Mdac) on chromosome 13 and dactylin on chromosome 19 [56, 78]. Mice must be homozygous for the recessive Mdac allele, regardless of the mutant dactylin alleles present, to exhibit the phenotype [78, 79]. At the dactylin locus, mice heterozygous for the mutation lack the central digits of the feet, whereas homozygotes present with only the most posterior digit [76]. The dactylin region is syntenic to human chromosome 10q24, making the Dac mouse the current model for SHFM3 [78].

There are two known mutant alleles of dactylin, both resulting from insertions in the dactylin gene, equally sensitive to the effects of a homozygous recessive Mdac allele, and have indistinguishable phenotypes [56]. The Dac$^{1J}$ allele is the result of an insertion of an early transposon repeat element upstream of the dactylin gene [56, 79]. The Dac$^{2J}$ allele is either an insertion or a small inversion in intron 5 of dactylin [56, 79]. It appears that the phenotype is caused by an inability to maintain the AER, though the exact mechanism remains largely unknown [56, 76, 79].

Populations of Dac$^{1J}$ and Dac$^{2J}$ mice were being maintained for the Greenwood Genetic Center’s research; however, problems with inbreeding have caused those populations to be lost. Cryogenically frozen mice are commercially available if there is interest in reviving them for future studies.
Purpose

The purpose of this study is to gain a better molecular characterization of SHFM, specifically at the SHFM3 locus, and through pilot studies into the SHFM5 locus. Despite what is already known about the BTRC, POLL, DPCD, FBXW4, FGF8, and SUFU genes from the SHFM3 critical region, this data is insufficient to identify a causative gene. Studies of each gene’s expression pattern in the limb during the course of autopod development are limited. This study aims to rectify this gap in our knowledge. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) will be used to sequentially study the timing of expression for each gene in the developing chicken limb. Analysis will be done from the emergence of the limb bud, at E3, until digit formation, at E13, in both forelimbs and hindlimbs. If these genes are expressed in the developing limb at this time, then in situ hybridization analysis will be employed to determine the spatial expression pattern in the limb.

Even with multiple identified or proposed loci, there are still patients with SHFM for whom the molecular cause is unknown. Given that the causative mechanism at some of these loci involves complex chromosomal rearrangements, it cannot be ruled out that such rearrangements remove a gene from its regulatory element(s). It also stands to reason that a mutation in a regulatory element could prevent proper gene expression. The Lawrence Berkeley National Laboratory is currently using a mouse model to identify non-coding elements that have enhancer activity. So far two such elements, which have been found to have enhancer activity in the limb, are located in SHFM loci. One is
within an intron of *BTRC* in the SHFM3 locus; the other is in the SHFM5 critical region.

This study will also screen a cohort of patients with SHFM for which the molecular cause has not been identified in either of these elements.
CHAPTER TWO
MATERIALS AND METHODS

Collection of Tissue and Isolation of RNA

White Leghorn eggs were incubated at 38.5°C until embryonic day 3 (E3) through E13. At the appropriate stage, forelimb and hindlimb tissue was collected and stored separately in AllProtect Tissue Reagent (Qiagen). Numbers of embryos harvested were as follows: E3-5, 24; E6-8, 12; E9-11, 6; E12-13, 4. RNA was isolated using the RNeasy® Lipid Tissue Mini Kit (Qiagen), and treated with Turbo DNA-free DNase kit (Ambion) to remove DNA contamination.

RT-PCR

cDNA was synthesized from 10µg of RNA from each stage using the SuperScript II First Strand Synthesis kit (Invitrogen). Intron spanning primers for the homologous genes from the SHFM3 locus as well as the 18S rRNA gene used as a control in chicken were designed using NCBI Primer BLAST (see Appendix). PCR was performed with GoTaq polymerase as follows: 95°C for five minutes, then 30 cycles (40 cycles for fgf8) of 95°C for 30 seconds, the determined annealing temperature for the gene (see Appendix A-1) for 30 seconds, 72°C for 35 seconds. The final extension step was 72°C for seven minutes. The resulting products were visualized on a 1% agarose gel.
Quantitative PCR (qPCR)

25 µl PCR reactions were set up as follows: 12.5 µl iQSYBR Green Supermix (Bio-Rad), 0.5 µl each of the appropriate forward and reverse primers (See Appendix A-2), 9.5 µl water and 2 µl cDNA (50 ng/µl). Reactions were run on the Biorad iCycler iQ Multicolor Real Time Detection System under the following conditions: 50°C for 10 minutes, 95°C for 5 minutes, 45 cycles of 95°C for 10 seconds; 55°C for 30 seconds (camera on), followed by the melt curve generation: 95°C, one minute, 50°C, one minute, and 80 cycles of 50°C + 0.5°C increments, 10 seconds (camera on). Results were analyzed using Gene Expression Macro ™ (Bio-Rad).

Paraffin Embedding and Sectioning

E6 and E8 limbs were harvested and fixed in a 4% Paraformaldehyde (PFA)/Phosphate Buffered Saline (PBS) solution overnight and rinsed twice with PBS for five minutes each. Limbs were then dehydrated in 35%, 70%, 95% (2x), and 100% (2x) ethanol, five minutes each, before washing twice in NeoClear (Harleco), ten minutes each. After washing in 1:1 NeoClear:Paraplast solution for 30 minutes at 60°C under vacuum, tissue was washed in Paraplast three times, 30 minutes each at 60°C under vacuum, before embedding in peel away molds. 10 µm sections were made on a microtome and placed in order on glass slides.
In Situ Hybridization Probes

For dpcd, fbxw4, and sufu probes, PCR products, generated as stated above, from E5 limbs were cloned into the pCR®2.1 vector and transformed into One Shot® competent cells using the TA Cloning® Kit (Invitrogen). The btrc and fgf8 probes were a gift from Dr. Susan Chapman. Plasmids were minipreped using either the QIAprep Spin® Miniprep Kit (Qiagen) for btrc, dpcd, fbxw4, and sufu probes or FastPlasmid Mini Kit (Eppendorf) for the fgf8 probe. Purified plasmids were linearized by digestion with Xhol (New England Biolabs) for dpcd, fbxw4 and sufu, or BamHI (New England Biolabs) for btrc, and BamHI (Promega) for fgf8, using the buffers supplied for each enzyme.

The transcription labeling reaction for each probe was mixed on ice as follows: 10 µl of 5X transcription buffer (Promega), 3 µl FITC RNA Labeling mix (Roche), 0.5 µl RNAse Inhibitor (Fisher), 2 µl T7 RNA Polymerase (Fisher), 1 µg of linearized template, and water to a volume of 50 µl. The reaction mix was incubated for two hours at 37°C. Two microliters of DNaseI (Fisher) was added to the reaction mixture, for 15 minutes of incubation at 37°C. Each product was cleaned using illustra™ ProbeQuant™G-50 Micro Columns (GE Healthcare), and checked for concentration on a 1% agarose gel.

In situ hybridization on Limb Sections
Sections were baked on the slides for one hour before washing in NeoClear twice, for ten minutes each, and then rehydrated in 100% (2x), 95%, 70%, and 50% ethanol for three minutes each. After rinsing with water for one minute, sections were fixed in PFA/PBS for ten minutes followed by 3% hydrogen peroxide in PBS solution (pH 7.4) for 20 minutes to remove peroxidases, and then washed with PBS for ten minutes. Five minute active Diethyl Pyrocarbonate (DEPC) wash was used to remove RNases before treating slides with Proteinase K (4µg/ml) for 15 minutes to remove proteins. Slides were washed three times, 10 minutes each, in PBS. Tissue was permeabilized with a 10% Tween-20 in PBS solution for 30 minutes and washed three times, 10 minutes each, in PBS. Sections were dehydrated in 70%, 95%, and 100% ethanol for two minutes each before placing in a prehybridization solution (50% formamide, 5x SSC, pH4.5, 2% SDS, 2% BBR, 250 µg/ml tRNA, 100 µg/ml Heparin) and incubated in a 63°C water bath for one hour. Ten micrograms/ml of the appropriate probe were added to the slides and incubated overnight in a 63°C water bath.

Unbound probe was removed by two 30 minutes washes in 1X SSC and two 30 minute washes in 0.5X SSC, all in a 63°C water bath. Slides were then rinsed with PBS for 5 minutes, and treated with SlowFade (Invitrogen), before fluorescence was visualized using a FITC filter on a Nikon SMZ 1500 fluorescence microscope.

**Screening of Candidate Enhancer Element Located in Intron 4-5 of BTRC**
PCR: Four pairs of overlapping primers were designed using Primer3 to cover the enhancer sequence provided by the Vista Enhancer Browser from the Lawrence Berkeley National Laboratory (see Appendix A-3 for sequences and annealing temperatures). PCR was performed on genomic DNA from 57 patients with SHFM of unknown cause with GoTaq polymerase as follows: 95°C for five minutes, then 40 cycles of 95°C for 30 seconds, the determined annealing temperature for the primer pair (see Appendix A-2) for 30 seconds, 72°C for 35 seconds. The final extension step was 72°C for seven minutes. The resulting products were visualized on a 1% agarose gel.

Sequencing: Excess primers and dNTPs were removed from PCR products using ExoSAP-IT PCR Clean-Up Reagent (USB). Sequencing reactions were carried out using the DYEEnamic™ ET Dye Terminator Kit (MegaBACE™) (GE Healthcare) under the following conditions: 95°C 20 seconds, 50°C for five seconds, 60°C for one minute, for 27 cycles. Products were analyzed on a MegaBACE and results examined using DNAStar software.

RsaI digest to test for C391T change: PCR was performed as described above, using BTRC EN1 primers (see Appendix A-2), on 18 normal control samples and 38 patients showing C391T change by sequencing stated above (27 heterozygous, 9 homozygous). Products were cut with RsaI (New England Biolabs) using the buffer supplied by the company. Results were visualized on a 1% agarose gel.

Screening of the 3’ Sequence of Enhancer Element within SHFM5 Critical Region
PCR and sequencing were performed as stated above, with an annealing temperature of 53°C used in the PCR. See Appendix A-4 for primer sequences.
CHAPTER THREE

RESULTS: POLYMERASE CHAIN REACTION

RT-PCR

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) was performed to determine if and when the genes from the SHFM3 region are expressed during limb development. RNA was extracted from several forelimbs and pooled, similarly for hindlimbs.

During normal limb development of the chicken, the wing and leg buds emerge approximately 66 hours after the egg is laid [54]. The apical ectodermal ridge (AER) becomes visible at E3, and the buds continue to grow along the proximal-distal axis, with the angle between the limb and the flank decreasing through rotation, from 90° to 45°. Demarcations of the future regions of the wing become apparent at E4, becoming more defined by E6. At E5.5, the digital plate with the interdigital grooves begins to emerge. The groves become continue to deepen from E6 through to E7.5. Digit II is separate from digit III by E8, with interdigital webbing completely removed by apoptosis by E9, with the appearance of the claw by E10 [80].

RT-PCR results indicate that btsc is present throughout the stages tested, dropping dramatically at E7. Band intensity is strong again at E8, thereafter weakening in the forelimb and similarly weakening at E10 in the hindlimb (Figure 3.1). The forelimb
sample at E3 has greater expression intensity than the hindlimb sample, which can be explained by the forelimb emerging ahead of the hindlimb (Figure 3.1).

First detected at E3, sufu expression is strong at E3-6, E8 and E11-13, with weak expression detected at E7 and E10, with no expression at E9 in the forelimb (Figure 3.1). Hindlimbs show expression of sufu from E3-E10, and again at E12-13 (Figure 3.1), with E6-8, E10, 12, 13 being weaker. This expression pattern is similar to that of brtc.

Expression of fbxw4 is detected in forelimb samples at E3-6, E8, and at E12 and E13 (Figure3.1). In the hindlimb samples, expression is detected at all stages, except E7 and E11, with weak band intensity at E9, 12, and 13 (Figure 3.1).

dpcd expression is evident at all stages in forelimb samples, with the exception of E10 (Figure 3.1). In hindlimb, the only stage lacking expression is E11. Again, this is likely due to the delayed development of the hindlimb.

Both forelimb and hindlimb samples had poll expression that was barely detectable at all stages, except for a jump in band intensity at E6 (Figure 3.1).

In both forelimbs and hindlimbs, fgf8 expression was detected for all stages, with bands of equal intensities, with the exception of undetectable expression at E9 in the hindlimb (Figure 3.1).

In summary, the dynamic expression of brtc, fbxw4, sufu, and dpcd, suggested the need for quantitative approaches to better determine expression levels of these genes at each stage. To do this we performed quantitative PCR (qPCR).
Figure 3.1: RT-PCR results from E3-E13

Agarose gels of PCR products from cDNA isolated from forelimb (left column) and hindlimb (right column) of chick embryos at various embryonic stages. Forelimb (left column) and hindlimb (right column). Lanes are labeled with the embryonic stage number, ladder (L) and negative control lane using water (W) in the reaction mix, rather than cDNA. btrc is present at all stages tested, with stronger band intensity at stages E3-E6 and E8 in forelimb samples and E4-E6, E8-E10, and E13 in hindlimb. The pattern for sufu is similar to btrc with expression present at all stages except E9 forelimb samples, and E11 hindlimb, where expression is undetectable. Band intensity in fbxw4 samples is stronger in E3-E4, E6, E8, and E12-E13 in forelimb samples. In the hindlimb, expression is stronger in E3-E6 and E8-E10 samples. Bands were not present in E7 and E9-E11 forelimb samples, or weakly at E7 and undetectable in E11 hindlimb samples. For dpcd, expression was detected at all stages except E10 in forelimbs and E11 in hindlimbs. poll was only detected at E6 in both forelimb and hindlimb samples. All stages shown, fgf8 expression was observed, except for E9 in the hindlimb. 18SrRNA was used as a control to measure the quality of the cDNA.
Quantitative PCR (qPCR)

To better understand how the expression levels of btrc, sufu, fbxw4, and dpcd change over the course of limb development, qPCR was performed on cDNA from forelimb (Figure 3.2) and hindlimb samples (Figure 3.3). The expression level of each gene was compared to that of 18S rRNA at the same stage.

In forelimb samples, the expression ratio of the test genes to the control gene is below one, indicating that 18S rRNA is an extremely robust gene. Other control genes such as actin or GAPDH may be preferential as control genes, as they are not as robustly expressed. Expression of btrc, fbxw4, and dpcd is greater during stages E4-8 than sufu, with a reversal from E9 through to E13, when sufu expression is dominant. From E3-8 dpcd expression is higher than btrc, sufu, fbxw4 at E3, peaking at E4, then gradually down regulates to an undetectable level by E9. Expression of btrc and fbxw4 is highest at E4 and E6, with fbxw4 remaining robust at E7, whereas sufu dominated between E9-11.

In hindlimb samples, the expression ratio for the test genes for most stages is below 0.5. Exceptions to this are the expression of btrc is greater than 0.5 at E12, dpcd at E5, sufu at E9. sufu exceeds 0.5 at E9, its peak and again exceeds 0.5 at E12. The expression ratio for fbxw4 exceeds 0.5 at the most stages, first at E5, again at E9-10, peaking at nearly 2.5 at E12 and remaining above 0.5 at E13.
Figure 3.2: Quantitative PCR results of cDNA from E3-E13 forelimb samples

The expression ratio of all of the genes tested to the control gene remains below one throughout the stages tested. Expression of btrc, fbxw4, and dpcd is greater during E4-8 than sufu expression, which is greater from E9-E13. btrc expression is present at all stages, being highest at E4 and E6. fbxw4 is also expressed at all stages, with the highest expression at E4, E6 and E7. dpcd expression is lower than btrc and fbxw4 at all stages except E3, is highest at E4, and dropping to an expression ratio of zero from E9. Expression of sufu is lower than the other genes early on, surpassing them and peaking from E9-E11, remaining higher than the other genes through E13. 18S rRNA was used as a control.
Figure 3.3: Quantitative PCR results of cDNA from E3-E13 hindlimb samples

The expression ratio of the tested gene to the control gene for btrc is below 0.5 through all stages tested except at E12 when btrc peaks at one. sufu also stays below 0.5 except at E9 and E12, rising higher at these two stages. Expression of fbxw4 increases above 0.5 at E5, E9-10, peaks to almost 2.5 at E12, remaining above 0.5 at E13. Similar to btrc, dpcd is below 0.5 at all stages, except E5 when it rises to a ratio of one. 18S rRNA was used as a control.
CHAPTER FOUR

RESULTS: *IN SITU* HYBRIDIZATION

The Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) results indicate that all of the genes from the SHFM3 critical region, with the exception of poll, are expressed in the limb from the appearance of the limb bud through to the end of digit formation. *In situ* hybridization was employed to determine where in the limb btrc, dpcd, fbxw4, fgf8, and sufu are expressed. Weak or zero band intensity in the RT-PCR for nearly all of the genes at embryonic day seven (E7) suggests that a major change in developmental patterning is occurring at this stage. Chondrogenesis of the nascent limb skeleton mesenchymal condensations begins at E6, with the onset of ossification detectable at E7. Thus, down regulation of btrc, fbxw4 and sufu at this critical stage may be due to the onset of endochondral ossification of the skeleton elements in the limb. To detect potential changes in expression *in situ* hybridization using probes for btrc, dpcd, fbxw4, fgf8, and sufu was performed on paraffin sections of E6 and E8 chicken forelimbs and hindlimbs.

**btrc expression**

btrc expression in E6 forelimbs is in specific mesoderm regions around the presumptive digits (Figure 4.1 A, C), and is weakly detectable surrounding the radius and ulna (Figure 4.1 A, B). By E8, expression increases distally in the wrist and digit areas (Figure 4.1 E, F), localizing to the mesoderm underlying the surface ectoderm (Figure 4.1 E, F).
In E6 hindlimbs, expression surrounds the patella and femur (Figure 4.2 A), and is similar to the equivalent forelimb stage, with expression in the tibia and fibia (Figure 4.2 B). Expression is also detectable surrounding the forming digits II-IV (Figure 4.2 C, D). In the E8 hindlimbs expression is mainly in the proximal region of the foot (Figure 4.2 F), but not observed in the tibiotarsus or the distal foot (Fig. 4.2 E, G).
**Figure 4.1: btrc forelimb sections labeled by fluorescence in situ hybridization.**

Level of section, at 10 µm, indicated by lines on schematic. Fluorescence (left) and bright field (right) images of in situ hybridization on E6 (A-C) and E8 (D-F) forelimb sections. (A) Lateral, (B-F) Cross-sections: B) section through radius (r) and ulna (u), C) section through the presumptive digits (pd), D) section through radius and ulna, E and F) section through carpometatarsus (cm) and digit I (dI). (A-C) Specific expression is observed in cells between the presumptive digits (C, arrowed) in E6 sections, with some expression between the radius and ulna (B). (D-F) At E8, expression is localized to the area of mesoderm underlying the surface ectoderm.
Figure 4.2: btrc hindlimb sections labeled by fluorescence in situ hybridization.

Level of section (10 µm) indicated by lines on schematic. Fluorescence (left) and bright field (right) images of in situ hybridization on E6 (A-D) and E8 (E-G) hindlimb cross-sections. (A) Section through femur (f) and patella (p), (B and E) sections through tibiotarsus (tt), (C-D, F-G) sections through the digits (d), numbered accordingly. Equivalent images are matched for E6 and E8 sections. Restricted expression is detectable in cells mainly around the digits (D) at E6. By E8, expression in down regulated, being mainly in the proximal foot (F).
**dpcd expression**

Expression of dpcd, in E6 forelimbs, is restricted to the area immediately surrounding the radius, ulna. Low levels of expression appear in the presumptive digits. Higher levels of expression are observable in the proximal regions, than in the distal regions (Figure 4.3 A-B). At E8, the expression level is only around the carpometacarpus in the distal region (Figure 4.3 C-D).

Distally, E6 hindlimbs (Figure 4.4 A-B) also show greater expression than forelimbs at the same stage (Figure 4.4 A-B). Expression in the distal hindlimbs, at E8, is also in the area immediately surrounding the bones, with no expression detectable in the distal most regions (Figure 4.4 C-E).
Figure 4.3: dpcd forelimb sections labeled by fluorescence *in situ* hybridization.

Level of section (10 µm) indicated by lines on schematic. Fluorescence (left) and bright field (right) images of *in situ* hybridization on E6 (A-B) and E8 (C-D) forelimb cross-sections. (A) Section through radius (r) and ulna (u), (B) section through presumptive digits (pd), (C-D) section through digit II (dII) and carpometacarpus (cm). Equivalent images are matched for E6 and E8 sections. (A) Expression is detected in the region surrounding the radius and ulna at E6. (B) The bright spots surrounding the digits in B are a result of wax contamination, with low levels of expression found within the presumptive digits. (D) By E8, in the distal region expression in the carpometacarpus is restricted to the outer edges, and absent in digit II compared to more proximal section (C), where little expression is detected.
Figure 4.4: dpcd hindlimb sections labeled by fluorescence in situ hybridization.

Level of 10 µm sections indicated by lines on schematic. Fluorescence (left) and bright field (right) images of in situ hybridization of E6 (A-B) and E8 (C-E) hindlimb cross-sections. (A-B) Sections through presumptive digits (pd), (C-E) through digits (d) numbered accordingly. (A-B) At E6 expression is found surrounding the presumptive digits. (C-E) Expression is observed in the peripheral edges of the digits by E8.
**fbxw4 expression**

In the E6 forelimb, fbxw4 expression is detected only around the presumptive digits, mainly around posterior two digits (Figure 4.5A). By E8, expression is restricted to the surround muscle masses of the wing and in the mesoderm underlying the surface ectoderm (Figure 4.5 B-C).

The E6 hindlimb shows a similar expression pattern for fbxw4 as the forelimb, surrounding the presumptive digits (Figure 4.6A-B). E8 hindlimbs also show dispersed expression throughout the mesoderm around the tarsometatarsus, but in the distal most regions expression is detected in the mesoderm underlying the surface ectoderm (Figure 4.6 C-E)
Figure 4.5: fbxw4 forelimb sections labeled by fluorescence in situ hybridization.

Level of 10 µm sections indicated by lines on schematic. Fluorescence (left) and bright field (right) images of in situ hybridizations of E6 (A) and E8 (B-C) forelimb cross-sections. (A) Section through presumptive digits (pd), (B-C) sections through digit II (dII) and carpometacarpus (cm). (A) Expression is observed in tissue around the presumptive digits at E6. (B-C) By E8, expression continues to be detected around digit II, and also around the carpometacarpus and in underlying mesoderm adjacent to the surface ectoderm.
Figure 4.6: fbxw4 hindlimb sections labeled by fluorescence in situ hybridization.

Level of section, at 10 μm, indicated by lines on schematic. Fluorescence (left) and bright field (right) images of in situ hybridization on E6 (A-B) and E8 (C-E) hindlimb cross-sections. (A-B) Sections through presumptive digits (pd), (C) section through tarsometatarsus (tm), (D-E) sections through digits (d) numbered accordingly with digit I (dI) in (D) turned laterally. Equivalent images are matched for E6 and E8 sections. (A-B) At E6, expression is detected in the areas surrounding (A) and between the presumptive digits (B). (C-E) By E8, expression is dispersed through the mesoderm around the tarsometatarsus (C), reduced around digits II-IV, and is now detected in the surface ectoderm associated underlying mesoderm (D-E).
fgf8 expression

The most expansive expression pattern was that of fgf8. In E6 forelimb, expression was localized to the ventral portion of the distal limb and in between the presumptive digits. More distally, expression was localized to the anterior and posterior regions, with discrete expression around the future digits (Figure 4.7 A-C). By E8, the expression became localized adjacent to the bones (Figure 4.7 D), and distally in the central mesoderm beside the digits (Figure 4.7 E).

Expression was not as widespread in the hindlimb, with the pattern more completely surrounding the digits in E6 limbs. Expression was stronger in the ventrally side (Figure 4.8 A-B). Expression down regulated in the proximal tissue (bright spots are wax contamination), with stronger expression in the distal most region surrounding the digits, by E8 (Figure 4.8 C-E).
Figure 4.7: fgf8 forelimb sections labeled by fluorescence in situ hybridization.

Level of section, at 10 µm, indicated by lines on schematic. Fluorescence (left) and bright field (right) of in situ hybridizations of E6 (A-C) and E8 (D-E) forelimb cross-sections. (A-C) Sections through presumptive digits (pd), (D) section through carpals (c), (E) section through digit II (dII) and carpometacarpus (cm). Equivalent images are matched for E6 and E8 sections. (A-C) Expression at E6 is widespread throughout the ventral mesoderm, and becoming more refined in between the presumptive digits distally. (D, E) By E8, expression is more specific to the areas around the carpals (D), digit II, and carpometacarpus (E).
Figure 4.8: fgf8 hindlimb sections labeled by fluorescence in situ hybridization.

Level of section, at 10 µm, indicated by lines on schematic. Fluorescence (left) and bright field (right) images of in situ hybridization on E6 (A-B) and E8 (C-E) hindlimb cross-sections. (A-B) Sections through presumptive digits (pd), (C-E) sections through digits (d), numbered accordingly. Equivalent images are matched for E6 and E8 sections. (A-B) Expression at E6 is in the ventral mesoderm and in the area in between the presumptive digits. (C-E) By E8, proximal expression is less expansive, remaining strong around the digits in the distal region.
sufu expression

Finally, the expression of sufu in E6 forelimb (Figure 4.9 A) was minimal, appearing to localize around the central presumptive digit (digit III). At E8, this limited expression is now detected in the mesoderm cells underlying the surface ectoderm (Figure 4.9 B-D). This is reminiscent of *btrc* E8 forelimb expression (Figure 4.9 D-F).

In the hindlimb, expression appears restricted to the more distal regions in both stages, and remains very minimal (Figure 4.10 A-G). The several bright spots in these sections are the result of wax contamination.
Figure 4.9: sufu forelimb sections labeled by fluorescence in situ hybridization.

Level of section, at 10 µm, indicated by lines on schematic. Fluorescence (left) and bright field (right) of in situ hybridization of E6 (A) and E8 (B-D) forelimbs cross-sections. (A) Section through presumptive digits (pd), (B) section through radius (r) and ulna (u), (D) section through digit II (dII) and carpometacarpus (cm). Equivalent images are matched for E6 and E8 sections. Expression is barely detectable, appearing to localize around the presumptive digits at E6 (A). (B-D) At E8, faint expression is detected in the underlying mesoderm of the surface ectoderm (B-C) and around digit II (D).
Figure 4.10: *sufu* hindlimb sections labeled by fluorescence *in situ* hybridization.

Level of section, at 10 µm, indicated by lines on schematic. Fluorescence (left) and bright field (right) of *in situ* hybridization of E6 (A-C) and E8 (D-G) forelimbs cross-sections. (A) Section through tibiotarsus (tt), (B-C) sections through presumptive digits (pd), (D-G) sections through digits (d) numbered accordingly. Equivalent images are matched for E6 and E8 sections. (A) No visible expression was detected around the tibiotarsus. (B, C) Expression is barely detectable, appearing to localize around the digits at E6, and by E8 only in the subjacent mesoderm, underlying the surface ectoderm (D-G).
CHAPTER FIVE

RESULTS: SCREENING OF ENHANCER ELEMENTS IN SHFM3 AND SHFM5 LOCI

Using the Vista Enhancer Browser, (http://enhancer.lbl.gov/) to search for enhancer expression in the limb, revealed an enhancer within the SHFM5 locus as well as one within the SHFM3 locus. Thus, patients with SHFM having no known molecular cause were screened for mutations in these sequences.

**Screening of the enhancer within the SHFM3 Locus**

Screening of 57 patients with SHFM of unknown molecular causes using the BTRC EN1 primers found a change in 36 patients. For the enhancer within intron 4-5 of BTRC a C-T change was observed. Of these 36 patients, 27 were heterozygous for the change, while 9 were homozygous (Figure 5.1 A, B). The change was confirmed in all of the patients using digesting with the restriction enzyme RsaI (Figure 5.1 C).

Screening of 18 unaffected controls with RsaI digests found 6 that were heterozygous for the change and 9 that were homozygous, indicating that the change is not significant and most likely represents a single nucleotide polymorphism normal variant.

When screened with the BTRC EN1 primers, one patient had a G insertion at position 398 of the enhancer sequence (Figure 5.2 A). However, this initial sequencing result was of poor quality and further screening with BTRC EN4 primers showed no insertion (Figure 5.2 B).

**Screening of the Enhancer within the SHFM5 locus**
57 patients with SHFM of unknown molecular cause were screened. Screening the 3’ sequence of the enhancer element, in the SHFM5 locus, including the ultra conserved region, showed no sequence changes (data not shown). Thus this line of enquiry was not pursued further. The 5’ sequence was screened in a separate study and is not reported here.
Figure 5.1: C-T change found in 36 patients with SHFM of unknown molecular cause.

(A) Sequence trace data showing a heterozygous C-T change. (B) Sequence trace data showing a homozygous C-T change.
(C) *RsaI* digest confirming C-T change. Normal digest fragments of 115 bp, 214 bp, and 242 bp; C-T change digest resulted in 214 bp and 357 bp fragments. Patients heterozygous for the change had bands at 115 bp, 214 bp, 242 bp, and 357 bp; patients homozygous for the change only had bands at 214 bp and 357 bp.
Figure 5.2: Sequence trace data for patient with apparent insertion

(A) Sequencing trace data from BTRC EN1 forward and reverse primer showing what appears to be a G insertion in both directions.  (B) Sequencing trace data of the same region using BTRC EN4 forward and reverse primers, showing no insertion.
A tandem duplication at chromosome 10q24 has been identified as the causative mutation for SHFM3 [28]. Patients heterozygous for the duplication have three full copies of the genes BTRC, DPCD, and POLL, as well an additional copy of exons 6-9 of FBXW4 [28, 47, 48]. Lymphoblasts from these patients over express the duplicated gene BTRC, and SUFU, a gene that is downstream of the duplication [48]. FGF8, a gene also in the critical region, plays a key role in AER formation and maintenance [4]. Gene expression is time and tissue specific, and with the exception of FGF8, it was unknown if the genes of the SHFM3 critical region were even expressed in the developing limb. Even with the knowledge of FGF8, expression of this gene has not been studied from the emergence of the limb bud through digit formation.

This study used both Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and in situ hybridization in chick embryos as effective ways to identify candidate genes in and around the duplicated region. Using this approach, a better understanding of the normal mRNA expression of the genes from the SHFM3 critical region, in the developing chicken limb, was achieved. Moreover, these data provide the basis for pursuing these candidate genes in future studies, initially using the chick as a model system for gain- and loss-of-function studies of single genes and combinations of the candidate genes, and then moving to the mouse for transgenic studies.
**Genes in the duplicated region are expressed during chick limb development**

All genes analyzed showed varying expression levels throughout stages E3-E13 and, with the exception of poll, were further tested by *in situ* hybridization on sections from E6 and E8 forelimbs and hindlimbs. Expression of btrc was very specific to mainly the mesoderm cells between the presumptive digits at E6. *BTRC* has been implicated in apoptosis [50, 51], and if its expression marks the cells destined to undergo apoptosis, over expression of *BTRC* has the potential to expand this region between the presumptive digits. It is plausible that expansion of the region of apoptotic cells could prevent the central digits from developing properly, leading to the SHFM phenotype.

The localization of dpcd expression around the bones of the distal limb indicates a potential role in digit development or patterning. The pattern of expression appears to be in the perichondrium sheath of the developing bones. To better determine the type of tissue in which dpcd is expressed, sections should be stained with Alcian blue and Chlorantine fast red to distinguish between cartilage and ossified tissue, respectively. Deletion of *Dpcd* in mouse leads to the pulmonary ciliary dyskinesia (PCD) like phenotype, but no limb anomaly was reported [58]. Zariwala and coworkers suggest that *DPCD* is responsible for the formation or function of ciliated cells [58]. At the time of limb development in the chicken, over half the mesenchymal cells are ciliated, which correlates with the mitotic stage of the cell [59]. Cartilage cells in the developing limb are ciliated, thus, dpcd may be responsible for the proper formation of the cilia. An over-
expression of $DPCD$ during limb development has the potential to affect patterning of the limb skeleton leading to the SHFM phenotype.

Mutations in the mouse homolog of $FBXW4$ have been implicated as the causative mutation in the $Dactylaplasia (Dac)$ mouse mutant, which is the current model for SHFM3 [56]. Over the course of limb development, RT-PCR results indicate that $fbxw4$ expression fluctuates. $In situ$ hybridization of E6 sections shows expression around the presumptive digits. At E8, $fbxw4$ expression is around the muscle masses of the limbs. The mutations in the mouse model and human are different, but both involve the $FBXW4$ gene, and is expressed around the presumptive digits during chick limb development.

This suggests that the gene is a candidate for a role in the SHFM phenotype. It is possible that the duplicated exons code for a protein that competitively interacts with the target protein of $FBXW4$. This interaction could disrupt the normal pathway leading to the SHFM phenotype.

$FGF8$ is well documented as being crucial to AER formation and maintenance [1, 49], but this study extends the reported stages of expression and shows that $FGF8$ is expressed well after the AER has disappeared. $In situ$ hybridization analysis shows localization of expression around the digits in E6 limbs. Localization in E6 forelimbs to the ventral side suggests a role for $fgf8$ in dorsal ventral patterning of the forelimb only. Despite the lack of evidence implicating $FGF8$ in the manifestation of the SHFM phenotype, the crucial role $FGF8$ plays in limb development and the expression pattern found in this study, are sufficient evidence to study $FGF8$ further. Although $FGF8$ is not
in the duplicated region, the duplication, found in SHFM3 patients, may remove FGF8 from a regulatory element, thus affecting its expression levels and/or pattern.

Although not duplicated, SUFU is over expressed in SHFM3 patient lymphoblasts [48]. This could be a result of the over expression of BTRC, as the two genes, or their proteins, are believed to interact [49]. RT-PCR results demonstrate a similar time course to btrc, although expression in the sections is not as strong. btrc is thought to act upstream of sufu, and further in situ hybridization studies using double staining with the btrc and sufu probes, over several substages, will be required to determine if this is indeed the case. Similar to btrc, the expression pattern of sufu around the presumptive digits and its involvement in apoptosis [72, 73] indicate that an over expression of the gene may prevent the central digits from forming and lead to the SHFM phenotype.

RT-PCR showed that poll was detected at E6 in both forelimbs and hindlimbs, but not detected at any of the other stages tested. Given the amount of rapidly proliferating cells in the developing limb, it is not surprising to find trace amounts of a DNA repair polymerase. At E6, mesenchymal condensations undergo chondrogenesis, and this spike in expression of poll may be a result of the number of cells differentiating into osteoblasts during this process. Such limited expression likely eliminates POLL as a key player in limb development.

In summary, the results of this study indicate that BTRC, DPCD, FBXW4, FGF8, and SUFU are candidate genes for limb development and patterning, and that altered expression of one or more of these genes may cause or contribute to the SHFM phenotype in patients who have duplications at the SHFM3 locus.
Quantitative gene analysis

In order to better understand the role of each gene in normal development, and possibly how errors in the expression of these genes can lead to the SHFM3 phenotype, quantitative studies were necessary. Expression levels of btrc, dpcd, fbxw4, and sufu change dynamically over the course of limb development. To address these issues quantitative PCR (qPCR) was performed on E3-13 cDNA from forelimbs and hindlimbs. The expression of each gene was compared to that of control 18S rRNA at the same stage. A number of general trends were identified, such as the difference of sufu expression in the fore limbs compared to the other genes tested. The expression ratio of the test gene to the control gene rarely exceeded 1.0 for any stage tested. In retrospect, the robust expression of 18S rRNA makes it a poor choice of a control gene for qPCR. These tests should be repeated with another control gene such as actin or GAPDH, to provide results that may be more conclusive.

This study focused only on the expression of the genes in the limb, and did not compare the expression to that in the rest of the embryo. Key stages in the development of the autopod such as the emergence of the limb bud, demarcation of presumptive digits, and end of digit formation, could be tested using the rest of the embryo from the same stage as a positive control sample. If a particular gene has greater expression in the limb than the remainder of the embryo during key stages in limb development, it is possible that altered expression of that same gene can lead to an abnormal limb phenotype.
**Future studies using the Dactylaplasia mouse mutant**

After determining which genes are up regulated in the limb at these critical stages, it would be beneficial to examine the expression of each gene in the developing limb of a Dac mutant mouse compared to that in a control wildtype mouse limb. Since the mutation in the Dac mouse involves the Dactylin gene, it is expected that its expression would be altered. However, because both forms of the mutation are chromosomal rearrangements, changes in the Dactylin gene might impact the expression of nearby genes. Kano and coworkers studied the mRNA levels of Btrc, Poll, and Dpcd in wildtype and Dac embryos, but found no significant changes [79]. The study did not specify if the samples used were from the limbs or the whole embryo, or what stages were studied. Instead limb tissue from wildtype and both homozygous and heterozygous Dac embryos should be collected from key developmental stages and the expression of the candidate genes analyzed using qPCR. Genes that show a different expression level between the three genotypes should be considered candidate genes for further study.

**Future studies using the chick embryo**

One current hypothesis is that the over expression of duplicated genes from this locus is the causative mechanism for the SHFM3 phenotype [48]. Genes that are found to be up regulated in Dac mouse can could be cloned into an over expression vector that is electroporated into a developing chicken embryo [81]. The embryo can then be
incubated until the end of limb development and the injected limb studied for a change in phenotype [81]. Given the location of btrc expression found in this study, and that Lyle and coworkers showed an over expression of BTRC in patient lymphoblasts, this is a promising candidate. SUFU has also been shown to be over-expressed in patient lymphoblasts [48] and has a similar expression pattern to btrc. These two genes have been hypothesized to interact with each other [71]. It stands to reason that although SUFU is not duplicated in SHFM3 patients, if BTRC acts upstream of SUFU then an over expression of BTRC would lead to an over expression of SUFU. Merely over expressing BTRC in the chicken model may lead to an increase in SUFU as well; however it may be necessary to over express SUFU independently and within a BTRC model system.

In SHFM3 patients, there is also a duplication of exons 6-9 of FBXW4. It is possible that these three exons encode a protein that prevents the proper formation of the limb. Transcription of these exons could also result in a short-interfering RNA (siRNA) that prevents proper translation of a gene transcript crucial for patterning of the hand or foot. In vitro translation of the duplicated fragment in either orientation should determine if a protein is made. If so, the fragment should also be cloned into an over-expression vector to test for any affect on limb phenotype in the chick.

**Other experimental approaches**

When comparing the expression of genes between the Dac limbs and the wildtype, it is possible that expression of some of these genes is reduced. To test if the
lower expression of a gene results in the SHFM phenotype, knock-down morpholinos in zebrafish could be employed to lower expression of the candidate gene and observe the result on the phenotype.

A better experimental method to study the effect of this duplication on limb phenotype is to create a mouse model with the full duplication seen in SHFM3 patients. A conditional knock-in method is required to insert the duplicated region under the control of an inducible limb specific enhancer.

As stated above, the mouse model for SHFM3 is the result of insertion or inversion mutations of the Dactylin gene, the mouse homolog of FBXW4. It is possible that the duplication seen in SHFM3 patients causes the phenotype by separating a gene, either within the critical region or further displaced, from a regulatory element. This region should be studied for highly conserved, non-coding regions that could be candidate regulatory elements for genes involved in limb development. Candidate elements can be cloned into a vector that when electroporated into the developing chick limb will express GFP in the tissue in the same pattern as the gene that the element enhances. If such an element is found, patients with SHFM of unknown cause should be screened for mutations in these elements.

In summary, most of the genes in the SHFM3 critical region are expressed during the time of limb development in the chicken. In situ hybridization suggests that any or all of these genes may play a role in the formation and patterning of the digits.
Screening of VISTA enhancers

The Lawrence Berkeley National Laboratory is currently using a mouse model to identify non-coding elements with enhancer activity. Two such elements, with enhancer activity in the limb, are located in SHFM loci. One element located within intron 4-5 of \textit{BTRC} in the SHFM3 locus (Figure 6.1), shows enhancer activity in the central region of the autopod, the same region that lacks digits in SHFM patients (Figure 6.1). The second element, located within the SHFM5 locus, shows activity in the AER (Figure 6.2).

Although these sequences do not code for protein, changes in the sequences could inhibit enhancer function. Screening of both elements in 57 patients (this study) with SHFM for which the molecular cause is unknown found no significant sequence changes.

Microduplications of the \textit{SHH} enhancer, ZRS, have been associated with the limb malformation triphalangeal thumb polysyndactyly [50]. Since \textit{BTRC} is duplicated in SHFM3 patients, it is possible that the extra copy of the enhancer is responsible for the SHFM3 phenotype. Patients that do not have a molecular diagnosis should be screened using qPCR for duplications of the enhancer within \textit{BTRC}. This technique was used to screen for deletions of the enhancer from the SHFM5 locus in a separate study and is not discussed here.
Figure 6.1: Enhancer element within *BTRC*

Image from the VISTA enhancer browser of the expression of a reporter gene driven by an enhancer within intron 4-5 of *BTRC* in a mouse embryo. This enhancer shows activity in the central region of the autopod, in the same region where digits are absent in SHFM patients.
Figure 6.2: Enhancer element within SHFM5 locus

Image from VISTA enhancer browser of the expression of a reporter gene driven by an enhancer within the SHFM5 locus in a mouse embryo. This enhancer shows activity in the AER.
Conclusion

RT-PCR and in situ hybridization analysis identified candidate genes in and around the SHFM3 critical region. This study provided a better understanding of the normal mRNA expression patterns of btrc, poll, dpcd, fbxw4, fgf8, and sufu in the developing chicken limb, suggesting that with the exception of POLL, mis-expression of all of these genes are candidates for the causative agent of Split Hand/Foot Malformation. These data provide a baseline for pursuing future studies, initially using the chick and zebrafish as model systems for gain- and loss-of-function studies of the candidate genes both individually and in combination. Mouse experimental approaches will further expand our understanding of the role of these genes in normal limb development and the SHFM3 phenotype.
APPENDICES

Appendix A Primer Sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Annealing Temperature (°C)</th>
</tr>
</thead>
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<tr>
<td>btrc</td>
<td>GGC TGT GGG ATA TCG AGT GT</td>
<td>TGA GGA TGG TGT CAT CGT GT</td>
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</tr>
<tr>
<td>poll</td>
<td>GTG TGG CAT GTG GCT CCT AC</td>
<td>CAG AGT CAC CAG TCC CGT TC</td>
<td>59</td>
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<tr>
<td>dpcd</td>
<td>ACG GGA AGC GGA AGA TCC</td>
<td>CTC CTT TGG CTT CTT GTA CG</td>
<td>58</td>
</tr>
<tr>
<td>fbxw4</td>
<td>GAG AAC ATC CAG GCC TAC CA</td>
<td>CGC AGT TCA CCT CTT GTT CA</td>
<td>52</td>
</tr>
<tr>
<td>fgf8</td>
<td>CAG AGC CTG GTG ACA GAT CA</td>
<td>TTT CCT TTG CCG TTA CTC TT</td>
<td>50</td>
</tr>
<tr>
<td>sufu</td>
<td>GAA TGG GTT GAA CCA TGA CC</td>
<td>TCA GGC CAG CTG GTA CTC CTT</td>
<td>55</td>
</tr>
<tr>
<td>18SrRNA</td>
<td>CGG CGG CTT TGG TGA CTC TA</td>
<td>CGC CGG TCC AAG AAT TTC AC</td>
<td>55</td>
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</table>

**Figure A-1 Primers Used in RT-PCR and Annealing Temperatures:** Primer sequences for RT-PCR of each gene and the annealing temperature used. All sequences are reported 5’ to 3’.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
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<tr>
<td>btrc</td>
<td>GGC TGT GGG ATA TCG AGT GT</td>
<td>TGA GGA TGG TGT CAT CGT GT</td>
</tr>
<tr>
<td>dpcd</td>
<td>CAG TTT GAG GAT GGG AAG GA</td>
<td>CAA TGG TCC CAG GAG AGG TA</td>
</tr>
<tr>
<td>fbxw4</td>
<td>GAG AAC ATC CAG GCC TAC CA</td>
<td>CGC AGT TCA CCT CTT GTT CA</td>
</tr>
<tr>
<td>sufu</td>
<td>ACG AAG ACA GCA GGA GCA TT</td>
<td>GGT CAT GGT TCA ACC CAT TC</td>
</tr>
<tr>
<td>18 S rRNA</td>
<td>CAT GGC CGT TCT TAG TTG GT</td>
<td>GAC ACA AGC TGA GCC AGT CA</td>
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</tbody>
</table>

**Figure A-2 Primers Used in qPCR**: Primer sequences of primers used in qPCR of each gene, annealing temperature was 55°C for all primer pairs. All sequences are reported 5’ to 3’.
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<th>Reverse Primer</th>
<th>Annealing Temp (°C)</th>
</tr>
</thead>
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<tr>
<td>EN 1</td>
<td>TCT AGC TGC AGG GGT TTT TG</td>
<td>GAA CTT GAT CGA TGG CTG CT</td>
<td>59</td>
</tr>
<tr>
<td>EN 2</td>
<td>GAA ACA TAA ACA CAG CGC TCA</td>
<td>GCA CTG CAG CAA GCT CTA AA</td>
<td>58</td>
</tr>
<tr>
<td>EN 3</td>
<td>GAA AGG CTG TGT CCT GAA ATG</td>
<td>TGC AGT CAT TCC AAT TCC TTT</td>
<td>58</td>
</tr>
<tr>
<td>EN 4</td>
<td>TCC CCA AGT CTG TAT CAC TGG</td>
<td>ACC CCC TGG CAA ATT GTA A</td>
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</table>

**Figure A-3 Primers Used to Sequence Enhancer Located in Intron 4-5 of BTRC and Annealing Temperatures:** Sequencing and annealing temperatures for primers used in sequencing of enhancer elements within intron 4-5 of BTRC. All sequences are reported 5’ to 3’.
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<th>Name</th>
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<th>Reverse Primer</th>
</tr>
</thead>
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<td>EN 4</td>
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</tr>
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<td>CCT GAT GGA GGA TAC TGA CCA</td>
<td>CAA ATC AAA ACT ATC CAA AGC AAA</td>
</tr>
<tr>
<td>EN 6</td>
<td>TGG AAA ATT GAA ACC ATG TGC</td>
<td>TTT TAA AAT CCA GAT TGA ATG CTT</td>
</tr>
</tbody>
</table>

**Figure A-4 Primers used to Sequence Enhancer within PTD004 in the SHFM5**

**Critical Region:** Primers used to sequence the 3’ portion of the enhancer within *PTD004*. The annealing temperature was 53°C for all primer pairs. All sequences are reported 5’ to 3’.
REFERENCES


