Chapter 6

Novel Cellular and Molecular Interactions During Limb Development, Revealed from Studies on the Split Hand Foot Congenital Malformation

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Abstract

The embryonic development of the limbs is widely used as a paradigm for the comprehension of the cellular processes and molecular mechanisms underlying organogenesis and pattern formation. The chick, mouse and (recently), zebrafish embryos are excellent models, for the ease of experimental manipulation and the availability of several mutant strains with limb malformation defects.

Knowledge on the molecular circuits that control cell expansion and position-dependent cell differentiation in the developing limb bud is rapidly expanding. Recently, a set of human congenital malformations known as split hand foot malformations (SHFM) together with the corresponding animal models have revealed novel molecular players and regulations, important for the function and maintenance of the apical ectodermal ridge, the structure that coordinates limb outgrowth with digit pattern.

In this chapter we illustrate the pathways centred on the master transcription factor p63, and discuss the mechanisms by which these pathways impact on the regulation of signalling molecule controlling growth and shape of the normal limb. Finally we indicate how the signalling networks are misregulated in SHFM, and point to emerging functions of the FGF8 and Wnt5a signalling molecules.

Keywords: Limb, Embryonic ectoderm, AER, SHFM, EEC, p63, Dlx5, Wnt5a, FGF8

1. Introduction

The limbs are projecting paired appendages of an animal body used especially for movement and grasping, for example, wings, arms, and legs. The development of the limb bud is often taken as a paradigm for a cellular and molecular comprehension of the common principles of organogenesis and pattern formation. Embryonic patterning implies that cells acquire positional information, usually by interpreting concentration gradient of signalling molecules. Accordingly, limb pattern is specified along three principal axes: anterior-posterior (A-P) (e.g., thumb to little finger), dorsal-ventral (D-V) (e.g., back of hand to palm) and proximal-distal (P-D) (e.g., shoulder to nails). Digit pattern across the A-P axis is a classic example of a signalling gradient that specifies positional values, linked to a gradient of Sonic-Hedgehog (SHH). D-V patterning is less studied and involves signals from dorsal and ventral ectoderm. The specification of P-D positional values has long been considered to involve a timing mechanism, under the control of ligands of the fibroblast growth factor (FGF) family. A concentration gradient of molecules can also give cells polarity information, recently shown to be critical for patterning and morphogenesis.

The limbs are not essential for life, thus a large number of mutant strains are available for studies on the genetic determinants of limb development, in normal and pathological conditions. Manipulation of chicken limb buds has been widely used in the past, mainly because of the ease of examination and manipulation, to postulate the first models of limb bud development leading to the identification of important regulatory genes and interactions. In addition to the chicken model, functional genetics has made great advances thanks to spontaneous and engineered loss- and gain-of-function mutant mouse strains, and recently with the advent of the zebrafish embryos as animal models.

In this chapter, we illustrate the pathways centred on the master transcription factor p63, and discuss the mechanisms by which these pathways impact on the regulation of signalling molecules controlling growth and patterning of the normal limb bud. Based on available knowledge, we propose how signalling networks are misregulated in the split hand foot malformation (SHFM) and related developmental conditions, and indicate emerging functions of the FGF8 and Wnt5a diffusible molecules.

1.1. Limb initiation

Around the embryonic age E8.0 in the mouse, limb buds are initiated as four lumps of mesenchymal cells covered by ectoderm, protruding from the main body axis at approximately the position of somites 6–11 (the forelimbs, FL) and somites 24–27 (the hindlimbs, HL). The limb buds are paired along the cephalo-caudal axis and develop at the same fixed locations on this body axis (Figure 1A). How are their positions defined?

It has been proposed that the position of several dorsal organs along the cephalo-caudal axis, their identity and timing of appearance depend on the expression of specific sets of *Hox* genes. The 39 vertebrate *Hox* genes code for homeodomain transcription factors, homologous with the genes of the Drosophila *HOM-C* complex, and are combinatorially expressed along the

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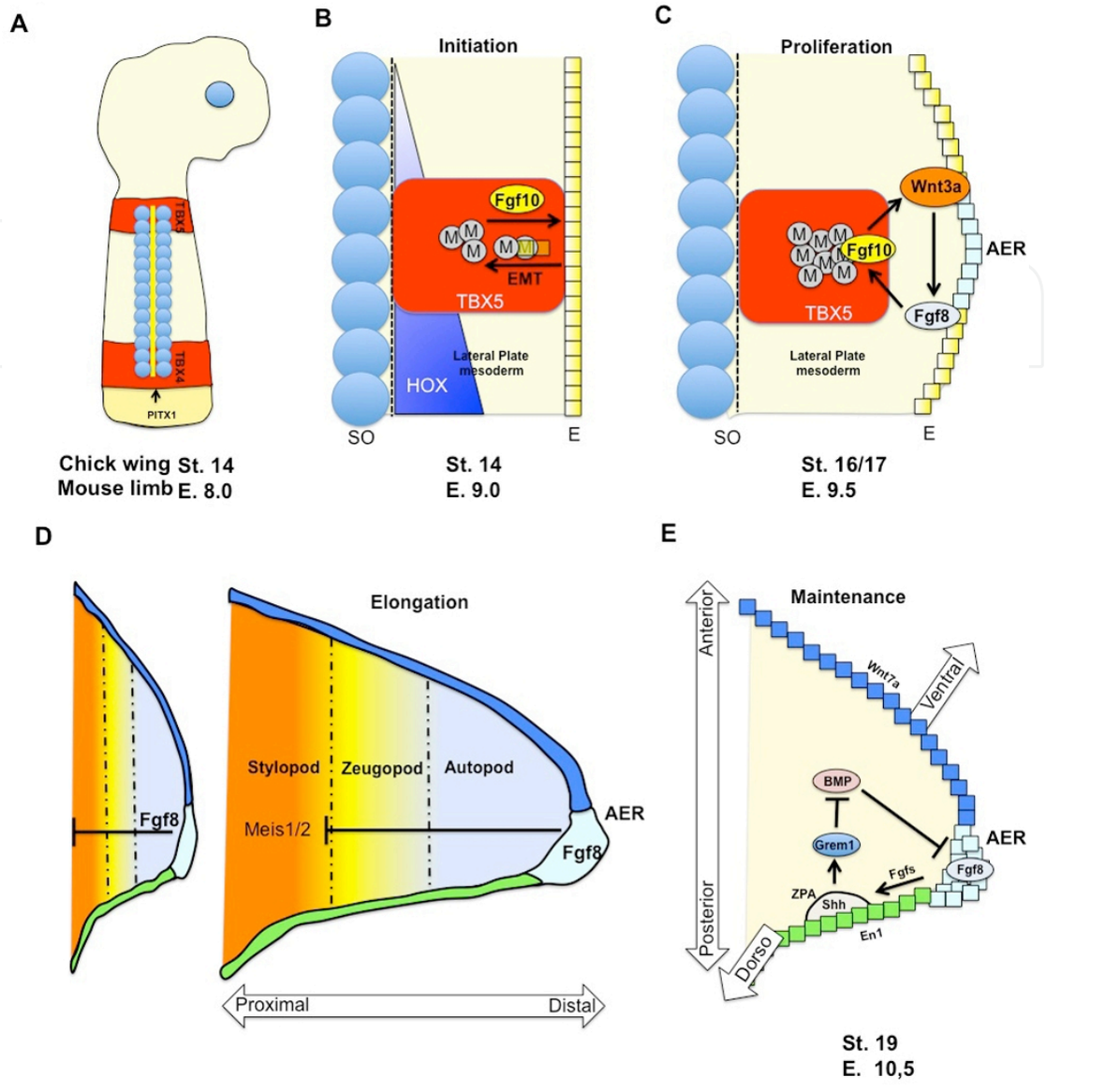

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Figure 1. Schematic representation of limb development with embryonic timeline for chick wing and mouse forelimb. A) Representation of the prospective limb territories in a stage 14/8 chick (Hamburger-Hamilton stages, HH))/mouse embryo. The forelimbs (FL) and hindlimbs (HL) derive from discrete regions of the lateral plate mesoderm (LPM). *Tbx5* (red) is expressed in the prospective FL, whereas *Tbx4* (red) is expressed in the prospective HL. In this stage, *Pitx1* (yellow) is expressed in a caudal domain that overlaps with *Tbx4*. The somites (blue) are numbered and serve as reference for the axial position of the FL and HL fields. B) Model of initiation of FL bud. Hox protein gradients establish the condition for the synthesis of retinoic acid (RA) in the LPM. RA causes induction of the transcription factors Tbx5 (or Tbx4 for the HL). *Tbx5*-expressing mesechymal cells express *FGF10* and induce the ectodermal cells of the surface (yellow square) to activate epithelial-mesenchymal transition (EMT). C) Newly generated mesenchymal cells express *FGF10* that induces the overlaying ectodermal cells to express *FGF8* giving rise to the apical ectodermal ridge (AER). Expression of *FGF8* by the AER induces the mesenchymal cells to express *FGF10*, thus establishing a positive feedback loop for the initial phases of limb outgrowth. D) Schematic representation of the progress zone (PZ) model. The AER maintains cells of the PZ in an unspecified state. For a detailed description of the proposed models of P-D patterning see the text. E) AER-derived FGF8 maintain the expression of *SHH* in the ZPA cells, which in turn gives feedback on the AER cells to maintain *FGFs* expression, via Grem1 and BMP inhibition. This signalling between the AER and ZPA contributes to co ordinate the patterning along the P-D and A-P axes.

main body axis. *Hox* genes are serially organized in four clusters (a, b, c, and d), each located on a different chromosome. Within each cluster, *Hox* genes are organized in a physical order collinear with the cephalo-caudal axis of the growing organism so that the genes lying at the 3′ end are expressed earlier and are localized in the most anterior domains. Moving toward the 5′ direction, each next gene is expressed in a progressively more posterior territory. Thus, each *Hox* gene has a specific anterior limit of expression, and each A-P embryonic territory expresses a specific combination of *Hox* genes, utilized as positional information.

A key signalling molecule for limb initiation is FGF10, a member of the FGF family of diffusible peptides. The *FGF10* mRNA is detected quite early in the presumptive limb mesenchyme and promotes AER induction (a key organizer and regulator of the P-D limb extension; see below) and initiation of ectopic limb development when applied exogenously [1]. Conversely, in *FGF10*-null mice, limb buds are initiated but the AER does not form, resulting in complete truncation of all four limbs [2 - 4].

The expression of *FGF10* coincides with the time when the trunk is only competent to form an ectopic limb, for example, the time at which the trunk mesenchyme becomes determined and can no longer be redirected to a limb fate (the HH stage 16–17 of the chick embryo) [5, 6]. The initial assumption was that limbs originate from a pre-existing mesenchymal population undergoing a localized regulation of proliferation. In fact, at the HH stage 17–18, there is a substantial decrease in proliferation of the flank mesoderm, while instead higher rates are maintained within the emerging limb buds.

The current model considers that, shortly after gastrulation, a re-epithelization of mesodermal cells occurs so that the entire embryo is essentially epithelial, including also the notochord, the somites, the intermediate mesoderm and the lateral plate mesoderm (LPM). At stage HH 13 in the chick, before limb initiation, the somatopleure displays epithelial rather than mesenchymal features. The LPM of the limb field starts out as an epithelium and ultimately generates limb-bud mesenchyme through a process termed epithelial-mesenchymal transition (EMT) [7] (Figure 1B). In embryos null for *FGF10* and *Tbx5* the proportion of mesenchymal cells compared to the proportion of epithelial cells was significantly lower than that of wild-type (WT). These mutants show hyperplasia of the somatopleure epithelium, in support of failure of these cells to undergo EMT. These new data show the time in which the trunk is competent to form an ectopic limb, is precisely the time at which the trunk mesenchyme is initially generated. The old experiments of ectopic application of FGF10 need to be re-interpreted, as induction of limb-bud formation from epithelial trunk somatopleure cells and not from mesenchymal cells of the same A-P level.

1.2. T-box genes and limb-type specification

The FL and HL of all vertebrate species are evidently different (e.g., wing vs. leg in the chick embryo, pectoral vs. pelvic fins in fish embryos, arms vs. legs in primates, etc.). The specification of limb-type identity and morphology is established before overt limb initiation. A large body of evidence indicates that two transcription factors of the T-box family participate in the early definition of limb-type identity: *Tbx5* for the presumptive territories of the mouse FL (wing of the chick, pectoral fins of fishes) and of *Tbx4* in the presumptive territory of the mouse

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HL (leg of the chick, pelvic fins of fishes) (Figure 1A). These genes are expressed very early in the prospective limb mesoderm and, in addition to define limb-type identity, also appear to be necessary and sufficient for early limb induction [8 - 15].

However, although only expressed in FL and HL, respectively, *Tbx5* and *Tbx4* appear not to be the master directors of limb-bud identity/morphology design. Instead, the *Pitx1* gene, which codes for a paired-type homeodomain transcription factor and which is expressed in the HL bud, is the upstream regulator of *Tbx4*, and is directly implicated in HL specification. Multiple independent *cis*-regulatory elements of *Tbx4* expression have been identified, including the HL-specific enhancers [16]. Both may be targets of Pitx1 and other unknown upstream factors. Structural changes of these regulators might be some of the multiple factors responsible for the hind/lower limb morphology specification. However, limb-bud identity/morphology determination remains to a large extent unexplained.

1.3. Role of retinoic acid

A signalling molecule known to act upstream of *Tbx5* during forelimb/pectoral fin development is retinoic acid (RA) [17 - 21]. According to old observations, patterning along the proximal-distal axis of the vertebrate limb is controlled by opposing diffusible signals, in which RA functions as the proximal signal and FGF as the distal one [22, 23].

The mechanism through which RA controls limb development has been widely debated [21], but clear results have only been produced in recent years [17, 19, 22, 24 - 27]. Mouse and zebrafish embryos null for the gene *aldehyde dehydrogenase-1a2 (aldh1a2*) fail to synthesize RA and do not develop limbs/fins. These mutants fail to express *Tbx5*, and the exogenous application of RA can rescue both limb/fin development and *Tbx5* expression [18, 19, 25, 27, 28]. However, a recent paper shows that RA signalling is not required for P-D limb patterning, and instead provides genetic evidence that RA-FGF antagonism occur only along the trunk lateral plate mesoderm, prior to FL budding, to permit induction of *Tbx5* [29]. This study shows that RA controls limb development in a manner much different than that originally envisioned (see below).

1.4. Proximal-distal axis

The limb skeleton is laid down as five cartilage skeletal elements, not just the three referred to as stylopod (humerus/femur), zeugopod (radius-ulna/tibia-fibula), and autopod (digits); in fact two carpal regions between zeugopod and autopod are present, that initially have the same size as the other segments but then grow substantially less.

P-D extension and patterning is strictly linked to the signalling activity of the apical ectodermal ridge (AER), a morphologically distinct ectodermal thickening, extending along the entire A-P length, and lining the D-V border. The AER is present between E9 and E11 in the mouse embryo, consists of a pseudo-stratified epithelium in the chick and pluri-stratified epithelium in the mouse, and is a dynamic structure constantly undergoing morphogenetic changes [30 - 32].

The AER plays a fundamental role in promoting and regulating the outgrowth and patterning of the P-D limb axis. Experimental removal of the AER in chicken limb buds, causes a developmental arrest, and truncation of wing skeleton [33], meanwhile grafts of an AER to a recipient limb bud induces ectopic P-D outgrowth [34]. In 1993, Niswander identified FGFs as the relevant signals produced by the AER to induce P-D limb axis formation and extension. P-D extension and outgrowth is rescued by exogenous application of FGFs on AER removal [35]. This study provided the first molecular insights into how AER-FGF signalling controls in P–D extension and patterning. Four FGF ligands (4, -8, -9 and -17), are expressed by the AER cells with redundant functions during P–D patterning of mouse limb buds. Inactivation of the three FGFs expressed predominantly by the posterior AER (FGF4, -9, -17) does not alter limb-bud development [36]. In contrast, loss of *FGF8*, which is the first and only FGF ligand expressed by the entire AER from early stages onward, disrupts formation of the proximal-most limb skeletal element [37 - 40]. This early and transient disruption of P-D extension is rescued by the activation of FGF4 in the *FGF8*-deficient AER, which results in almost normal development of the more distal limb skeleton [41, 42]. Combined inactivation of both *FGF8* and *FGF4* causes a complete arrest of limb-bud development and limb agenesis [39, 42]. In addition, transient expression of *FGF8* and *FGF4* during initiation of limb-bud outgrowth is sufficient for specification of the entire PD axis, but the progressive proliferative expansion of such specified limb segments is disrupted [42].

Other AER-expressed FGFs, in particular *FGF9*, contribute to the proliferative expansion of the limb mesenchymal progenitors in a P-D sequential order, so that higher AER-expressed FGF levels are required for formation of more distal limb structures [36]. Taken together, this genetic analysis reveals an instructive role of AER-FGF signalling in the specification and proliferative expansion along the P–D axis.

The AER is first induced by the expression of *FGF10* in the prospective limb-bud mesenchyme. *FGF10* is expressed in the same territories as *Tbx4 and Tbx5* and interestingly, *FGF10* is a direct transcriptional target of these transcription factors. The expression of *FGF10* is essential to establish AER-expressed FGF signalling, which in turn is required to maintain *Ffg10* expression [1, 42]. The reciprocal induction of *FGF10-FGF8* requires the expression of *Wnt3a*, coding for a ligand of the Wnt family acting through the β-catenin pathway (described in the following sections). The activation of *Wnt3* expression couples *FGF8* and *FGF4* expression from cells of the AER with *FGF10* expression [43, 44]. Thus, in these early phases, a positive feedback loop between *FGF10* and *FGF8* is established in adjacent territories, and is required for reciprocal maintenance (Figure 1A-C).

1.5. Limb extension: The progress zone

Old experiments showed that removing the AER at progressively earlier stages resulted in truncations of the limb skeleton at progressively more proximal levels [33]. Thus, the acquisition of a P-D positional identity seemed to depend on the time that proliferating/unspecified cells spend near the AER (the progress zone, PZ) under the influence of AER signals. According to the model proposed by Summerbell and Wolpert [45] the mesenchymal progenitor cells

leaving the PZ early would acquire proximal identities, whereas the same cells leaving the PZ later would acquire progressively more distal identities (Figure 1D).

The great merit of this model has been to introduce the notion of time as an important factor in morphogenetic signalling; however, as a result of extensive molecular and cellular analyses, the original PZ model has been largely abandoned. First, the loss of proximal but not distal skeletal elements in *FGF8*-deficient mouse limb buds [40] are difficult to reconcile. Second, fate mapping studies in chicken embryos provide good evidence for the presence of pools of progenitor cells with distinct P-D identities, specified very early and then expanded sequentially by proliferation [46]. Removal of the AER at progressively later stages simply eliminates the distal mesenchyme containing the specified but not yet expanded progenitor pools. These studies provide a straightforward alternative explanation for the loss of distal skeletal elements following AER removal [46]. These and other results led to the proposal of the early specification/expansion model as an alternative to the PZ model [42, 46]. This model proposes that AER-expressed FGF signalling controls survival and sequential proliferative expansion of a pool of progenitor/stem cells in P-D territories, in a dose- and time-dependent highly regulated fashion.

A third model has been proposed, in which P-D patterning is controlled by opposing diffusible signals, with RA functioning as a proximal signal and FGF acting as a distal signal [26]. Chick FL or HL ectopically exposed to RA or FGF8, or to antagonists of RAR or FGF receptor, display P-D fate changes that either expand or contract expression of proximal limb markers [23]. Further evidence has been recently provided, indicating that RA is needed for P–D patterning of both FL and HL [47, 48]. Using recombinant heterotopic chick limb transplantations they propose that the exposure to the activities of Wnt3a, FGF8 (distal molecule), and RA (proximal molecule) maintains the potential to form both proximal and distal structures. While these studies report the ability of RA treatment to reprogram distal limb mesenchyme to a proximal fate and to maintain early limb mesenchyme in a *Meis1*-expressing proximal fate alongside Wnt and FGF treatment [47], they do not address a requirement for endogenous RA in proximal limb mesenchyme. The ability of RA to increase *Meis1/2* (a proximal marker) could be a consequence of loss of FGF signalling, known to repress *Meis1/2* distally [36].

Recently, Cunningham and colleagues [29] provide convincing evidence that RA is not required for limb patterning and that RA-FGF antagonism does not occur along the limb P-D axis, as originally proposed [26]. They suggest that both the initial expression of *Meis1/2* in the LPM and later in the proximal limb bud do not require RA signalling, while the downregulation of *Meis1/2* expression in the distal limb requires AER-derived FGF8. They suggest that since *FGF8* expression in the AER appears after limb-bud formation [37], the proximal most limb domain is out of range of early AER-derived FGF signals, leading to maintenance of proximal *Meis1/2* expression and restricted distal *Meis1/2* expression (Figure 1D).

1.6. Anterior-posterior pattern

The mammalian limb bud is typically pentadactylous, for example, the autopodium gives rise to five skeletal elements. The digit organization, from anterior (pre-axial, the thumbs) to posterior (post-axial, the little finger) is referred to as the A-P pattern. It has long been

recognized that the embryonic tissue mainly implicated in the regulation of the A-P pattern is the zone of polarizing activity (ZPA) (Figure 1E). In 1956, a region within the posterior-proximal limb mesenchyme was identified, that when grafted in the anterior margin of host chicken wing buds results in mirror image duplications of all digits [49, 50]. The ZPA acts as a signalling centre and specifies positional information in the limb-bud mesenchyme by secreting the diffusible molecule Sonic Hedgehog (SHH). Within the limb bud mesenchyme, SHH is present in a posterior (high) to anterior (low) gradient [51, 52]. Genetic studies indicate that the time spent expressing *SHH* provides cells with a kinetic memory relevant to specification of their A-P identities [53 - 56].

SHH signalling is translated into an intracellular, anterior (high) to posterior (low), gradient of the transcriptional repressor Gli3R within the limb mesenchyme [67]. Upon binding to the receptor Patched, SHH counteracts the conversion of Gli3 full-length into its cleaved repressor form. The Gli3R gradient is then required to establish the polarized expression of other genes involved in A-P patterning, and ultimately is translated into digit pattern, in ways not fully clarified [24, 57 - 61].

Further genetic studies in mouse and zebrafish embryos have implicated also HAND2 in the activation of *SHH* expression in both limb and fin buds [62]. Moreover, in the mouse embryo a mutual antagonistic interaction between Hand2 and Gli3, prior to *SHH* expression, establishes a A-P pre-pattern [60, 61, 63]. Finally, at later stages of development, the expression of the 5' most members of the *Hoxd* gene cluster is activated within the posterior limb-bud mesenchyme. Cell biochemical studies have revealed a direct interaction of Hoxd proteins with the *cis*-regulatory limb-bud enhancer region of the *SHH* gene [64].

1.7. Hox genes and digit identity

An exhaustive illustration of this topic is beyond the scope of this chapter. Digit patterning has commonly been interpreted in the context of a gradient of expression of *SHH* preventing the processing of Gli3 to its repressor form (Gli3R) [65, 66].

Thus, a SHH gradient is translated into an inverse Gli3R gradient [24, 67]. However, between *Gli3* and *SHH;Gli3* null mutant mice display identical polydactylous limb phenotypes, indicating that an iterative series of (non-patterned) digits can form in the absence of SHH [24, 60], suggesting the existence of a SHH-independent prepatterning.

This observation, rather than supporting the SHH gradient model, is consistent with a Turing-type model of digit patterning [68 - 70]. According to this model, dynamic interactions between activator and inhibitor molecules produce periodic patterns of spots or stripes, serving as a molecular pre-pattern for chondrogenesis. Although the core molecules of a self-organizing mechanism remain poorly known, potential candidates for molecular modulators of the system include the *Hox* genes [70]. Distal *Hoxa* and *Hoxd* genes have a well-known impact on digit number, though their specific role remains unclear. *Hoxd* genes interact with the SHH-Gli3 pathway; these include the mutual transcriptional regulation between Hox genes and SHH and the binding of Hoxd12 to Gli3R, resulting in a blockage of Gli3R repressor activity [71 - 73]. In general, gain- and loss-of-function experiments suggest a positive relation between *Hox* genes and digit number [72, 74] that is also indicated by the ectopic anterior up-regulation

of distal *Hoxd* genes in the Gli3-dependent polydactyly [24, 61]. Interestingly the disruption of various *Hox* genes combined with loss of *Gli3* results in polydactyly; more *Hox* genes are removed – more digits are formed [75]. Thus, losing *Hox* genes seemed to shorten the spacing between digits – the wavelength in Turing's mathematical language.

The Turing's model implies the activity of two diffusing and interacting molecules; however, *Hox* genes code for non-diffusible transcription factors, for example, they cannot directly participate. However, evidence that distal *Hox* genes are necessary for correct limb development is overwhelming. Indeed, in addition to a correct digit formation via a Turing-type regulation of SHH signalling, a second role of *Hox* genes in limb P-D patterning has been studied in depth. Caudal, late-expressed paralogs of the *Hox* gene clusters display a P-D as well as A-P gradient of expression within the limb mesenchyme [76, 77] suggesting a combinatorial role of these genes in patterning the limb skeletal elements. Experimental evidence leads to the conclusion that the paralogs 9–13 of the *Hox* gene clusters *-a* and *-d* specify individual limb segments [78]. Indeed *Hoxa11 -/-;Hoxd11 -/-* double mutant embryos lack radius and ulna [79] while *Hoxa13 -/-;Hoxd13 -/-* double mutants lack digits [80].

Finally, in spite of the major role played by posterior *Hox* genes, little is known about the cellular and/or molecular bases for the observed developmental defects. Attempts in this directions [81] show that malformation of the FL zeugopod in *Hoxa11/Hoxd11* double mutant mice results from multiple defects during the formation of the zeugopod, including reduced *FGF8* and *FGF10* expression, formation of smaller mesenchymal condensations, and failure to form normal growth plates at the proximal and distal ends of the zeugopod bones. As a consequence, growth and maturation of these bones is highly disorganized.

1.8. AER and ZPA interaction

The maintenance and propagation of *SHH* expression requires AER-derived FGF signalling as part of a positive epithelial-mesenchymal (E-M) feedback loop operating between the ZPA and the AER [82, 83] (Figure 1E). The BMP antagonist Gremlin1 (Grem1) was identified as a crucial mesenchymal component in this E-M feedback signalling system [59, 66, 84]. Grem1 is required to up-regulate AER-FGF signalling and to establish SHH/Grem1/FGF E-M feedback signalling. In *Grem1*-null limb buds, the establishment of this E-M feedback signalling loop is interrupted, and this in turn interferes with specification and expansion of the distal compartment (zeugopod and autopod) [59, 84].

1.9. Dorsal-ventral axis

Dorsal-ventral (D-V) patterning is mainly organized via signalling by Wnt7a, a diffusible molecule of the Wnt-family expressed in the dorsal ectoderm. Wnt7a is both necessary and sufficient to dorsalize the limb, indeed the loss of *Wnt7a* causes the dorsal side of limbs to acquire a ventral side identity, accompanied by missing posterior digits. Wnt7a is required to maintain expression of *SHH,* explaining the digit loss. Restoring the Wnt7a signal rescues both of these defects [85, 86].

Wnt7a induces the expression of *Lmx1*, coding for a Lim-family homeodomain-containing transcription factor. Lmx-1 is involved in dorsalization of the limb, which was shown by

deleting *Lmx1* in mice: *Lmx1* null embryos produced ventral skin on both sides of their paws [87, 88]. There are other factors known to control the D-V patterning; on the ventral side the transcription factor-coding *Engrailed-1* gene, exclusively expressed in the ventral ectoderm, has been shown to repress the dorsalizing effect of Wnt7a in this territory [89] (Figure 1E).

2. Distal Limb Malformations in Human

Congenital limb malformations are relatively common, and are genetically and clinically heterogeneous, with a diverse spectrum in their epidemiology, aetiology and anatomy. They are often difficult to diagnose and categorize, because of their complex phenotypes and their association with other malformations and clinical symptoms. Many etiological factors have been suggested for limb anomalies, including inheritance of mutated genes, teratogenic drugs, environmental chemicals, ionizing radiation (atomic weapons, radioiodine and radiation therapy), infections, metabolic imbalance (e.g., maternal diabetes), or mechanical factors like amniotic band syndrome. With the advent of functional genetics, molecular pathways centred on disease genes are being unravelled.

A wide set of human congenital limb malformations can be attributed to defects in P-D development. In this chapter, we will attempt to link known disease-causing genes with their known or presumed function in the maintenance of the AER. We will focus on the genes for which more functional data are available: namely *Dlx5*, *FGF8*, *p63* and *Wnts*. These genes are co-expressed in the AER cells of the mouse limb [90] as well as in the fins of the zebrafish embryos [91, 92] and are known or proposed diseases-genes for the SHFM and EEC congenital limb malformations.

P-D defect refer to absence or hypoplasia of distal structure of the limb with more or less normal proximal structures. The spectrum of P-D limb reduction anomalies ranges from very mild disorders, such as syndactyly, to very severe forms, such as phocomelia or amelia. The most frequent congenital limb malformations are syndactylies, characterized by the fusion of the soft tissues of fingers and toes with or without bone fusion. Syndactylies are due to the lack of apoptosis in the interdigital mesenchyme and may also occur isolated or with other symptoms in a syndrome [93].

Polydactylies are distinguished by the appearance of supernumerary digits or parts of them, which may be present as a complete duplication of a whole limb or as a duplication of single digits [94]. Pre-axial polydactyly with extra digits located on the side of the hand or of the thumb or postaxial polydactyly where the extra digit is found on the side of the hand or foot of the fifth digit are common isolated limb malformation traits. On molecular level, many forms of polydactyly have been shown to be more or less directly linked to the SHH signal transduction pathway, which play a major role in A-P patterning of the limb [95, 96].

Brachydactylies are defined by shortened digits and are classified on an anatomic and genetic background into five groups from A to E [93]. Isolated brachydactylies are often inherited in an autosomal dominant manner and are characterized by a high degree of phenotypic

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variability. Type-B brachydactylies are associated to mutation in the *Ror2* gene, and *Ror2* mutations are also associated with the Robinow syndrome in which brachydactyly is a common feature [97 - 101].

A severe P-D arrest of the developing limb bud gives rise to phocomelia, characterized by undeveloped limbs [102]. Usually the upper limbs are not fully formed and sections of the "hands and arms may be missing". Short arm bones, fused fingers and missing thumbs will often occur. Legs and feet are also affected. Individuals with phocomelia will often lack thigh bones, and the hands or feet may be abnormally small or appear as stumps due to their close attachment to the body. Phocomelia is a known negative effect of the administration of thalidomide to pregnant women, in use in the late 1950s/early 1960s, to treat morning sickness, although the mechanism of action of this teratogen remains controversial [103, 104].

Failure of formation of limb buds gives rise to amelia, the complete absence of one or more limbs. The most severe form of amelia is the tetra-amelia, characterized by the absence of all four limbs, associated with craniofacial, pulmonary and urogenital defects. This autosomal recessive disorder has been linked to mutations of the *WNT3* gene (see the following section).

2.1. SHFM and EEC

SHFM, also known as ectrodactyly or lobster-claw malformation, is a congenital defect affecting predominantly the central rays of hands and/or feet. It may manifest either as an isolated trait or as part of syndromic conditions comprising other developmental disorders [105]. SHFM occurs with the incidence of about 1 in 18,000 live born infants and accounts for 8–17% of all limb malformations [106, 107]. SHFM is clinically heterogeneous, ranging from a relatively mild defect, such as hypoplasia of a single phalanx or syndactyly, to aplasia of one or more central digits (i.e., classical cleft, also known as lobster-claw anomaly).

Inter-individual and intra-familial variability of the SHFM is very high. Furthermore, variable expressivity of this feature can be so significant, that a different pattern of anomaly is seen in each limb of the same individual patient [93]. SHFM is mostly sporadic, although familial forms are known: in these cases an autosomal dominant transmission with reduced penetrance is the most common mode, but autosomal recessive and X-linked forms have been reported.

SHFM has been linked to (at least) six distinct loci [106] (Table 1). SHFM-I (MIM #183600) is the most frequent type and is linked to mutations and/or deletions/rearrangements of the *DLX5;DLX6* bigenic locus. Deletions, inversions and rearrangements affecting chromosome 7q21 have long been reported [108 - 112]. The smallest region of overlapping deletions encompasses several other genes in addition to *DLX5*, and *DLX6*: as *DYNC1l1, SLC25A13, DSS1,* but only *Dlx5* and *Dlx6* have been shown to be specifically expressed in the AER of the developing mouse limbs [113 - 115]. Recently, a point mutation in the DNA-binding domain of DLX5 (Q178P) has been reported in a SHFM-I family with a recessive transmission, co-segregating with the limb malformations [116]. In the mouse, the combined disruption of *Dlx5;Dlx6* leads to an ectrodactyly phenotype affecting the HLs [114, 115], fully confirming that the human orthologs *DLX5* (and presumably *DLX6)* are the disease genes for this malformation. Interestingly, SHFM type-V (MIM #606708) is linked to deletions of a region on chromosome 2 encompass-

ing the *HOXD* gene cluster, near *DLX1* and *DLX2* [117 - 119]. Although no clear evidence for the involvement of *DLX1* and *DLX2* in this malformation is available, it is tempting to imagine that similar to *DLX5*, misregulated expression of *DLX1/2* in the human embryonic limb bud could be the molecular mechanism leading to SHFM type-V.

SHFM type-III (MIM# 600095) is associated with duplications/rearrangements around the *DACTYLIN* (*FBXW4*) locus on chromosome 10q and the synthetic one in mice [120, 121]. The genomic lesion involves the *DACTYLIN*, *LBX1*, *βTRCP* and other more distant genes, but none of these is directly disrupted by the rearrangement and no point mutation has been reported. Interestingly, the *FGF8* locus is located in the proximity of the rearrangement breakpoints [122], and considering its importance for P-D limb development, it represents a valid candidate SHFM type-III disease gene.

Mutations of p63 are associated to SHFM type-IV (MIM #605289), a condition in which ectrodactyly appears as an isolated non-syndromic disorder linked to mutations or chromosomal anomalies in the DBD or in the C-terminal domain of p63*α* [123 - 125]. The *α* tail of p63 contains a sumoylation site, inactivated by *p63* mutations found in SHFM-IV (E639X). Sumoylation can modulate p63 half-life [126] and naturally occurring mutated p63 proteins often display altered stability, suggesting that the final effect of the mutations could be the persistence of the mutated protein and consequent misexpression of p63 targets [125, 127, 128]. p63 mutations also cause the ectodermal dysplasia-ectrodactyly-cleft lip/palate syndrome type-III (EEC-3) syndrome (MIM #604292) [129] in which ectrodactyly is a common feature.

SHFM type-VI (MIM #225300) is the only autosomal recessive form of this malformation, and is due to homozygous point mutations of the *WNT10B* gene [130 - 133]. Finally, the X-linked SHFM type-II form (MIM #313350) has been mapped to chromosome Xq26.3 [134] but no disease gene has yet been identified.

SHFM locus	Chromosome/gene affected	Case reported	Inheritancepattern	Limb phenotypes	Additional phenotypes	References
SHFM-I	Rearrangements 7q21.3-q22.1*DLX5* mutation	1 family	Autosomal dominant Autosomal recessive	SHFM	EEC, mental retardation, sensorineural deafness	Crackower et al. (1996) Marinoni et al. (1995) Shamseldin et al. (2012)
SHFM-II	Xq26	1 family	X-linked recessive	SHFM, syndactyly, metacarpalhypo plasia, phalangeal hypoplasia		Faiyaz ul Haque et al. (2005)

SHFM locus	Chromosome/gene affected	Case reported	Inheritancepattern	Limb phenotypes	Additional phenotypes	References
SHFM-III	Duplication 10q24	20%	Autosomal dominant	SHFM, triphalangeal and/ orduplicated thumbs		de Mollerat et al. (2003)
SHFM-IV	*TP63* mutations	10% non syndromic93% EEC syndromes	Autosomal dominant	SHFM	EEC, ADULT, LADD, CHARGE,VATER/mental retardation	van Bokhoven et al. (2001) Ianakiev et al. (2000)
SHFM-V	Deletion 2q31		Autosomal dominant	SHFM	Mental retardation, ectodermal and craniofacialfindings, orofacial clefting	Goodman et al. (2002) Del Campo et al. (1999)
SHFM-VI	*WNT10B* mutation	3 family1 sporadic case	Autosomal recessive	SHFM, tibial aplasia/ hypoplasia		Ugur and Tolun (2008) Blattner et al. (2012) Khan et al. (2012)

SHFM – Split Hand/Foot Malformation, EEC – Ectrodactyly-Ectodermal dysplasia-Cleft lip/palate, ADULT – Acro-Dermato-Ungual-Lacrimal-Tooth syndrome, LADD – Lacrimo-Auriculo-Dento-Digital syndrome,

CHARGE syndrome (Coloboma of the eye, Heart defects, atresia of the nasal choanae, Retardation of growth and/or development, Genital and/or urinary abnormalities, Ear abnormalities and deafness), VATER association - vertebral anomalies, anal atresia, cardiovascular anomalies, tracheoesophageal fistula, renal and/or radial anomalies, limb defects.

Table 1. Genetic alterations and SHFM-related phenotypes

2.2. p63-Dlx5;Dlx6 Regulation

SHFM type-IV and EEC are caused by mutations in the *p63* gene, which codes for a highly conserved transcription factor related to the *p53* and *p73* tumour-suppressor genes [129, 135 - 137]. A common feature of these disorders is ectodermal dysplasia, consisting in abnormal maturation and stratification of the skin and abnormal development of hairs, teeth, nails, exocrine glands and cornea. The other two consistent features of *p63*-linked disorders are cleft lip/palate and ectrodactyly.

p63 is expressed in the basal or progenitor layers of many epithelial tissues [138, 139], and is able to promote the epithelial stratification program typical of the mammalian skin, as well as to control proliferation and exit from the cell cycle of epidermal stem cells. For these activities

p63 has been proposed as a master regulator of epidermal stem cell maintenance, proliferation and stratification [140]. The *TP63* gene is translated into ten protein isoforms [141]: the trans-activating (TA) isoforms, closely resembling p53, and the delta-N (ΔN) isoforms, devoid of the TA-domain-1 (TA1). Although the TA isoforms were initially thought to be the ones to possess transcriptional regulatory functions, it has well been established that the ΔN isoforms can activate transcription of a distinct set of target genes via a second TA-domain-2 (TA2) [142]. Five TA and ΔN isoforms are generated by two transcripts which are subjected to alternative splicing, thus the final protein products differ at the carboxyl termini (α, β, γ, δ and ε). In addition to TA1 or TA2 domain, the p63 proteins contain a DNA-binding domain (DBD) and an oligomerization domain (OD). The α-isoforms (either TA or ΔN) also contain a sterile alpha motif (SAM) domain, a protein-protein interaction module found in developmentally relevant proteins [143, 144]. Recent studies have identified a transcriptional inhibitory (TI) domain located between the SAM domain and the C-terminus of p63α; this domain is believed to be responsible for the lower transcriptional activity of TAp63α compared to the -β and the -γ isoforms [145]. ΔNp63α is the most expressed isoform in the embryonic ectoderm.

Attempts to establish genotype–phenotype correlations are hampered by the variable clinical expressivity observed within families: SHFM type-IV and the EEC syndromes are due to mutations in the DNA-binding domain of p63 [129]. In these cases, all p63 isoforms are affected by the mutations. DBD mutants usually act as dominant-negative effectors and render the WT protein unable to bind DNA [129], explaining the dominant transmission of EEC. In contrast, the Hay Wells or ankyloblepharon-ectodermal dysplasia-cleft palate syndrome (AEC, MIM #106260) manifests with normal limbs but severe skin defects, and is typically associated with heterozygous missense mutations in the SAM domain of p63. The acro-dermato-ungual lacrimal tooth (ADULT, MIM #103285) syndrome is associated with a specific gain-of-function mutation R298Q/G in exon 8, affecting the DNA-binding domain of p63. Finally, both limb-mammary syndrome (LMS, MIM #603543), very similar to ADULT and EEC syndromes, and Rapp-Hodgkin syndrome (RHS, MIM #129400), resembling AEC, are due to p63 mutations. *p63* mutations causing EEC are usually not found in AEC, LMS and SHFM [146 - 148].

Mice null for *p63* have been generated by two groups independently [136, 137]; at birth these mice show severe defects affecting their skin, limb and craniofacial skeleton, teeth, hair, and mammary glands. Specifically, the skin appears thin, mostly single layered and translucent, unable to prevent water loss. The HLs fail to form altogether, while the FLs are severely truncated and lack most of their distal skeletal elements. The altered phenotypes observed in these mutant mice are a direct consequence of altered cellular properties affecting the same tissues and organs as in EEC patients [90, 136, 137, 149, 150]. While in the null embryos the *p63* protein is missing altogether (i.e., both the TA and ΔN isoforms), in EEC, AEC, LMS, and SHFM-IV patients the mutated p63 protein coexists with half of the normal dose of the wild-type protein. To better model the disease, the group of Dr. A. Mills (CSHL, USA) has generated mice bearing the *R279H* mutation (found in EEC patients). Homozygous embryos and newborn animals show a global phenotype similar, but not identical, to that of *p63-/-* [90], consisting in the absence of the HL, severely truncated FL, a thin translucent skin and cranio-facial and palatal defects. The HL defects in both the *p63* null and in the *p63-R279H* homozy-

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gous embryos are evident as early as E9.5, and are accompanied by loss of AER stratification and *FGF8* expression [90, 136, 137]. Interestingly, mild limb defects are observed in heterozygous p63-R279H mice, the mouse model closer to EEC.

Mouse models of the AEC syndrome have also been generated. Compared to EEC patients, AEC patients suffer of extreme skin fragility but have normal limbs. The AEC-mutant p63 proteins appear to act in a dominant-negative fashion. Mice were generated in which either ΔNp63a is down regulated in the skin, as a way to mimic the dominant negative action of mutant p63 in the AEC patients, or an AEC-mutant p63 was introduced [151 - 153]. These mice show severe skin erosion resembling the AEC phenotype, characterized by suprabasal epidermal proliferation, delayed terminal differentiation and altered basement membrane.

p63 mutations cause limb congenital phenotypes due to their impact on the AER Animal models show p63 is essential for epidermal stratification [90, 139, 154 - 156]. Considering that the AER is one of the earliest attempt of the embryonic (non-neural) ectoderm to organize into a multilayered epithelial tissue [157], it is not surprising that in *p63* null or *p63 R279H* homozygous mice the AER is thinner and poorly stratified. Failure to maintain AER stratification and FGF8 expression is a common feature of various ectrodactyly phenotypes [90, 157 - 159].

p63 is expected to control AER functions via transcriptional regulation of AER-restricted target genes [122, 154 - 156], indeed failure of AER stratification has also been associated with loss of expression of key morphogens for limb development, such as *FGF8* and *Dlx5;Dlx6* [122]. *Dlx* genes are the vertebrate homologs of Drosophila *Dll*, a homeodomain transcription factors required for the specification of distal limb elements in the fly embryo. In *Dll* hypomorphic mutant flies, a variable set of phenotypes is observed depending on the mutation, ranging from fusion of the distal segments (weak mutants) to complete loss of distal and medial leg segments (severe mutants) [160, 161]. In mice *Dlx* genes have a prominent role in specifying the mandible and maxillary skeletal structures [162, 163], as well as controlling normal limb development [114, 115]. Point mutations of *DLX5* have been found to co-segregate in familiar cases of SHFM [116] and the combined deletion of *Dlx5* and *Dlx6* leads to ectrodactyly of the HLs, that is, a true mouse model of SHFM type-I. There is evidence that until E11.5 the AER appears and functions normally, including a normal morphology and normal expression of AER markers (*FGF4, FGF8, Msx2*). On the contrary, at E11.5-E12 the expression of AER markers indicate that the central wedge of the AER fails to function. At about the same time the first signs of dysmorphology are visible. The expression of *FGF8* and other markers declines in the central sector of the limb bud, accompanied by loss of stratification in the same territory [158], while the expression of *SHH, Hand2* and *Tbx4* in the mutant limbs is unchanged. Considering the expression pattern of *Dlx* genes in the limb, the *Dlx5;Dlx6* null defect can be summarized as a cell-autonomous failure of the central AER to maintain and express morphogenetic molecules.

p63 and Dlx proteins are co-expressed in the AER cells [90] as well as in the fins of the zebrafish embryos [91, 92]. In homozygous *p63* null and *p63EEC* (R279H) mutant limbs, the expression of four *Dlx* genes is strongly reduced. Functionally, when the *p63+/EEC* (heterozygous) mutation is combined with an incomplete loss of *Dlx5* and *Dlx6* alleles, severe limb phenotypes

are observed, not present in mice with either mutation alone [90]. Together, there is a clear evidence for p63-Dlx regulatory cascade that is functional for distal limb development.

In vitro, ΔNp63*α* induces transcription from the *Dlx5* and *Dlx6* promoters, an activity abolished by EEC and SHFM-IV mutations, but not by AEC-associated mutations. ChIP analysis shows that p63 occupies the *Dlx5* and *Dlx6* promoters. This regulation takes place both at the proximal promoter level [90] and via a conserved *cis*-acting genomic element, located 250 kb centromeric to DLX5, an element that is specifically deleted in few SHFM patients [164]. Recent studies have identified a tissue-specific enhancer located within the coding exons 15 and 17 of the *Dync1/1* gene (near the *Dlx5;Dlx6* locus). This genomic element is characterized by an enhancer-type chromatin signature and physically interacts with a *DLX5/6* promoter region 900 kb distal to *DYNC1/1*, specifically in the limb [165, 166]. Using copy number variation (CNV) analyses in SHFM patients, combined with whole genome sequencing to map deletion and translocation breakpoints, a recent study shows that the *DYNC1I1* enhancers are also critical for limb development in humans [167]. An additional enhancer was identified in an intron of the *Slc25a13* locus, close to *Dlx5;Dlx6*, and was shown to drive *Dlx* gene expression in the otic vesicle, forebrain, branchial arch and limbs of the developing embryo [165, 166]. It is plausible that the SHFM phenotype linked with mutations in these enhancers is caused by an altered regulation of Dlx5/6 transcription.

2.3. Downstream of Dlx5;Dlx6

Sp8 is a transcription factor of the Sp1 zinc-finger family [168, 169], homologous to the Drosophila *D-Sp1* gene that has been implicated in appendage development [170]. In the developing limbs *Sp8* shows restricted expression in the ectoderm, including the AER cells [168]. Mouse embryos null for *Sp8* show severe developmental defects affecting the distal portion of the limbs, associated with a strongly reduced expression of *FGF8* [168, 169, 171], *Sp8* is co-expressed with *Dlx* genes in the murine AER and forebrain [172] and appears in the top 1% of a list of conserved/co-expressed genes in microarray data [173]. Furthermore, conserved Dlx5 DNA-binding sites are predicted near the *Sp8* locus, thus *Sp8* is a likely direct *Dlx5* transcriptional target. A Dlx5-Sp8 transcriptional cascade could be upstream of *FGF8* expression, which in turn maintains p63 protein stability.

A number of observations suggest that p63 and Dlx proteins may regulate *FGF8* expression by acting directly on the genomic region corresponding to the SHFM type-III critical region [120, 121]: indeed p63-binding sites are present within the region, as demonstrated by ChIP-seq screening [164], and several predicted *Dlx5* binding sites cluster around the *FGF8* locus, in genomic regions conserved across mammalian species [158] (unpublished data).

Considering that the AER of *Dac* heterozygous embryos shows reduced *FGF8* expression and defective cell layering [159], and considering that rearrangements/duplications around *Dactylin* do not disrupt or interrupt the gene, and since *Dactylin* is ubiquitously expressed in mouse tissues, the role of *Dactylin* as disease-gene is doubtful [122]. In alternative, *FGF8* and components of the NFkB pathway might be the disease-genes. It is tempting to speculate that the complex duplication rearrangement modifies the position/organization of *cis*-acting

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control elements, which in turn affect expression of *FGF8* and components of the NFkB pathway. Thus SHFM type-III could be a genome-misorganization type of genetic disease.

In further support of this, genome-wide CNV analyses on a Chinese family with SHFM type-III revealed a micro-duplication on chromosome 10q24 co-segregating with the SHFM phenotype [174]. This novel duplication contains two discontinuous DNA fragments: the minimal centromeric duplicated segment involves *LBX1*, *POLL* and a disrupted *BTRC*; the telomeric duplication encompasses *DPCD* and part of *FBXW4*. No coding and splice-site mutations of candidate genes in the region were found. Interestingly, the second duplicated fragment comprises Dlx5 and p63 DNA binding sites [164].

Another pathway that links p63 and Dlx5 in the regulation of the *FGF8* locus implicates the gene *IKKα*, a direct transcriptional target of p63 relevant for ectoderm development and limb morphogenesis [175 - 177]. Interestingly, while mutations of *p63* and loss of *Dlx5;6* lead to a reduced *FGF8* expression in the AER, in *IKKα* mutant embryos the AER shows an increased *FGF8* expression [178], which nevertheless results in distal limb truncations and severe malformations.

From the above considerations, it appears that numerous players in the p63-Dlx5 cascade may contribute to regulate *FGF8* expression in the AER. The possibility that the *FGF8* locus is a common target of the p63 and the Dlx5 networks during limb development is in agreement with the well-known functions of FGF8 to sustain epithelial mesenchymal signalling and assure the timely generation of mesenchymal progenitors [36].

2.4. Post-translational p63 protein regulations

Several biochemical observations indicate that the ΔN- and TAp63 proteins are tightly regulated at post-translational level, via protein modification (phosphorylation, sumoylation and ubiquitination) and protein-protein interactions [126, 158, 179, 180]. Such modifications modulate the stability of the p63 protein, regulate its transcriptional activity and ultimately modulate its ability to orchestrate the timing of exit from the cell cycle and the dynamic of stratification of mammalian ectoderm [156, 181, 182].

Among the interacting or modifying proteins, MDM2 and p53 have been previously recognized [179, 180]. Recently we have shown that the peptidyl-prolyl *cis*/*trans* isomerase NIMA-interacting-1, Pin1, is a regulator of ΔNp63*α* protein stability, inducing its proteasome-mediated degradation [158] resulting in diminished transcription of two p63 targets [183] (Figure 2).

Another modification is acetylation, catalyzed by histone acetyl-transferase on lysine residues, and known to finely regulate p53 and p73 stability and transcriptional activity [184 - 189]. p73 is acetylated by p300 on lysine residues in the DBD and Oligomerization Domain [190] enhancing p73 ability to bind and activate proapototic target genes [191]. The p73-p300 interaction requires the prolyl-isomerase Pin1, which induces conformational changes following phosphorylation by the tyrosine kinase c-Abl [192]. Acetylation of p53 correlates with its stabilization and activation by antagonizing the activity of the MDM2 ubiquitin-ligase. It is interesting to note that a naturally occurring p63 mutation found in SHFM type-IV patients

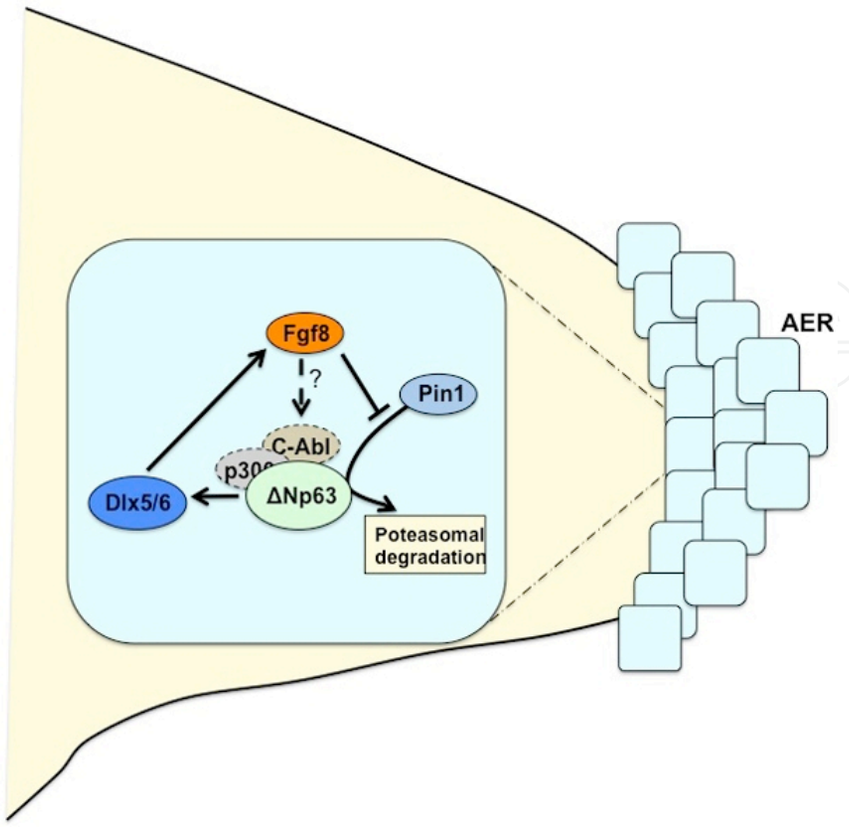

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Figure 2. Schematic representation of the molecules and their interactions that regulate the stability of ΔNp63 during the AER stratification. p63 regulates its own stability via to the expression of *FGF8*; this pathway includes the *Dlx5/Dlx6* disease genes. Fgf8 stabilizes p63 by counteracting the activity of Pin1 to induce proteasome-mediated degradation of ΔNp63α. Novel results indicate that FGF8 activates a signalling cascade leading to activation of c-Abl that promotes phosphorylation of ΔNp63α on tyrosine residues. This phosphorylation event is required for the interaction of ΔNp63α with the p300 acetyl-transferase, which modulated ΔNp63α stabilization and transcriptional activity. Although only shown in vitro, we speculate that regulation may also occur in the AER cells (dotted line).

changes lysine 193 into glutamic acid (K193E) [125, 146, 147, 193]. Our unpublished data show that ΔNp63α is acetylated by p300 on the K193 residue, and that the K193E mutation prevents this modification (Guerrini and Restelli, unpublished) (Figure 2).

2.5. Emerging roles of FGF8

Expression of *FGF8* is strongly reduced in the AER of the *p63* null, *R279H p63* mutant, and *Dlx5;Dlx6* mutant embryos [90, 115] as well as several other mouse strains with distal limb defects. The AER of these mutants appears poorly stratified. Thus, loss of AER stratification and reduced *FGF8* expression are a common theme during the onset of this specific class of malformations. The link between *FGF8* expression and AER stratification is not totally clear. When *FGFR2* gene is deleted in the AER cells, via conditional genetics, the AER loses stratification as well as Fgf*8* expression. In this case, the AER cells cannot respond to (AER-derived?) FGFs [194] and it can be concluded that AER-expressed FGFs are needed for AER maintenance, apparently in an autocrine fashion.

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An emerging role of FGF8 is the control of p63 stability in the AER cells. The AER of *Dlx5/Dlx6* null mice shows poor stratification as well as reduced *FGF8* expression, similar to what is seen in *p63* mutant mice. We have documented that *Dlx5;Dlx6* are transcriptional targets of p63, and that in turn *FGF8* is a target of Dlx5. As already said, ΔNp63α protein stability is negatively regulated by the interaction with Pin1, via proteasome-mediated degradation. Recently we have shown that FGF8 counteracts Pin1-ΔNp63α interaction, thus indicating that FGF8 participates in a feedback loop which involves the p63-Dlx5 cascade [158] (Figure 2).

p63 stability might also be regulated by another post-translational modification, namely acetylation by the p300 histone acetylase. c-Abl is a key regulator of the p53 family members and is known to be activated by treatment with FGF2 [192, 195 - 198]. Recently we have collected new data showing that FGF8 is able to stabilize ΔNp63α also via a novel pathway that requires the c-Abl tyrosine kinase and the protein acetylation by p300 (Guerrini and Restelli, unpublished). Thus, Dlx5, p63, Pin1, p300 and FGF8 participate in a time- and location-restricted regulatory loop that seems to be able to self-maintain and whose normal functioning is necessary for AER stratification, hence for normal extension and patterning of the limb buds. These results shed new light on the general molecular mechanisms at the basis of the SHFM and EEC limb malformations (Figure 2).

In an interesting set of experiments using cultured embryonic limbs, it was recently shown that the FGF/MAPK pathway establishes a high-distal to low-proximal gradient that controls the migration velocity of mesenchymal cells [199]. These cell movements enable continuous rearrangement of the cells at the distal tip of the limb bud. The effect of FGF/MAPK signalling emanating from the AER is different than the effect induced by Wnt5a in the limb bud. While Wnt5a induces directional movement of cells, FGF8 acts to induce rapid, yet disorganized, movements. Ultimately, the activity of both Wnt5a and FGF results in distal elongation (Figure 3). These observations suggest that FGF8 acts by inducing random movements, but with a higher velocity as cells move close to the source. A study proposes that the FGF pathway drives tail-bud elongation in the chick embryo by promoting random cell movements [200]. According to these authors FGF creates a gradient of cell motility and that the tail bud elongates by mass action of random cell movement at the posterior end of the embryo. Although this data indicate a similar mode of FGF action, cells in the limb bud additionally undergo oriented processes of cell division and directional movements under the influence of Wnt5a. This study indicates that it is the combined action of non-canonical WNT and FGF that integrates orientation and movement, consequently driving limb-bud elongation and thereby establishing a progenitor field of the proper dimensions for the subsequent patterning and morphogenesis of limb anatomy.

2.6. Wnt signalling and limb development

Wnt molecules are the vertebrate homologs of the Drosophila *wingless* gene, required for wing development. Wnt molecules are involved in all aspects of embryonic development, from patterning to morphogenesis and cell-tissue interactions [201 - 203].

Several members of the Wnt family of ligands are expressed in the ectoderm and mesenchyme of the developing limbs. At early stages, *Wnt8c* and *Wnt2b* are transiently expressed in the

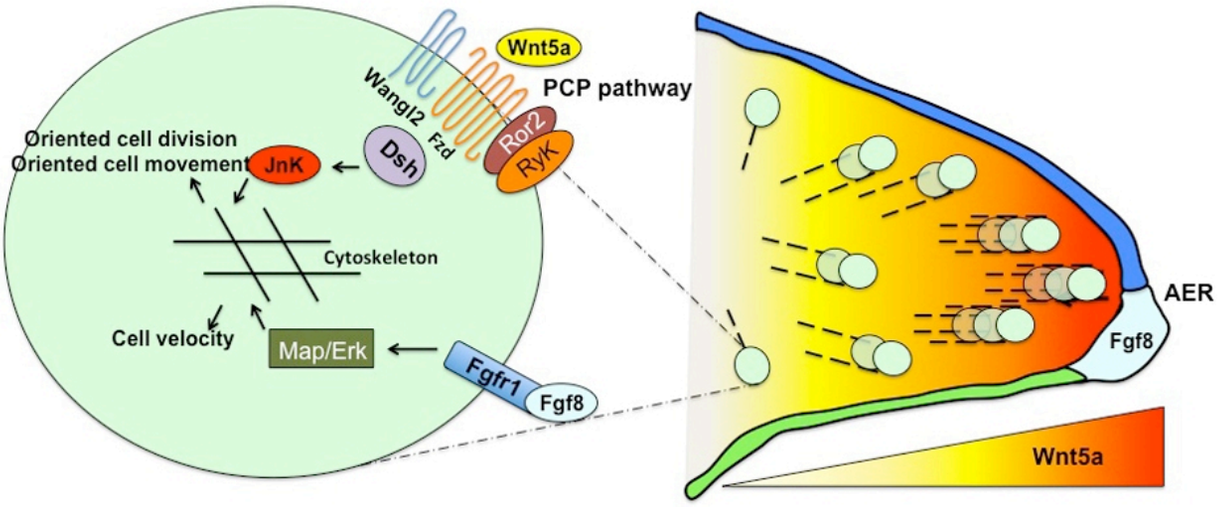

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Figure 3. Schematic representation of the mesenchymal cells orientation and organization in the early limb bud. These cellular events are regulated by the combined activities of the WNT and FGF pathways. Wnt5A/Jnk/PCP pathway is necessary for the proper orientation of cell movements and cell division. In contrast, the FGF/MAPK signaling pathway, emanating from the AER establishes a gradient of cell velocity. The combination of oriented cell divisions and movements drives the P-D extension of the limb bud necessary for subsequent morphogenesis.

LPM and participate in the initiation of HL and FL outgrowth, respectively [43]. At later stages, *Wnt3*/*Wnt3a* and *Wnt5a* are expressed by the AER cells while *Wnt7a* is expressed in the dorsal ectoderm.

Wnt ligands signal through the Frizzled (Fz) seven-pass trans-membrane receptors. In the "canonical" pathway, binding of Wnt ligands to Fz receptors represses the axin/glycogen synthase kinase-3β (GSK3β) complex, which in the absence of the ligand promotes the degradation of β-catenin via the ubiquitin pathway (reviewed in reference [204]). In Wnt-activated cells, cytoplasmic β-catenin accumulates and translocates to the nucleus where, in conjunction with T cell-specific factor/lymphoid enhancer binding factor-1 (Tcf/Lef1) transcription factors, activates transcription of target genes.

A role of "canonical" Wnt signalling in limb development has long been recognized [205]. In the chick limb bud, the Wnt/β-catenin pathway is essential for the induction and maintenance of the AER. Indeed, ectopic expression of Wnts in the interflank region prior to limb outgrowth induces ectopic *FGF10* expression and limb formation. FGF10 subsequently induces *Wnt3a* expression in the AER, which in turn switches on the expression of Fgf8, again via the β-catenin pathway, and promotes AER formation [43, 44, 206].

In the chick embryo *Wnt3a* mediates the Wnt/β-catenin signalling required for establishment of the AER. In the mouse, old data indicate that mouse embryos lacking the Wnt/β-catenin pathway component *LRP6*, or simultaneously lacking *Lef1* and *Tcf1*, exhibit defective AER formation and limb defects, indicating that his pathway is indeed essential for AER formation [207, 208]. However, *Wnt3a* is not expressed in the limb ectoderm of the mouse embryo, and *Wnt3a* null embryos do not show limb defects [209, 210]. Instead, the closely related *Wnt3* gene

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is expressed in the limb ectoderm [210] and the conditional removal of *Wnt3* in the limb ectoderm leads to severe distal limb truncations and AER malfunction. Similar results were obtained by the conditional removal of β-catenin in the limb ectoderm [211], strongly suggesting that the murine *Wnt3* is functionally homologous to chick *Wnt3a*, and that a pre-AER active Wnt3/β-catenin pathway in the embryonic ectoderm is essential for AER formation and maintenance. Notably, homozygous mutations of *WNT3* in human are associated with a rare autosomal recessive congenital disorder known as tetra-amelia [212] characterized by the absence of all four limbs.

Wnt signalling has been implicated in removing "excess" tissue by programmed cell death and sculpting the limb shape. Indeed, the ability of BMP4 to induce cell death in the developing limb appears to be mediated by Dkk1 [213]. Loss of function of Dkk1 in mice results in the downregulation of *Msx1*, a component of the cell death pathway, in the anterior and posterior necrotic zones and the interdigital mesenchyme, whilst gain of Dkk1 function in chicks causes excessive cell death via activation of the c-jun pathway [213, 214]. The decrease in cell death in the mouse mutants contributes to the polydactyly and fusion of digits that occur in *Dkk1* mutant mice [214]. In addition, Fz2, –3 and –4, and Dkk2, and –3, are expressed in the interdigit mesenchyme, suggesting that a fine balance of Wnt signalling controls cell death/survival in this region [215, 216].

2.7. Emerging role of Wnt5a and non-canonical signalling

Wnt ligands can also activate two other branches of "non-canonical" pathways; one of these is known as the planar cell polarity (PCP) pathway, involves Fz receptors and dishevelled (Dvl), which interact with a distinct set of "PCP proteins" such as Van Gogh (Vang) and Prickle [217]. The PCP pathway recruits the small GTPases Rho and Cdc42 and the c-Jun N-terminal kinase (JNK) [218 - 220]. Initially identified in Drosophila, PCP establishes cellular polarity in the plane of an epithelium, perpendicular to the apical-basal orientation [217]. Studies in vertebrate model systems, including Xenopus and zebrafish, indicate that the PCP pathway also regulates a morphogenetic process known as convergent extension (CE). CE was first demonstrated in gastrulating *Xenopus* embryos in which mesodermal cells underwent mediolaterally oriented intercalation, leading to concomitant tissue lengthening and narrowing [221]. Imaging experiments in zebrafish indicate that, in addition to polarized cell intercalation, the PCP pathway also regulates directional cell migration and oriented cell division underlying CE [222 - 225]. A second branch of "non-canonical" Wnt transduction pathways leads to the release of intracellular Ca2+ and the activation of protein kinase C (PKC) and Ca2+/Calmodulin-dependent Kinase-II (CamKII) [226 - 229]. The choice of the pathway being activated by a Wnt ligand appears to depend mostly on the receptor profile and on the intracellular signalling molecules available in a given cell type, and little on the Wnt ligand itself.

A role of "non-canonical" Wnt signalling during limb development has been recognized, although the cellular and molecular mechanisms are not fully clarified. The vertebrate *Wnt5a* gene, the homolog to Drosophila *Dwnt-5* gene essential for limb and appendage development, is considered the typical non-canonical Wnt, involved in the establishment of PCP [230 - 232]. Wnt5a together with Wnt11 mediates the activation of PCP during the CE in frogs and

zebrafish [223, 233, 234], and during mouse limb development *Wnt5a* is expressed in a gradient from the AER to the proximal mesechymal cells, is regulated by FGF signalling from the AER and has been shown to inhibit β-catenin degradation [235, 236].

In addition to the PCP pathway, Wnt5a has been shown to activate at least two other non-canonical pathways. The first is known as the Wnt–Ca2+ pathway, in which Wnt5a stimulation induces Ca2+ release and subsequent activation of the Ca2+-sensitive kinases protein kinase C and Ca2+/calmodulin-dependent kinase [226, 227, 237, 238]. Over-expression of the core PCP proteins, Dvl and Pk, can also activate the Wnt–Ca2+ cascade in zebrafish and *Xenopus*, suggesting that the Wnt–Ca2+ and PCP pathways either overlap substantially or are components of the same signalling network [229, 239, 240]. Second, in mammalian cells Wnt5a has been shown to antagonize the canonical Wnt pathway by either promoting GSK3β-independent β-catenin degradation [236] or by inhibiting β-catenin-dependent transcription [241].

Wnt5a can signal through different Fz receptors and co-receptors, but also via non-conventional tyrosine-kinase like receptors (Ror2 and Ryk) and can activate both the canonical and the non-canonical Wnt pathways [241, 242]. Activation of the canonical pathway entails the Lrp5 and Lrp6 co-receptors, which through cytoplasmic Dvl promote stabilization of β-catenin, its nuclear translocation and the activation of gene transcription [243, 244]. However, the distinct phenotypes observed between *Wnt3, β-catenin*, *Lrp5*/*Lrp6* and *Wnt5a* mutant mice [245] argues that during limb development Wnt5a does not signal through the β-catenin pathway [246].

In human, missense mutations in *WNT5A* have been documented in an autosomal dominant form of RRS (MIM #180700) [247 - 249] implying that a disruption of Wnt5a signalling may underlie both RRS and BDB1. Homozygous *ROR2* mutations have been linked to the autosomal recessive form of Robinow syndrome (or COVESDEM syndrome) (MIM #268310), while heterozygous *ROR2* mutations lead to type brachydactyly (MIM #113000) [250] and autosomal Dominant Brachydactyly type-B (BDB1, MIM #113000). BDB1 is the most severe form of brachydactyly and is characterized by loss of nails and varying number of phalanges [100, 251]. In contrast, RRS patients display broader skeletal dysplasia including mesomelic limb shortening and dwarfism, and may or may not display brachydactyly [97, 98, 101].

In mice, the disruption of *Wnt5a* results in short metacarpal elements, absence of phalanges and truncations of proximal elements [236, 252, 253]. The remaining limb skeletal elements are significantly shortened and the severity of the phenotype follows a gradient, with distal bones more affected than proximal ones, reminiscent of mesomelic limb shortening in RRS patients. Interestingly, the AER appears normally stratified and expresses *FGF8* [252]. Strong evidence of the involvement of the Wnt5-dependent pathways in limb development is derived from phenotypes of mice with loss of Wnt5a receptors. In addition to Fz receptors, Wnt5a binds to both Ryk and Ror2 receptors and regulates PCP by promoting Vangl2 stability during limb extension [242, 254]. Ryk and Ror2 are single-pass tyrosine-kinase type of receptors [241, 255]. Ror2 (an orphan tyrosine kinase receptor) activates JNK [256] and in Xenopus has been shown to interact with Wnt11 and Fz7 to regulate CE, suggesting that it may be part of the PCP pathway [257]. Upon binding with Wnt5a, Ror2 inhibits the canonical Wnt signalling. Furthermore, Ror2 also plays an important role in chondrogenesis. *Ror2* is selectively expressed

in chondrocytes of cartilage anlagen, and is thus probably important in their initial growth and patterning. Mice mutant for *Ror2* and double mutants for *Ror1;Ror2* exhibit phenotypes that correspond to human RRS malformation, and bear similarities with the *Wnt5a* mutant mice [258, 259]. *Ryk* is another unconventional Wnt5a receptor, consisting in a single transmembrane pass, catalytically-inactive, tyrosine kinase molecule. Ryk mutant mice show limb truncation similar to those of *Wnt5a* null embryos [260]. Finally, disruption of PCP signalling as Vangl2 in mice causes limb morphogenesis and skeletal defects and may underlie the Robinow syndrome and brachydactyly type B [261]. Together, these observations indicate that Wnt5a, Ryk and Ror2 molecules produce similar phenotypes when lost, for example, the disruption of components of the Wnt non-canonical pathway causes similar limb developmental defects.

2.8. Wnt5a controls aspects of PCP and CE in limb development

Recent data [199] shed light on the cellular functions of Wnt5a during limb development. Inspired by the CE process and the PCP pathway, first described in lower organisms, the authors examined the proliferative expansion and migration of mesenchymal cells of the mouse limb bud; in particular, they examined the orientation of cell division and movements in response to Wnt5a. The combination of oriented cell divisions and movements drives the P-D elongation of the limb bud necessary to set the stage for subsequent morphogenesis. They show that Wnt5a via the JNK PCP pathway is needed for the proper orientation of mesenchymal cell movements and cell division reminiscent of CE in *Xenopus* and zebrafish [222 - 225] (Figure 3).

Although these recent studies implicate Wnt5a in the oriented migration and cell division of the mesenchymal cells, little is known about the ectoderm cells, and in particular the AER cells, in which *Wnt5a* is expressed. It is conceivable that the AER cells might be the prime (autocrine) cellular target of Wnt5a, and that the acquisition of a correct planar orientation is a requisite for correct AER formation. Wnt5a and Dlx5 have an overlapping expression pattern, and the phenotype of *Wnt5a* null mice, although not identical, is quite similar to that of *Dlx5*/*Dlx6* mutant. One possibility is that a deregulation of *Wnt5a* expression, secondary to the disruption of *Dlx5;Dlx6* may underlie ectrodactyly of the *Dlx5;Dlx6* mutant embryos (Figure 4). In support of this, we have evidence that *Dlx* genes promote neuronal differentiation via *Wnt5a*, and that Dlx2 and Dlx5 physically occupy conserved genomic elements near the *Wnt5a* locus and activate its transcription [262]. This interaction and regulation is likely to occur also in the AER cells, a possibility that remains to be investigated.

2.9. Quantitative and dynamic gene expression in limb development

An emerging theme in developmental biology is the importance of gene dosage and dynamic gene expression for correct morphogenesis [56]. Several *Dlx* (*1, 2, 3, 5* and *6*) and *FGF* (*4, 8, 9* and *17*) genes are co-expressed in the AER, and their expression is dynamically regulated, both with respect to time (embryonic age) and location (territory of expression). In addition, there is evidence that *Dlx* and *FGF* genes are functionally redundant, at least in part. For example, no limb phenotype is observed in mice null for only one *Dlx* gene, while ectrodactyly is

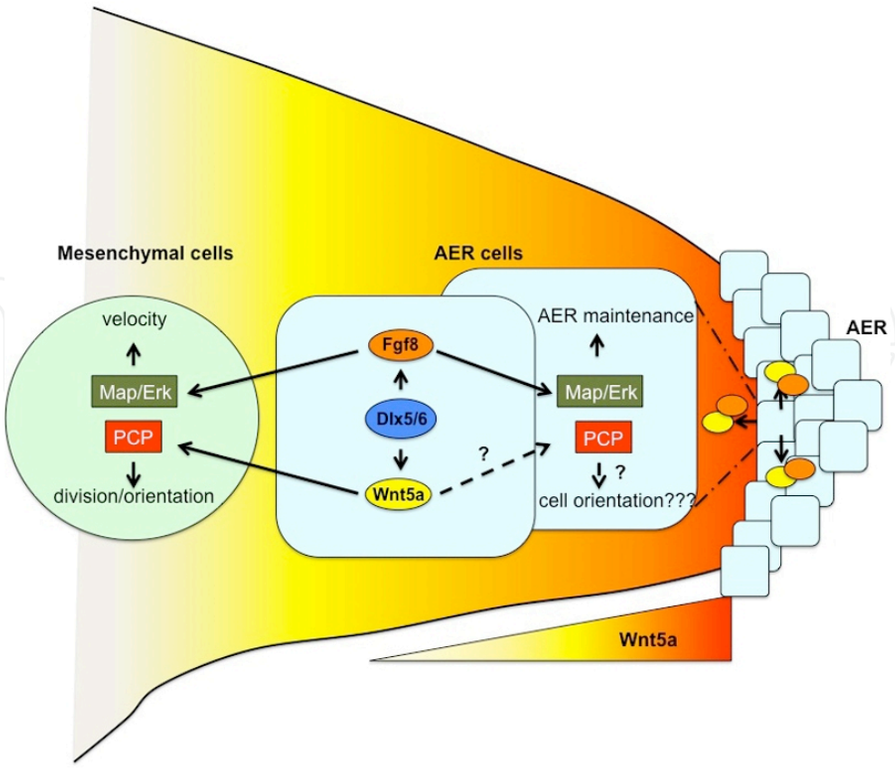

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Figure 4. Proposed model of regulation of the AER cell orientation. Dlx5 is known to regulate the transcription of both *FGF8* and *Wnt5a*. In turn, FGF8 is required for AER maintenance and stratification, via p63, while the function of Wnt5a for AER maintenance is still poorly known. Recent data of the regulation of orientation and velocity of mesenchymal cells by, respectively, the Wnt5a/PCP and the FGF8-MAP-Erk pathways open the possibility that Wnt5a may regulate the orientation/motility of the AER cell and assure a correct stratification (dotted line).

observed in *Dlx2;Dlx5* null mice [161] and the ectrodactyly of *Dlx5;Dlx6* null mice is fully rescued by the re-expression of *Dlx5* alone [115]. Likewise, an increased severity of craniofacial phenotypes correlates with progressive loss of *Dlx* gene [263, 264]. All these are indications of gene-dosage effects between functionally redundant genes.

We propose that the portion of the p63 network that (direct or indirect) regulates *FGF8* expression is exerted in a quantitative and dynamic mode. To support this, we should consider that although *p63* null and *p63EEC* homozygous mice show severe limb truncation or absence, the heterozygous mice appear to be normal. When heterozygous EEC mice are bred with heterozygous *Dlx5;Dlx6* ones (the latter have normal limbs), anomalies are clearly observed [90].

A gene-dosage effect combined with the co-expression of functionally redundant genes implies the existence of a threshold level to be maintained to assure AER stratification and signalling functions. Indeed, we have noted that the expression of *Dlx2* and *Dlx5* is lower in the central portion of the AER, compared to the anterior or posterior segments [122]. Thus, the central AER might be more sensitive to reduced *Dlx* expression due to intrinsic lower expression. On the same line, there is evidence that certain amount of AER-derived pan-FGF is required to

induce and maintain the underlying mesenchymal progenitors [36, 56, 157]. In fact, in the *Dlx5*; *Dlx6* mutant limbs, the reduction of *FGF8* expression is restricted to the central AER, the region where epithelial-mesenchymal signalling is primarily defective and the region where morphogenesis fails [114, 115]. Thus, the entire p63-Dlx-FGF cascade is sensitive to gene dosage and position of expression.

3. Concluding remarks

p63 is a master regulator of ectodermal cell proliferation, differentiation and stratification, and has a key role in the establishment of a positive loop that maintains *FGF8* expression. In turn, our recent data reveal a novel role of FGF8 to (directly and indirectly) stabilize the p63 proteins and modulate their transcriptional activity. Thus, in the biology and development of the ectoderm, p63 post-translational modifications are as important as *p63* gene expression and may reveal novel targets to be used in p63 modulation.

We illustrate that the p63-Dlx5 transcriptional regulation is at the centre of a pathway relevant for the SHFM malformation. The stability of p63 and the activation of the pathway appear to be under the regulation of FGF8, which in turn is regulated by the pathway. In addition to decipher this positive regulatory loop, these data support a model to attempt to explain the SHFM-III pathogenesis in terms of genome positional effects on the *FGF8* locus.

FGF8 and Wnt5a provide instructions for mesoderm cells as to which direction and orientation to take, at the basis of AER formation and proper migration of mesenchymal cells. This instruction adopts molecules of the PCP pathway, most likely inducing convergent extension. While this has been recently demonstrated for the mesenchymal cells, the possibility that a Wnt5a-dependent PCP pathway is also functional for the organization and stratification of the AER cells remains to be addressed. Notably, data from the human malformation diseases and the corresponding animal models clearly suggest so.

The study of animal models of EEC and SHFM diseases has provided much of this knowledge, and will continue to do so. The big hope is that, once the pathways will be elucidated, we might be able to exploit diffusible molecules and attempt to correct the limb malformation defects. Preliminary attempts are being conducted on whole-organ cultured limbs.

Nomenclature

A-P, anterior-posterior

D-V, dorsal-ventral

P-D, proximal-distal

SHH, sonic hedgehog

FGF, fibroblast growth factor

FL, forelimb

HL, hindlimb

ZPA, zone of polarizing activity

AER, apical ectodermal ridge

PZ, progress zone

KO, knock-out

PCP, planar cell polarity

CE, convergent extension

LPM, lateral plate mesoderm

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