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Genetic regulatory pathways of split hand-foot malformation

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Running title: Genetic network associated with SHFM

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Abstract

Split-hand/foot malformation (SHFM) are caused by mutations in *TP63, DLX5, DLX6, FGF8, FGFR1, WNT10B*, and *BHLHA9*. The clinical features of SHFM caused by mutations of these genes are not distinguishable. This implies that in normal situations these SHFM-associated genes share an underlying regulatory pathway that is involved in the development of the central parts of the hands and feet. The mutations in SHFM-related genes lead to dysregulation of Fgf8 in the central portion of the apical ectodermal ridge (AER) and subsequently lead to misexpression of a number of downstream target genes, failure of stratification of the AER, and thus SHFM. Syndactyly of the remaining digits is most likely the effects of dysregulation of Fgf-Bmp-Msx signalling on apoptotic cell death. Loss of digit identity in SHFM is hypothesized to be the effects of misexpression of HOX genes, abnormal SHH gradient, or the loss of balance between GLI3A and GLI3R. Disruption of canonical and non-canonical Wnt signalling is involved in the pathogenesis of SHFM. Whatever the causative genes of SHFM are, the mutations seem to lead to dysregulation of Fgf8 in AER cells of the central parts of the hands and feet and disruption of Wnt-Bmp-Fgf signalling pathways in AER.

Keywords: SHFM, ectrodactyly, oligodactyly, malformation of limb, lobster claw deformity.

INTRODUCTION

Split-hand/foot malformation (SHFM) is a congenital limb malformation predominantly affecting the central rays of the hands and/or feet (MIM 183600, 313350, 600095, 605289, and 606708). The clinical features of SHFM are highly variable and asymmetrical.[1] They range from mild defects such as a hypoplasia of a single phalanx or syndactyly, to aplasia of one or more central digits. Intra-familial and inter-individual variability of the SHFM are very high (Fig. 1). Phenotypic variability is most likely the result of genetic background including modifier genes, epigenetic and environmental factors. The condition is clinically and genetically heterogeneous. It may be isolated (non-syndromic) or syndromic. Most cases show autosomal dominant inheritance with variable expressivity and reduced penetrance. Pathogenesis of SHFM is directly and indirectly related to maldevelopment of the apical ectodermal ridge (AER), a specialized stratified columnar epithelium locating at the distal rim of the developing limb bud. The AER is the transitory major signalling center for proximodistal growth and distal limb development.[2] It keeps the underlying mesenchyme in a proliferative and undifferentiated stage, preventing cell death, and subsequently allowing the developing limb to elongate. The AER is induced through the reciprocal interactions between the ectoderm and the underlying mesenchyme involving Wnt-Bmp-Fgf signalling pathways.[3,4] Disruption of these pathways in the AER generally lead to SHFM.

SHFM-ASSOCIATED SYNDROMES

Split-hand/foot malformation has been reported to be associated with ectrodactyly-ectodermal dysplasia-clefting syndrome (EEC; MIM 129900),[5] brachydactyly-ectrodactyly with fibular aplasia or hypoplasia (MIM 113310),[6] autosomal recessive *DLX5*-associated SHFM,[7] autosomal dominant *DLX5*-associated SHFM,[8] autosomal dominant *DLX6*-associated SHFM,[9] *FGFR1*-associated congenital hypogonadotropic hypogonadism with SHFM (MIM #147950),[10] *BHLHA9*-associated SHFM with long-bone deficiency (SHFMLD: MIM # 119100),[11,12] and autosomal recessive *WNT10B*-associated SHFM (SHFM6; MIM225300).[1] A patient with Kabuki syndrome with SHFM has been reported, but molecular testing was not performed.[13] Being associated with a number of syndromes implies that pathogenesis of SHFM involves a number of genes and these genes are likely to share the pathogenetic pathways involving in developing the central part of the hands and feet. It is important to note that "atypical" SHFM can be seen in patients affected with PORCN-associated Goltz-Gorlin or cohesin complex-associated Cornelia-de Lange syndrome, but here we focus only on the WNT-TP63-DLX-associated SHFM.

SHFM-ASSOCIATED GENES

Mutations in *TP63*, *DLX5*, *DLX6*, *FGFR1, BHLHA9,* and *WNT10B* are known to be associated with SHFM in humans, and interestingly the clinical features of SHFM caused by mutations of these genes are not distinguishable.[9,14,15] This implies that in a normal situation these SHFM-associated genes share in part an underlying regulatory pathway that is involved in development of hands and feet (Fig. 2). These genes are

found to be co-expressed in the AER cells of the developing mouse limb[2] and the developing fin of zebrafish.[16] Interestingly SHFM has also been observed in mice, amphibians, and chickens, indicating the similar and conserved genetic signalling pathways associated with SHFM in various species.[4]

***TP63* and SHFM**

p63 (MIM 603273), a homolog to p53 and p73, codes for transcription factor p63. *p63* is expressed in ectoderm-derived tissues. Its role is to initiate epithelial stratification program during early embryonic development. Mutations in *TP63* have been reported to be associated with various kinds of human malformations including SHFM-associated EEC syndrome and isolated SHFM.[5] *p63* has crucial roles in forming AER and controlling AER functions via transcriptional regulation of AER-restricted target genes including *Dlx5*, *Fgf8*, *Sp6*, *Sp8*, and *Msx1* (Fig. 2). The Np63± isoform, the most expressed isoform of embryonic ectoderm,[14] is the predominant p63 isoform expressed in developing limb. It is the main regulatory isoform that has been shown to induce transcription of the *Dlx5* and *Dlx6* promoters *in vitro* (Fig. 2).[2] *p63*$^{-/-}$ mice have abnormal expression of AER-restricted target genes, failure of AER formation, and subsequent absent hindlimbs and severely truncated forelimbs (Fig. 2).[3,17,18] Failure of AER stratification subsequent to disruption of p63-Dlx signalling pathway might be the result of dysregulation of cell adhesion-associated *p63* target genes including *Perp* and *CDH3*. Both genes have important roles in cell adhesion and maintenance of epithelial integrity and mutations in *CDH3* have been shown to cause SHFM in autosomal recessive ectodermal dysplasia-ectrodactyly-macular dystrophy syndrome (EEMS;

MIM #225280).[19] It is noteworthy that limb defects related to *p63* mutations or p63-associated pathways appear to be more severe on the hindlimbs than the forelimbs. However, only the *TP63* mutations that cause EEC syndrome and isolated SHFM but not ankyloblepharon-ectodermal defects-cleft lip/palate (AEC; MIM #106206) syndrome lose the ability to induce the transcription of the *Dlx5* and *Dlx6* by cis-acting regulation at the promoter level *in vitro*.[2] This implies that the SHFM caused by *TP63* mutations depends on the exact nature of the mutations.

***Dlx genes* and SHFM**

Dlx genes code homeodomain-containing transcription factors that are vertebrate homologs of Drosophila *distalless* (*dll*), a gene necessary for specification of the distal structures of appendages including legs. Mutations in *DLX5* (MIM 600028) have been reported to be associated with autosomal recessive SHFM[7] and autosomal dominant SHFM.[8] A heterozygous missense mutation in *DLX6* (MIM 600030) has been reported to be associated with SHFM (Fig. 2).[20] *Dlx5* and *Dlx6* genes, are known to regulate the development of the central portion of AER during early limb development. Both genes are essential for the maintenance of the central portion of the murine hindlimb AER. In the *Dlx5/6*$^{-/-}$ mouse embryos, the expression of *Fgf8* and *Dlx2* is downregulated specifically in the central portion of AER of the hindlimbs, the part that would have governed development of the central rays of the feet (Fig. 2).[14,21,22] This is supported by the SHFM observed in *Dlx2/5*$^{-/-}$ mice (Fig. 2).[23] The split hindlimbs in *Dlx5/6*$^{-/-}$ mice are the result of a cell-autonomous failure of the central AER to regulate *p63* and to

maintain and express morphogenetic signals (Fig. 2).[24,25] The degeneration of the AER in *Dlx5/6*$^{-/-}$ mice led to SHFM phenotype which is similar to the phenotype of *Dactylaplasia* mice.[26] The polarization of the ectodermal cells of AER of the developing limb is regulated by non-canonical Wnt5a signalling. Dlx-related SHFM is associated with the loss of basoapical and planar cell polarity (PCP) and abnormal non-canonical Wnt5a signalling, as a result of reduced expression of *Wnt5a*, a transcriptional target of Dlx5 (Fig. 2).[14,27]

Dlx5 and Dlx6 proteins are direct targets of Np63±.[2] *p63* mutations or combined loss of *Dlx5* and *Dlx6* lead to downregulation of *FGF8* and *Dlx2* expression in the central portion of AER, misexpression of genes important for limb development, and subsequent SHFM (Fig. 2).[2,21,22] *Dlx5* and *Dlx6*, and to a lesser degree *Dlx1* and *Dlx2,* are downregulated in heterozygous *p63*$^{-/+}$ and *p63*^{EEC} in the mouse hindlimbs, which have normal AER.[2] Like p63$^{-/-}$ mice the hindlimbs of *Dlx5/6*$^{-/-}$ mice are more severely affected than the forelimbs.[2,17,18] The *p63*^{EEC} mutation has different effects on forelimbs and hindlimbs because *Dlx* genes expression is reduced in the hindlimbs but increased in the forelimbs of the *p63* heterozygous mice.[2] SHFM in patients with *TP63*, *DLX5*, and *DLX6* mutations may be the result of failure of central AER formation and maintenance because *Dlx5* and *Dlx6* regulate the expression of the key AER controlling gene *Fgf8* in the central part of AER and p63 works upstream as their transcription factor (Fig. 2).

***WNT10B*, WNT signalling, and SHFM**

Initially limb buds form as the result of the interaction between Fgf and Wnt signalling.[28] Subsequently Wnt-² -catenin signalling has important roles in proximodistal outgrowth and dorsoventral patterning of the limb. Removal of ² -catenin from the limb ectoderm prior to the initiation of *Fgf8* expression completely prevents limb development while its removal after *Fgf8* expression results in limb truncations.[29] *Wnt8c,* a mediator of hindlimb bud initiation, is expressed in the presumptive hindlimb region and mediates the Fgf8-Fgf10 regulatory loop via canonical Wnt-² -catenin signalling.[30]

The association of *WNT10B* (MIM 601906) mutations and SHFM supports the important roles of *WNT10B* and canonical Wnt-² -catenin signalling pathway in the development of the central parts of hands and feet (Fig. 2).[1] *WNT10B* mutations have also been reported to be associated with isolated dental anomalies including hypodontia, microdontia, and taurodontism,[31] suggesting that the phenotypes of *WNT10B* and *TP63* mutations evidently depend on the exact nature of the mutations. Generally *Wnt10b* is expressed throughout the limb bud ectoderm in all stages of limb development. However, the expression in AER is only at E11.5.[32] Canonical and non-canonical Wnt signalling pathways have been known to be involved in several developmental processes at many stages of limb development.[28,33] Both pathways are required for AER formation and maintenance. Abnormal canonical Wnt-² -catenin signalling results in premature AER degeneration (Fig. 2). *Wnt5A* is expressed in the AER and the underlying mesenchyme at the same time as *p63*, *Dlx5*, and *Dlx6*. Loss of the non-canonical Wnt receptors Ryx and Ror2 causes abnormal limbs similar to those of *Wnt5a*

mutants suggesting the involvement of *Wnt5a* in non-canonical Wnt signalling.[34] *Wnt5a* via non-canonical Wnt/PCP signalling is necessary for driving proper limb morphogenesis by regulating cell organization and orienting cellular processes including mitosis and directional cell movements.[28,33] Inactivation of *Wnt5a* results in reduced proliferation of mesenchymal cells in the progress zone and subsequent distal limb truncation.[35] Evidently pathogenesis of SHFM involves both canonical and non-canonical Wnt signalling (Fig. 2).

Canonical Wnt signalling involves *Wnt10b* and *Wnt3a,* and acts upstream of FGFs in establishing AER gene expression.[29,36] *Wnt3a* acts upstream of *Fgf8* during chick limb bud outgrowth and AER formation through Wnt3a/² -catenin/Lef1 signalling.[36] Different *Wnt* genes are able to substitute for one another when they activate the same intracellular signalling pathways mediated by ² -catenin/Lef1 signalling.[36] The malformed digits in patients with SHFM might also be the result of abnormal *Shh* expression which is controlled by the combinatorial influence of Wnt and Fgf signalling.[28]

***Sp6* and *Sp8* and SHFM**

Sp6 (Epiprofin; MIM 608613) and Sp8 (Buttonhead; MIM 608306) are members of the Sp zinc finger transcription factor family that have important roles in AER induction and maintenance and dorsoventral patterning of the limb.[3] Both genes are expressed in the entire prospective limb ectoderm but subsequently confined to the AER during limb bud emergence. *Sp6* and *Sp8* share similar patterns of gene expression in limb bud ectoderm and AER and have redundant function downstream of Wnt/² -catenin

signalling in the induction of *Fgf8* (Fig. 2) and Bmp signalling in the induction of *En1,* coordinating the link between proximal-distal and dorsal-ventral patterning [Haro et al., 2014].[3] *Sp6*$^{-/-}$ mice had mild syndactyly[37] while *Sp8*$^{-/-}$ mice displayed severe limb truncation.[38] Sp6 and Sp8 work together in a dose-dependent manner and are indispendable mediators of Wnt-² -catenin and Bmp signalling in developing limb ectoderm.[3,39] Abnormal Wnt-² -catenin [30] and Bmp signalling [39] in AER have been reported to cause dysregulation of FGF signalling and subsequent SHFM.[4] *Sp6*$^{-/-}$; *Sp8*$^{+/-}$ mice had SHFM with hindlimbs more severely affected than the forelimbs (Fig. 2). The digits of forelimbs and hindlimbs showed dorsalization of the digit tips. Sp6 and Sp8 work together as necessary mediators of the Wnt-² -catenin-Fgf8 regulatory loop (Fig. 2). The observed phenotypes of *Sp6*$^{-/-}$; *Sp8*$^{+/-}$ mice indicate that the product obtained from one allele of *Sp8* in the absence of *Sp6* is not enough for proper *Fgf8* induction (Fig. 2). Complete absence of Sp6 and Sp8 transcription factors does not prevent the initiation of AER morphology indicating the independence of AER function and morphology.[3] *Tp63, Dlx5*, and *Dlx6* have normal expression in the early *Sp6*$^{-/-,}$ *Sp8*$^{-/-}$ limb bud implying that Sp6 and Sp8 act downstream of *Tp63* and *Dlx5* and *Dlx6*. It is suggested that Wnt-² -catenin signalling is upstream of Tp63-Dlx and the Sp-Fgf8 regulatory modules (Fig. 2).[3]

***BHLHA9* and SHFM**

Mutations in *BHLHA9* (Fingerin; MIM 615416) have been associated with SHFMLD (Fig. 2) [Malik et al., 2014].[12] BHLHA9, a member of the basic helix-loop-helix *(bHLH)* transcription factor family, has an important role in development of limb. At an

early embryonic stage (E10.5) *Bhlha9* is prominently expressed on the dorsal and ventral surfaces covering the progress zone near AER. It transiently regulates AER formation in the progress zone of developing limb by regulating AER formation-related genes, including *Trp63 and Fgf8* (Fig. 2). *Bhlha9*-knockout mice have various degree of syndactyly and poliosis of the limb,[40] and *Bhlha9*-knockdown zebrafish have severely truncated pectoral fins.[11] *Bhlha9*-knockout AER have overexpression and dysregulation of *Trp63* and *Fgf8*.[41] Mutations in the DNA-binding domain of BHLHA9 destroys the ability to fine tune control of regulatory pathways of limb development.[12] Abnormal expression of *BHLHA9* causes SHFM and SHFMLD via dysregulation of AER formation and AER-associated genes (Fig. 2).

ZAK and SHFM

A syndrome of split foot without split hand malformation with mesoaxial polydactyly (MIM #616890) has been reported to be caused by mutations in *ZAK* (Leucine-zipper and sterile alpha motif-containing kinase; MIM 609479) (Fig. 2). *ZAK* has an important role in limb development. ZAK is a direct target of Tp63 and the deletion of its SAM domain has been shown to be associated with down-regulation of *Tp63* in the developing limb bud (Fig. 2).[42] The absence of split hands but the presence split feet in patients with *ZAK* mutations supports the control of *ZAK* by *TP63* genes because the hindlimbs of the *Tp63* KO mice are more severely affected than the forelimbs.[17,18] In addition having split feet without split hands suggests the association of *ZAK*, *PITX1*, and *TBX4* genes in the formation of feet. The specification of limb identity and

morphology is established prior to limb initiation. The transcription factor Tbx4 and its upstream regulator Pitx1 are known to have important role in defining the hindlimbs in mice, legs of chicks, and pelvic fins of fishes.[14,43]

Fgf8, FGF signalling and SHFM

FGF signalling is known to be important in driving cell proliferation, cell survival, and specification of limb mesenchymal cells. It also promotes the velocity of cell movements within the developing limb bud, thereby promoting limb elongation.[33] FGF8 is a potent ligand of FGFR1 and mutations in *FGFR1* are associated with congenital hypogonadotropic hypogonadism with SHFM (Fig. 2).[10] Fgf8, a direct target of P63, is a key morphogen for limb outgrowth and patterning.[17] It is the first AER marker during chick limb development.[36] *Fgf8* is initially expressed in a broad area in the distal limb ectoderm and subsequently becomes restricted to the AER.[33] Fgf8 signalling is essential for AER induction and maintenance.[44] *Fgf8* functions are to sustain epithelial-mesenchymal signalling and assure the timely generation of the correct population of mesenchymal progenitors.[45] It in turn regulates function and stability of Np63± by increasing the binding of Np63± to the tyrosine kinase c-Abl and the level of Np63± acetylation.[25] Aberration of *Fgf8* expression is hypothesized to cause malfunction of Np63± (Fig. 2). In order for a normal limb to develop, *Fgf8* is crucial for the correct establishment of the signalling loop within the developing limb bud.[46] Alterations in *Fgf8* expression and signalling have been demonstrated to lead to altered reduced layer or altered adhesion in AER and subsequent limb malformations in mice.[44] Reduced

expression of *Fgf8* and its signalling is generally linked to abnormal AER morphology and subsequent SHFM (Fig. 2). However, increase of its expression in AER in *Ikk*± mutant mice is associated with distal limb truncations and overexpression of *Fgf8* has been demonstrated in *Bhlha9*-knockout AER.[41] It is noteworthy that AER-specific *Fgf8*$^{-/-}$ mice have a normal AER, suggesting that abnormal *Fgf8* expression alone does not lead to SHFM. In order for mice to have SHFM, alterations in *Fgf8* expression need to accompany abnormal expression of other genes in AER as well. Loss of *Fgf8* or mutations in *Fgf8* lead to abnormal Np63± protein stability,[24,25,44] failure of AER stratification, and subsequent aberrant limb development (Fig. 2).[45]

All lines of evidence suggest that abnormalities of *TP63*, *WNT10B*, *DLX5*, *DLX6, FGF8*, and *FGFR1* lead to dysregulation of *FGF8* in the central portion of the AER and subsequently lead to misexpression of a number of AER genes, failure of its stratification, and thus SHFM (Fig. 2).

Embryological mechanisms of digital patterning in SHFM

The anatomical defect in *Dlx5, Dlx6*-/- SHFM in the mouse has been shown to relate to a partial breakdown of the apical ectodermal ridge (AER) during the paddle stage of limb morphogenesis.[14] A typical phenotype in SHFM consists of a loss of one or more central digits, with the first and fifth digits typically remaining, but often malformed. Syndactyly of the remaining digits is common and may be related to the inability of the Fgf8-related disrupted AER to regulate the expression of *BMPs*, *Msx1,* and *Msx2* in the interdigital spaces. These molecules play important roles in stimulating normal

interdigital cell death. Reduced expression of *Msx1* and *Msx2* in developing autopod leads to premature differentiation of interdigital mesoderm into connective tissue, failure of apoptotic cell death of the interdigital mesoderm, and subsequent syndactyly of the remaining digits (Fig. 3).[47,48]

Recent reports have suggested that the putative transcription factor BHLHA9 is involved in SHFM.[41,49] Both the clinical report[49] and the study of *Bhlha9* knockout mice[41] emphasized the connection between lack of function of this gene and the presence of syndactyly that may or may not be associated with the missing or defective skeletal elements seen in SHFM. The knockout study showed that expression of *Bhlha9* occurs during the period when Conte et al. (2016) demonstrated a reduction of *Fgf8* expression in the central AER in the developing mouse limb, but the phenotypic effect appears to involve survival of interdigital soft tissue due to an diminution of apoptosis.[14]

Because of the malformation, it is not always possible to identify the remaining digits by digit number. Digit identity, the characteristic that allows one to determine specific digits 1-5, can be either embryologic or phenotypic. Embryologic identity would refer to the number of a specified digit, whereas phenotypic identity is based upon clinical examination. Unfortunately, there are presently no specific markers for mammalian digits 2-5 that would allow one to determine their embryological identity.

Two fundamentally different processes are involved in digit formation. One is patterning (specification), which occurs in the early outgrowing limb bud. Actual formation of digital primordia occurs later, under the influence of the AER and a putative digital organizing center (see below). Digit specification means that the fate of

mesenchymal cells in the hand or foot plate to develop into cartilaginous digital elements becomes fixed. By E11.5 in the mouse, when Conte et al. [2016] identified breakdown of the AER, all five digits in the mouse limb have already been specified,[14,50] and primordia of digits 2-5 are already identifiable.[51]

Early patterning of the limb and the digits is based on the secretion of the morphogen Shh from the zone of polarizing activity (ZPA), located on the posterior edge of the limb bud.[4] Patterning of the digits in a pentadactyl limb is largely based on the reactions of mesenchymal cells to various degrees of exposure to Shh. Digit 1 is generally conceded to be independent of an Shh influence, and it will form in the absence of a ZPA. Digits 2-5 are patterned on the basis of both time of exposure and concentration of mesenchymal cells to Shh. A low concentration of Shh specifies digit 2, whereas exposure to a higher concentration of Shh over a longer time specifies digit 3. Digits 4 and 5 are specified on the basis of length of exposure to high concentrations of Shh.[50,52] Loss of digit identity in SHFM has been hypothesized to be the effects of misexpression of *HOX* genes, abnormal SHH gradient, or the loss of balance between GLI3A and GLI3R as a result of *WNT10B*-associated abnormal FGF signalling.[53,54]

Individual digits begin to take shape through the appearance of pre-chondrogenic organizing centers that form the distal aspect of outgrowing digital rays.[55] Outgrowth of digits requires the action of a functional AER over the tips of the digits. While digits are taking shape, the AER over the future interdigital spaces becomes attenuated. In normal animals, mesenchymal cells accumulate, either by movements[33] and/or cell division, beneath the non-attenuated segments of the AER. Given a

sufficient number of mesenchymal cells, digital primordia begin to form. In the absence of sufficient cells, chondrogenesis in either limb or digital primordia fails to occur.

Based on the information available, the pathology commonly seen in the various types of SHFM suggests that the malformation does not affect the specification of the central digits because specification occurs very early, well before the earliest reported demonstration of pathology to the AER. Rather, it appears to be due in large part to the inability of the disrupted AER in mutants to support the outgrowth of the digital primordia in the central part of the hand or foot plate. It would be necessary to examine limbs at stages earlier than E11.5, as was done by Conte et. al. [2016], in order to determine whether or not the genetic defect affects digital specification. If affected hands or feet possess a recognizable non-digit 1 digit on the anterior side, it would suggest that full initial digital specification had, indeed, occurred prior to the development of the defect because digital specification occurs in a posterior-to-anterior direction, and it would be unlikely that a digit 2 could form in the absence of a specified digit 3 or 4. If only a digit 1 is present on the anterior side, it would not be possible to comment on possible specification issues.[14]

Mirror image morphogenesis in SHFM

It is noteworthy that the left-right abnormalities of hands and feet in patients affected with SHFM are almost always asymmetric. Although left-right asymmetry of appendages is set very early in development, very little is known about mechanisms that would be susceptible to the asymmetric genesis of abnormalities in processes that occur later in limb development.

Bilaterally symmetrical organisms exhibit mirror-image morphogenesis of many paired structures, such as limbs, eyebrows, palpebral fissures, nose, and teeth. Over the course of evolution it appears to provide mechanical advantage for the limbs. It provides balance for the feet and hands and a greater ability to approach things from opposite angles. The embryological basis for bilateral symmetry occurs very early in the embryo with the establishment of the primitive streak, which allows recognition of both the craniocaudal (anteroposterior) and right-left axes. SHFM is evidently a malformation affecting predominantly the anteroposterior axis of the limbs. Sequential expression of *Hox* genes along the central craniocaudal axis provides the basis for molecular events leading to the development of the left and right fore- and hindlimbs. Until establishment of the anteroposterior axis within the limb field, bilateral symmetry of limbs is not fixed. A century ago, Harrison (1921) and Swett, (1937) rotated limb disks or early salamander embryos. If done early enough, a left limb disk could be converted into a right limb by transplanting it onto the right side of the embryo. Later in development, after the anteroposterior axis was established, the same manipulation resulted in the formation of a left limb, although reversed in orientation. Fixation of the anteroposterior axis occurs very early, before formation of the AER. In birds and mammals, establishment of the ZPA is a concrete indication that the anteroposterior polarity of the limb bud has been fixed.[56,57]

It is noteworthy that most pathways that lead to SHFM are involved *Dlx5* gene and it is interesting to note that olfactory organs of *Dlx5*-/- mice are usually

asymmetric.[58] This suggests that *Dlx5* may affect the manifestation of mirror image morphogenesis

Concluding remarks

Split hand-foot malformation is associated with genes involved in AER function or maintenance. It is the consequence of maldevelopment of the central part of the AER, especially as a result of dysregulation of *Fgf8*. Dysregulation of the p63-Dlx transcriptional pathway appears to affect a centralized network relevant to SHFM. Mutations in *WNT10B*, *FGFR1*, or inactivation of *Fgf8* disrupt the p63-Dlx-Fgf8 transcriptional network leading to dysregulation of *Fgf8* in AER cells, misexpression of genes important for limb development, failure of AER stratification, and subsequently cause SHFM. It is noteworthy that p63-Dlx-Fgf8 transcriptional network is tissue-specific and sensitive to gene dosage, timing, and position. Another pathway leading to SHFM appears to be a Wnt/² -catenin-Sp8-Dlx-Fgf8 signalling pathway, which does not appear to involve p63. This review shows that a number of molecules are involved in the formation of hands and feet and every molecule seems to be connected. When mutation occurs, the process of limb formation is disrupted like “domino effect”, leading to SHFM.

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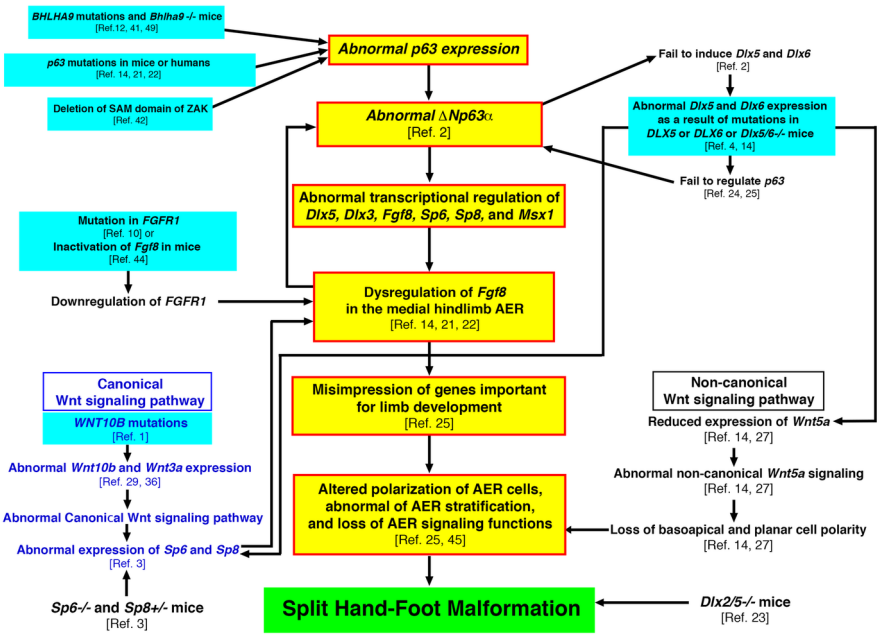
Figure Legends

Figure 1. Split hand-foot malformation caused by a mutation in *TP63*. A,B) Mother. C,D) Son. Note loss of mirror image morphogenesis or asymmetric manifestaitons of hands and feet. Note loss of phynotypic identity of digits.

Figure 2. Flowchart demonstrates genetic pathways involving SHFM. Mutations that cause SHFM in mice and humans. Disruption of p63-Dlx-Fgf8 transcriptional network leads to dysregulation of *Fgf8* in AER cells, misexpression of genes important for limb development, failure of AER stratification, and subsequently cause SHFM. Canonical and non-canonical Wnt signalling pathways are involved in the pathogenesis of SHFM.

Figure 3. Flowchart demonstrates genetic pathways involving syndactyly of the remaining digits in SHFM. Syndactyly of the remaining digits in SHFM involves abnormal Fgf-Bmp-retinoic acid signalling, reduced expression of *Msx* genes, premature differentiation of interdigital mesoderm into connective tissue, failure of apoptotic cell death, and thus syndactyly of the remaining digits.



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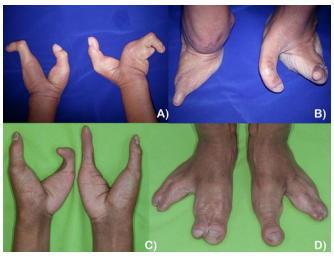

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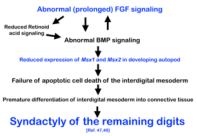

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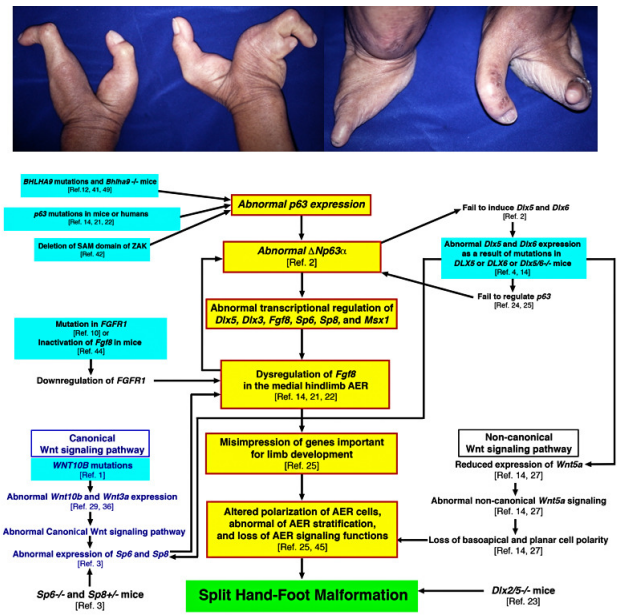

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