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Analysis of the upstream signalling pathway controlling ΔNp63α protein stability and function

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PhD Thesis

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Index

PART I

1. Abstract Page 7

2. State of the art Page 9

2.1 The p53 family members Page 9

2.2 The p63 transcription factor Page 10

2.2.1 Role of p63 in embryonic development Page 12

2.3 Limb development Page 16

2.3.1 Proximo-Distal patterning Page 17

2.3.2 Anterior-Posterior patterning Page 20

2.3.3 Dorsal-Ventral patterning Page 21

2.3.4 Survey on the molecular pathway controlled by p63 during limb development Page 25

2.4 Human syndromes and malformations associated to mutations in the p63 gene Page 27

2.4.1 Ectrodactyl-Ectodermal dysplasia Clefting Page 29

2.4.2 Ankyloblepharon-Ectodermal dysplasia Page 30

2.4.3 Acro-Dermato-Ungual-Lacrimal-Tooth syndrome Page 31

2.4.4 Limb Mammary Syndrome Page 31

2.4.5 Split-Hand/Split-Foot Malformation type IV Page 32

2.4.6 Genotype-phenotype correlation of p63 mutations Page 35

2.5 Regulatory post-translational modifications of the p53 family members Page 37

2.5.1 p300 acetyl-transferase Page 40

2.5.2 c-Abl tyrosine kinase Page 44

2.5.3 PIN1 prolyl-isomerase Page 45

3. Aim of the work and main results Page 49

4. Conclusion and future perspectives **Page 52**

5. References **Page 53**

PART II

Published paper

DLX5, FGF8 and the PIN1 isomerase control ΔNp63α protein stability during limb development: a regulatory loop at the basis of the SHFM and EEC congenital malformations

PART III

Manuscript under submission **Page 67**

FGF8, c-Abl and p300 cooperate in the regulation of ΔNp63α stability

PART I

1. Abstract

The p63 transcription factor, homolog to the p53 tumor suppressor, plays a crucial role in epidermal and limb development. Dominant mutations in the *p63* gene give rise to several human congenital syndromes characterized by skin, craniofacial and limb defects. One of the syndromes caused by *p63* mutations is the Split-Hand/Foot Malformation-IV (SHFM-IV) syndrome, characterized by the loss of central rays of hands and feet. These developmental defects are due to failure of Apical Ectodermal Ridge (AER) development. The correct limb outgrowth and patterning is guarantee by the expression of key molecules including Fybroblast Growth Factor 8 (FGF8), p63 and the DLX5 and DLX6 transcription factors. In this context, the study of the molecular mechanisms regulating p63 stability and function is fundamental for understanding the molecular bases of the SHFM-IV pathogenesis: indeed p63 as been proposed to be one of the crucial regulators of limb and epidermal development.

Little is known on the post-translational modifications and the upstream signalling pathway controlling ΔNp63α functions, one of the most expressed p63 isoform in epithelial tissues and in the AER cells. The projects performed during my PhD thesis achieved to the identification of FGF8 as a crucial regulator of ΔNp63α stability and activity in human osteosarcoma and keratinocyte cell lines. FGF8 determined also ΔNp63α protein stabilization in mice embryonic limb buds put in culture at Embryonic day 10.5 (E10.5). In particular, treatments with FGF8 of human osteosarcoma cell lines (U2OS) and human keratinocytes (HaCat), activate the tyrosine kinase c-Abl, leading to ΔNp63α phosphorylation and consequent acetylation by the p300 acetyl-transferase, promoting ΔNp63α stabilization and transcriptional activation. Moreover, I have found that p300 interacts with ΔNp63α determing its acetylation on lysine K193E, *in vitro*. Interestingly, this regulatory cascade is not active on the natural ΔNp63αK193E mutant associated to the SHFM-IV syndrome. Indeed, the ΔNp63αK193E mutant displays promoter

specific altered DNA binding activity that results in altered expression of ΔNp63α target genes involved in limb development (like *Perp*, *Ikkα* and *DLX5* gene) (Manuscript in preparation).

One of the mechanism by which FGF8 promotes ΔNp63α stability and activation, is inhibiting its interaction with Pin1, a prolyl isomerase known to positively regulate p53 and p73 in response to DNA damage stress. In particular, PIN1 has an opposite effect on ΔNp63α respect to p53 and p73: it promotes ΔNp63α degradation through the proteasome pathway. Moreover, ΔNp63α mutant proteins, associated with SHFM-IV or EEC syndromes, characterized by limb defects, are not degraded by PIN1 overexpression. These data were confirmed also by *in vivo* experiments on *PIN1* Knock-Out (KO) mice, where lack of *PIN1* expression caused the accumulation of p63 in the embryonic limbs and ectoderm compared to wild-type littermates. Moreover, I found that *FGF8* is a downstream target of the transcription factor Dlx5. Indeed, in the limb buds of both *p63* and *DLX5;DLX6* KO mice, the AER is poorly stratified and *FGF8* expression is severely reduced. All these data suggest that DLX5, ΔNp63α, FGF8 and PIN1 participate in a regulatory loop essential for AER stratification, normal patterning and morphogenesis of the limb buds (1).

The work performed during my PhD contributes to a better understanding of the regulatory mechanisms controlling ΔNp63α function and stability. We have identified FGF8 as a crucial upstream signal required for ΔNp63α activation and stabilization during limb development: mutations or altered expression of regulators in this pathway leads to abnormal limb development and onset of pathogenesis.

2. State of the art

2.1 The p53 family members

The p53 family is composed of three members: p53, p63 and p73. In humans, the *p53, p63* and *p73* genes are located on chromosomes 17 (17p13.1), 3 (3q27–29) and 1 (1q36), respectively. The proteins encoded by these genes display: an acidic amino-terminal Trans-Activation Domain (TAD), absent in the ΔN isoforms, a highly conserved core DNA-Binding Domain (DBD), and a carboxyl-terminal Oligomerization Domain (OD).

All three genes encode two primary transcripts that are controlled by distinct promoters (P1 and P2). The P1 promoter of each gene is embeded in a non-coding region of exon 1. The P2 promoter of p63 and p73 is located in intron 3 while the P2 promoter of p53 is localized in intron 4 (Fig.1). Transcripts generated from the P1 promoter produce proteins containing the TA, DBD and the OD domains (TAp53, TAp63, and TAp73). In contrast, transcripts generated from the P2 promoter are lacking the TA domain (Δ133p53, ΔNp63, and ΔNp73).

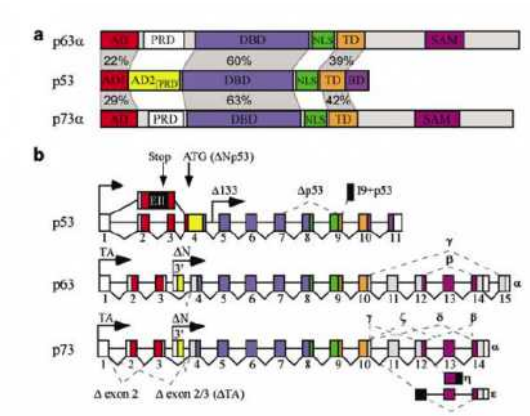

Figure 1. The functional domains in the p53 family proteins. **(a)** AD: Activation Domain; PRD: Proline-Rich Domain; DBD: DNA-Binding Domain; NLS: Nuclear Localization Signal; TD: Tetramerization Domain; SAM: Sterile-Alpha-Domain. % denote percent identity. **(b)** Genomic structure of p53, p63 and p73. Dotted lines denote alternative splicing (2).

Additional complexity to p53 family member isoforms is added by the fact that P1 and P2 transcripts can be spliced at the 3' end into several spliced variants. For p63 five TA variants (TAp63α, TAp63β, TAp63γ, TAp63δ and TAp63ε) and five ΔN variants (ΔNp63α, ΔNp63β, ΔNp63γ, ΔNp63δ and ΔNp63ε) have been identified (2-6).

While p63 and p73 demonstrate relatively little homology with p53 in their TA and OD, both share approximately 60% similarity with p53 in the DBD, including conservation of essential DNA contact residues. However, studies of Knock-Out (KO) mice have demonstrated that, even though these proteins clearly share some similarities with p53, their functions are very different. *p53* null mice (p53 -/-) are viable and develop normally, but are prone to spontaneus development of a variety of neoplasms by 6 months of age (7). *p73*-null mice are born viable, but show an high mortality rate within the first 2 months. The animals suffer from hydrocephalus, indicative of abnormal cerebrospinal fluid dynamics, immunological problems characterized by chronic infections and inflammation, and nervous system abnormalities related to hippocampal dysgenesis, olfactory neuron defects and the loss of sympathetic neurons (8) .

In contrast *p63* -/- mice are born alive but show the most severe developmental phenotype of all p53 family members and die soon after birth because of dehydration. They fail to develop limbs, stratified epidermis and most epithelial tissues (hair follicles, teeth, prostate, lacrimal and salivary glands, and mammary glands) (9,10).

2.2 The p63 transcription factor

The proteins encoded from the *p63* gene contain different functional domains (Fig.2):

- **Trans-Activation domain** (TA domain): important for transcriptional activation of p63 target genes (present in all TAp63 isoforms);

- **Second Trans-Activation domain** (TA2 domain) located between aminoacids 410 and 512. The identification of this domain revealed that also the ΔNp63 protein isoforms, generally regarded as dominant-negative isoforms, are able to directly activate gene expression (11);
- **DNA Binding domain** (DBD domain) located in the core of the protein and present in all isoforms, allows binding of p63 to its Responsive Elements (RE); some are in commons to p73 and p53 (p53 RE), the others are specific for p63 (p63RE) (3, 12, 13);
- **Oligomerization domain** (OD-ISO domain) is present in all p63 isoforms and allows the formation of omo and etero-tetramers among p63 isoforms but also with the other p53 family members (14). The formation of p63 tetramers is essential for p63 functions: it allows cooperative binding to DNA RE;
- **Steryl-Alpha-Motif** (SAM) domain is located in the carboxyl-terminal portion of the protein, present only in TAp63α and ΔNp63α. It displays a globular and solid structure with an highly conserved sequence of about 65-75 aminoacids organized into five helixes (15). This domain is thought to mediate protein-protein interactions, playing a crucial role during development and differentiation.
- **C-terminal Inhibitor domain** (TID) is present only in the α isoforms and has a negative effect on the transcriptional activity of p63. In particular, this domain binds the N-terminal region, masking the TA domain, giving a possible explanation for the reduction of the trans-activational capacity of TAp63α compared to TAp63β and TAp63γ isoforms (11,16,17).

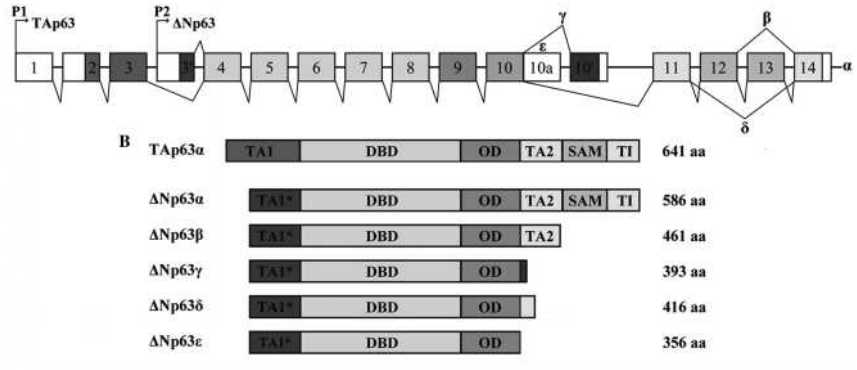

Figure2. Schematic representation of human *p63* gene structure: alternative promoters (P1 and P2), previously identified alternative splicing events (α, β, γ) and novel events (δ, ε) are indicated (6)

In recent years the identification of p63 targets genes has led to a better understanding of the developmental strategy sustained by p63. About 5800 target genes for p63 have been identified: p63 targets are enriched for genes involved in cell adhesion, proliferation, death and signaling pathways (18-20). Furthermore, it has been demonstrate that p63 associates with the promoter of *p53*, *p73* and of the *p63* gene itself, suggesting that among the p53 family members exists a complex cross-regulations (21).

2.2.1 Role of p63 in embryonic development

Immuno-histochemical analysis of mouse embryos, show high p63 isoform protein levels in epithelial cells, especially in progenitor or stem-cell populations of epithelial tissues (9,10,22). The main isotype expressed in these cells is the ΔNp63 protein, which likely acts in the maintenance of the proliferative capacity of such cells (13, 22-25). As these cells start to differentiate, ΔNp63 protein levels gradually drop, and the levels of TAp63 proteins increase. It thus appears that ΔNp63 protein is crucial for the maintenance of regenerative proliferation capacity of epithelial stem cells. Cells that no longer express ΔNp63 proteins loose this capacity and are committed to differentiate (25).

In mouse embryos, *p63* expression is first evident in nuclei of cells in the basal layer of the epidermis, which develop into the progenitor cells of the epidermis and related derivatives, such as hair and sweat glands. Basal cells of the cervix, tongue, esophagus, mammary glands, prostate, and urothelium also show high levels of p63 isoform expression. Early p63 protein expression is further evident in ectodermal cells of the limb buds and tail bud, branchial arches and oral epithelium (9, 10, 13, 26). In the developing limb buds, p63 expression is restricted to the Apical Ectodermal Ridge (AER), a key structure required for limb-bud emergence and progression in mice.

The sites of *p63* expression are well in line with the phenotypic consequences of homozygous *p63* inactivation in mice. *p63* deficient mice are viable at birth, but have strikingly developmental defects. Their limbs are absent or truncated: in particular the forelimbs (FL) are truncated and the distal part is missing, while hindlimbs (HL) are completely absent. The limb buds of *p63* deficient embryos are distinctly smaller and there is no morphologically distinct AER: indeed, mutant limb buds have a single layer epithelium at the dystal tip, indistinguishable from the surrounding ectoderm (9, 10) (Fig. 3). The limb defects observed in *p63* -/- mice results from a failure of the ectoderm to undergo growth and differentiation processes that give rise to a stratified epithelium and to a correct integrity of the AER structure, required for correct limb development (27). Indeed, *Fybroblast Growth Factor 8* (*FGF8*), a key signaling molecule expressed in AER cells, is not detectable in the limbs of *p63* homozygous mutant embryos at embryonic day 10.5 (E10.5) (9, 10, 28). Consistently, the stratified organization of the AER and the expression of morphogenetic molecules are dramatically compromised in *p63* mutant limbs.

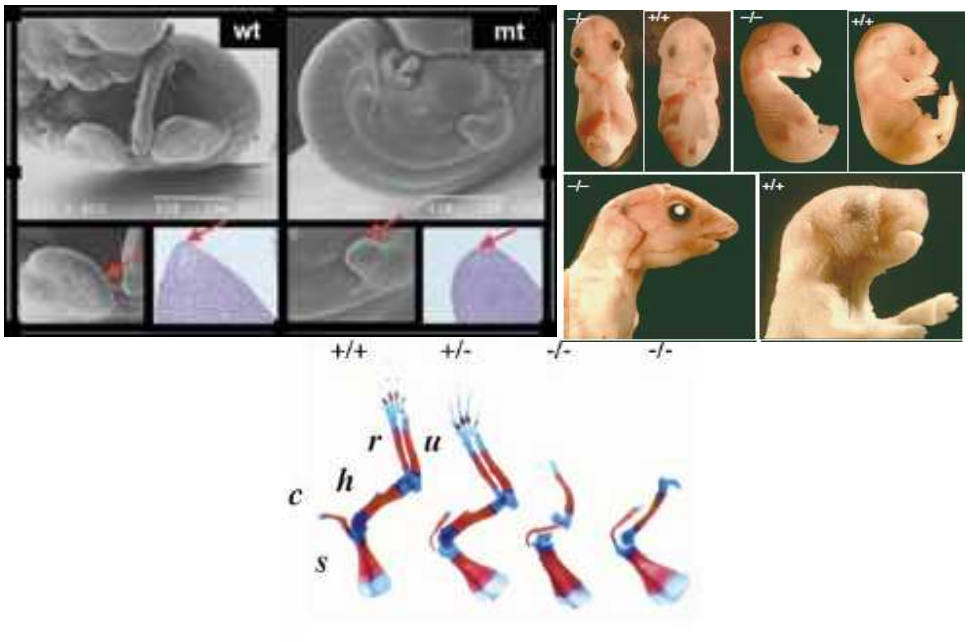

Figure 3. **(Left)** Analysis of p63 deficient limbs during embryogenesis. Scanning electron microscopy and histological analysis of wild-type (WT) and p63-deficient (mt) embryos, indicate that the AER is absent in p63 homozygous mutant embryos (arrows) (mills et al, 1999). **(Right)** Front and sagittal views of E17 control (+/+) an p63 -/- embryos. p63 -/- mice on post-natal day 1 (P1) have hypoplastic upper and lower jaws, and have no eyelids, wisker pads, skin and related appendages wich are present on the wild type control (Yang et al, 1999). **(Bottom)** FLs of p63-homozygous mutant mice are truncated: the phalanges, radius and ulna are absent and the humerus is deformed (9, 10)

Moreover, at birth *p63* deficient mice have severe skin defects. These mice die few hours later for dehydration: they looses thirty times more water than their wild-type littermates, as a consequence of alteration in the process of epidermal stratification. Histologycal analysis of neonatal *p63*-deficient skin revealed the absence of normal epidermal structure and complete lack of hair follicles. The surface of *p63* null skin is covered by a single layer of flattened cells, lacks stratification and does not express differentiation markers.

In addition to this well described phenotype of the *p63* null mice models, recently it has been discovered that *p63* ablation results also in severe defects of embryonic

cardiac development, including dilation of both ventricles, defects in trabeculation and abnormal septation. Indeed hystological sections of mice hearth at E14.5 revealed dilated cardio-myopathy with deficient trabeculation and thin ventricular wall (29).

Expression of the *p63* gene is required not only during embryonic development but also in adult tissues such as epidermis. Development of the epidermis requires a series of coordinated events, which regulate proliferation and differentiation of keratinocytes. Stem cells in the basal layer undergo asymmetric division to yield another identical stem cell, and a Transient Amplifying Cell (TAC) that is committed to differentiate. TACs are also capable of active proliferation (amplification) but eventually reach terminal differentiation (Fig. 4).

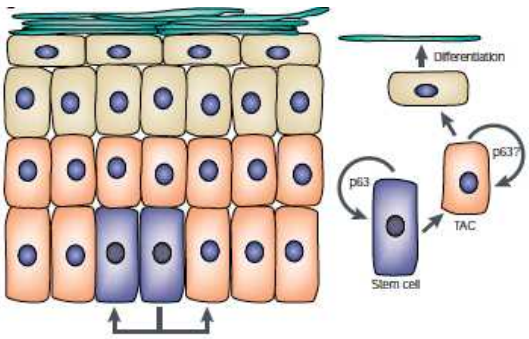

Figure 4. Model for p63 function in maintaining regenerative capacity in epithelial stem cells. On the left, representation of epidermal differentiation, showing a percentage of stem cells in the basal layer that undergo asymmetric division to yield another identical stem cell, and a Transient Amplifying Cell (TAC) that is commited to differentiation (5).

p63 transcript is expressed in the surface ectoderm prior to stratification and continues to be expressed during embryonic development. As the epidermis matures, *p63* expression becomes restricted to the basal layer of epidermis, indicating that it is required to maintain the proliferative potential of epidermis (30-32). In adult tissues, *p63* is expressed in stratified epithelia, whereas its expression is absent from single-layered epithelia. In the presence of lesions in the epidermis

and dermic tissues, the repair mechanism of damaged cells includes the formation of new epidermal layer. During this regenerative process different p63 protein isoforms are present, supporting the idea that *p63* expression is not important only during embryonic development but also in the regenerative process of epidermal layers (33).

2.3 Limb development

In mice, embryonic limbs are first visible as a small bud that protrudes from the body and contains morphologically homogenous cells covered by a layer of ectoderm. Limb buds emerge as a result of a thickening of the somatic layer of the Lateral Plate Mesoderm (LPM). As the bud elongates, a region of undifferentiated cells is maintained at the tip, while differentiated tissues are progressively laid down starting at the base of the limb bud. The developmental patterning of the limbs results from gradients of signaling molecules in three spatial dimensions: Proximo-Distal (shoulder-finger direction, Pr-D), Antero-Posterior (thumb-little finger direction, A-P), and Dorso-Ventral (back-palm direction, D-V). For correct development, three specialized cell clusters are of primary importance: the Apical Ectodermal Ridge (AER), the Progress Zone (PZ), and the Zone of Polarizing Activity (ZPA) (Fig. 5). These groups of cells express signaling molecules that determine the fate of neighbouring cells by instructing them to remain undifferentiated, to proliferate, or to differentiate into a particular cell type (34-38).

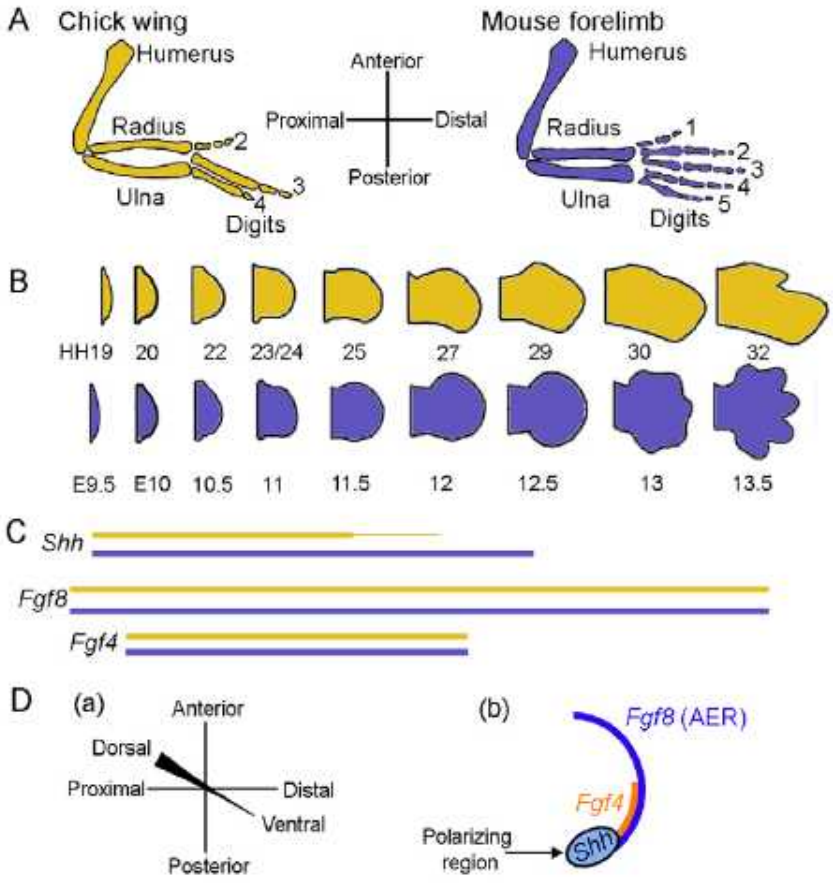

Figure 5. Chick wing and mouse FL development. **(A)** A schematic representation of fully developed chick wing (yellow) and mouse FL (blue) skeletons with antero-posterior (A-P) and proximo-distal (P-D) axes shown. **(B)** Schematics of equivalently staged chick wing (Hamburger-Hamilton stages, HH) and mouse FL buds (Embryonic day, E) from early stages to hand plate development. **(C)** Timeline of Shh, FGF8 and FGF4 expression in relation to embryonic stages shown in B. **(D)** **(a)** Orientation of A-P, Pr-D and D-V axes in early stage limb buds. **(b)** A schematic of expression of: Shh in the Polarizing region, FGF8 throughout the AER and FGF4 in the posterior AER.

2.3.1 Proximo-Distal patterning

Pr-D development depends on a strip of specialized epithelium at the distal tip of the limb bud, called AER. Recent studies on chick and mouse models, propose that

the AER controls the initial size of the limb bud, cell survival and proliferation in order to generate a limb buds of appropriate size. Removal of the AER in chick embryos results in limbs lacking distal skeletal elements.

The Fybroblast Growth Factors (FGFs), key factors required for AER function, are signaling molecules whose activities are mediated by a family of tyrosine kinase trans-membrane receptors (39-42).

FGFs that are specifically expressed in the AER are: FGF8, FGF10, FGF9 and FGF17. *FGF8* transcript is expressed in the prospective AER cells of nascent limb and subsequently throughout the AER until it regresses (43-46). By contrast, *FGF4, FGF9* and *FGF17* expression starts after the AER is formed, is restricted to the posterior AER, and ceases at least a day before AER regression. When AER *FGFs* are individually eliminated through the generation of KO mice, only loss of *FGF8* perturbs skeletal patterning. The other AER-FGFs have been proposed to be essential, but functionally redundant, components of a positive feedback loop between the AER and the patterning center in the posterior limb bud mesenchyme that produce Sonic-Hedge-Hog (SHH), a crucial factor required for AP patterning (46-48).

In particular, *FGF8* KO mice display a substantial reduction in limb bud size, a delay in *SHH* expression, mis-regulation of *FGF4* expression, and hypoplasia or aplasia of specific skeletal elements. In particular, *FGF8* null mice display limb abnormalities: stylopod are severely reduced in HLs, but only mildly affected in FLs. Zeugopod elements are mildly hypoplastic in both HLs and FLs. The autopod is missing a digit: digit I in HLs and digit II or III in FL (49, 50) .

The phenotype of *FGF8* null mice resembles the phenotype of the *p63* KO mice, indeed both KO models display limb defects that are affecting more seriously HL rather than FLs (mills and yang 1999) (Fig. 6).

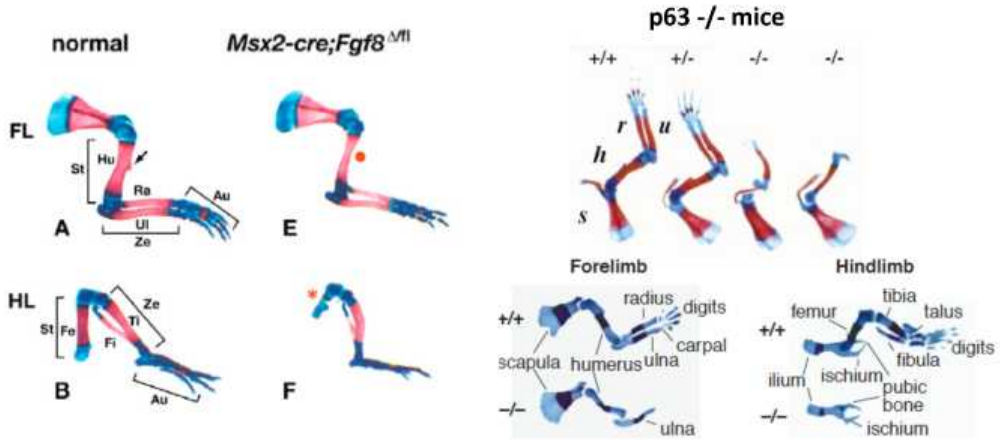

Figure 6. (Left) Skeletal preparation of newborn mouse limbs, with cartilage stained in blue and bone stained in red. In *Msx2-cre;Fgf8 Δ/fl* the femur is highly hypoplastic, one digit is missing and phalanges are absent from some of the remaining digits ([35,62]) (**Right**, **top**) Bone and cartilage of fore- and hindlimbs of E15 p63-/- and control littermates. Homozygous mutants lack distal components of the forelimb, including the radius, carpals and digits, as well as all components of the hindlimb. (**Right**, **bottom**) Forelimbs of *p63* homozygous mutant mice are truncated. The phalanges, ulna, radius are absent and the humerus is deformed (10, 47).

FGFs signaling from prospective AER cells is essential at early stages of limb development to establish an appropriately sized progenitor cell populations for the stylopod, zeugopod and autopod. During this period, FGF signalling might function by regulating cell fate determination, cell proliferation and/or cell survival. Early reduction in FGF signalling should therefore decrease the number of cells available to form each segment, resulting in hypoplasia or aplasia of elements in all segments.

Careful comparison of *FGF4* and *FGF8* expression in normal embryos reveals greater overlap in the HL AER rather than in the FL. This would allow FGF4 to better compensate for loss of FGF8 in the anterior HL AER. Indeed, inactivation of both *FGF4* and *FGF8* in the AER produced more severe phenotype than inactivating *FGF8* alone; the defects observed can be explained by the hypothesis

that a principal function of AER-FGF signaling during limb development is to ensure that enough progenitor cells are available to form each element of the limb skeleton (34, 51).

The limb mesenchyme produces FGF10, which signals to the limb ectoderm to initiate limb outgrowth. FGF10, together with other crucial signaling molecules of limb development, like *Wingless 3a* (*WNT3a*) and *Bone Morphogenetic Protein* (*BMP*), are required to induce *FGF8* expression of AER precursors in the ectoderm. The AER precursors are initially spread over a relatively broad region of the ectoderm, then became concentrated at the distal tip of the limb bud and compact to form a columnar epithelium. The compacted AER also serves as mechanical function to provide directed outgrowth and to maintain a dorso-ventrally flattened shape of the limb. After the AER has completed its function, it regresses, returning to a flattened cuboidal epithelium. This is accompanied by down-regulation of *FGFs* expression and a reduction of mesenchyme proliferation. Molecular experiments in the chick show that AER regression is mediated by BMP signaling (37, 52).

2.3.2 Anterior–Posterior limb patterning

The Anterior-Posterior limb development is assured by a posterior region of limb mesenchyme called the Zone of Polarizing Activity (ZPA). The ZPA is located in the posterior limb-bud mesenchyme and specifies A-P identities in the mesenchyme through the secretion of SHH signaling molecule. SHH is one of the three vertebrate homologs of *Hedgehog* segment polarity gene of the *Drosophila melanogaster*. Genetic analysis has revealed essential functions of *SHH* gene in a large number of morpho-regulatory processes. The active SHH signaling peptide is generated by auto-proteolytic cleavage of full-length protein, and is covalently modified by the addition of cholesterol and palmitate moieties. This modified peptide forms a posterior to anterior gradient in developing limb-bud. The formation of SHH gradient is fundamental for digit identity and specification.

Genetic inactivation of *SHH* in mice results in the dramatic loss of skeletal elements along the A-P axis: in particular loss of distal limb structures and complete absence of digits (35, 53)

2.3.3 Dorsal-Ventral patterning

The mesenchyme already contains the information for D–V limb patterning that occurs before limb-bud initiation. Just before the limb bud forms, the mesenchyme transfers this information to the overlying ectoderm; the molecular nature of this early mesenchymal signal(s) is unknown.

The transcription factor Lmx1b is expressed in the dorsal mesenchyme of the limb bud and is required for cells to adopt a dorsal character. Although the early regulator of *Lmx1b* expression is unknown, as the limb bud forms *Lmx1b* is induced by WNT7a, a signalling molecule that has been implicated in the regulation of cell fate and pattern formation during embryogenesis, expressed in the dorsal limb ectoderm (54). In the absence of *WNT7a*, the dorsal pattern of the distal structures (autopod) is not established and the limbs appear bi-ventral. Expression of *WNT7a* is restricted to the dorsal ectoderm because it is repressed in the ventral ectoderm by the transcription factor Engrailed1 (En1). In *En1−/−* limbs, Wnt7a is misexpressed in the ventral ectoderm, and the distal structures of limb bud develop with bi-dorsal character (52).

As described before, normal limb development occurs along three different axes: (Pr-D, D-V and A-P) controlled by different signaling centers: the Apical Ectodermal Ridge (AER), the Progress Zone (PZ), and the Zone of Polarizing Activity (ZPA). It soon became clear that each limb axes is not specified independently from the others and that the putative molecular determinants of the axes are mutually regulated. Indeed, complex feedback loops exist between SHH in the ZPA, BMPs and their antagonists in the adjacent mesenchyme, WNT7a in the dorsal ectoderm and FGFs in the AER. In particular, maintenance of *FGF*

expression seems to be regulated by a positive feedback loop between FGF signaling in the AER and *SHH* expression in the ZPA. There is also evidence that signaling from the dorsal ectoderm is required to maintain *SHH* expression (55) (Fig 7).

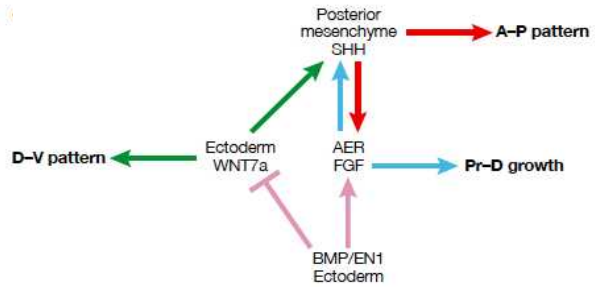

Figure 7. Signaling pathways in vertebrate limb development. molecular interactions that coordinate limb growth and patterning along the three limb axes: Pr-d is under the control of FGFs from the AER, the A-P is under the control of SHH in the posterior mesenchyme, and the D-V is under the control of BMP an En1from the ventral ectoderm and Wnt7 from the dorsal ectoderm (52).

However, for correct limb development and shaping is also required the establishment of programmed cell death (or apoptosis) process. The formation of free digits in vertebrates is accompained by massive apoptosis of the interdigital mesenchime and has the function of sculpturing the shape of the digits. In addition to mesodermal cell death, apoptosis is also an important feature of the ectoderm of the AER. In the chick limb buds ectodermal apoptosis appears to exert the function of controlling the extension of this structure (56,57).

Alteration in processes regulating apoptosis in limb buds causes an enlargement of the AER structure in limb buds, that results in polydactyly (57, 58).

The molecular machinery responsible for apoptosis exhibits a high degree of conservation in the course of evolution. During limb programmed cell death, members of the different groups of apoptotic regulators have been identified. As in other models of apoptosis, the final steps of limb programmed cell death consist in the activation of caspases, key mediators of the apoptotic response (57, 58) (Fig. 8).

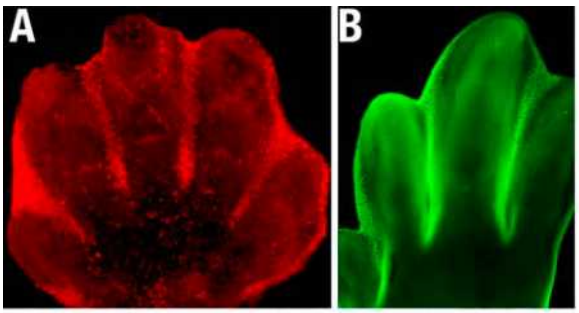

Figure 8. Interdigital cell death in the developing limb by TUNEL labeling and Acridine Orange Staining (58)

In developing limb buds, BMPs have been identified as the triggering apoptotic signal for both the ectoderm of the AER and the mesodermal cells. According to their pattern of expression BMP-2, BMP-4 and BMP-7, are the most likely physiological signals triggering apoptosis in the limb buds. These BMPs are also involved in the control of limb patterning and in the regulation of chondrogenic differentiation. There are evidences suggesting that the apoptotic effect of BMPs in the limb bud and in other developing model systems is mediated by the activation of the cytoplasmic kinase TAK-1 rather than by the canonical intracellular pathway of BMPs involving phosphorylation of Smad proteins, intracellular molecules that mediate the canonical signaling cascade of TGFbeta superfamily growth factors (35, 57, 58).

Two different models have been proposed to elucidate the mechanism of limb development (Fig. 9).

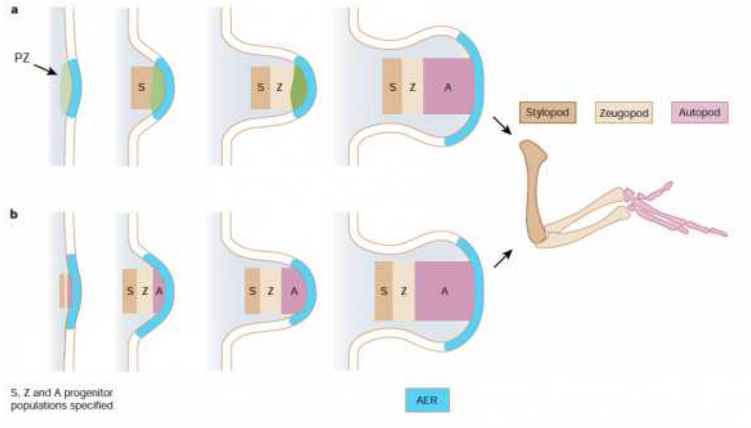

Figure 9. Representation of two models for proximo-distal limb skeletal patterning: progressive zone model (a) and early specification model (b) (35)

The 'progress zone' model proposed by Wolpert and colleagues postulates that cells acquire proximo-distal positional information progressively, in a proximal-to-distal sequence, by measuring time spent in a 'Progress Zone' (PZ). The longer the time spent in the PZ, the more distal the positional values they acquire. Cells that exit early are specified to form proximal limb structures, whereas those that exit late are specified to form distal ones. The other model proposed by Dudley et al., called 'early specification model', postulate that specification to form proximal, middle or distal limb structures does not occur progressively, but instead occurs very early, perhaps even before limb bud outgrowth has begun. According to this model, the specified populations subsequently expand as the limb bud grows, and become determined in a proximal-to-distal sequence. Cells are specified as progenitors of stylopod, zeugopod or autopod, and over time they expand and become determined to form particular structures in response to cell–cell interactions and signaling (35).

2.3.4 Survey on the molecular pathway controlled by p63 during limb development

It's well established that *p63* expression is fundamental for correct limb growth and development. The most abundantly protein isoform expressed in the AER of the limb buds is the ΔNp63α isoform and its expression increases from E10.5 to E12.5 (28, 59, 60). The expression of ΔNp63α protein is required to assure proliferation and stratification of AER cells in order to reach a correct process of limb outgrowth. In this context the identification of ΔNp63α target genes expressed during limb development is fundamental to understand which are the pathway by which ΔNp63α guarantee correct formation of the limbs.

Previously, it has been demonstrated that ΔNp63α co-localize in the AER with the DLX5 and DLX6 proteins and contributes to their transcriptional activation *in vivo* in AER cells (fig. 10).

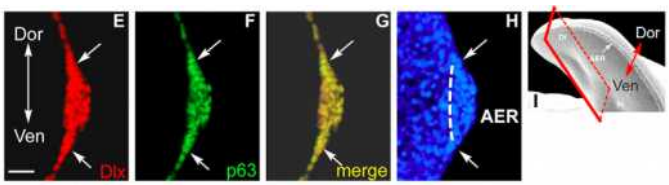

Figure 10. Double immunostaining for pan-DLX (red) and p63 (green) on sections of E10.5 HLs. The merged image is shown in G. (H) Nuclei were counterstained with DAPI. Dashed line indicates the border between the AER and the limb mesenchyme. Section plane and orientation A are indicated (**I**) (28).

DLX homeobox genes are mammalian homologs of the *Drosophila melanogaster Distal less (Dll)* gene and consist of six genes organized into the *DLX1/DLX2*, *DLX3/DLX7*, *DLX5/DLX6* bigene clusters (59-61). These genes are essential for proximo-distal patterning of insect limbs. In particular, the *DLX5* and *DLX6* genes encode for transcription factors playing a key role in the morhogenesis of head and limb skeleton in mice. Indeed, *DLX5;DLX6* -/- mice exhibited severe craniofacial abnormalities, bilateral ectrodactyly, resulting in shorter or missing digits, often

accompanied by malformation and syndactyly of the remaining digits and profound medial cleft of the posterior limbs (60). Interestingly, a number of cases of SHFM-I are associated with chromosomal aberrations involving the 7q21-22 chromosomal region. Indeed, extensive analysis to locate the minimal deletions associated to SHFM-I had narrowed down the SHFM-I critical region to 1.5 Mb and six breakpoints have been found within a 700 kb region. Three candidate genes are located in the common deletion interval: DLX5, DLX6 and DSS1 (62). Indeed, the *DLX5;DLX6* KO mice, displaying bilateral ectrodactyly with a severe defect of the central ray of the hindlimbs, phenocopy limb defects observed in SHFM-I patients (60, 61). Moreover, SHFM-IV, a phenocopy of SHFM-I, is caused by mutations in the *p63* gene. All these discoveries suggest that p63, DLX5 and DLX6 proteins might be components of a common signaling pathway regulating limb development. Furthermore, the severity of limb malformations displayed by the *p63* KO mice, respect to the *DLX5;DLX6* KO mice, suggest that p63 could be an upstream crucial regulator of signaling pathway during limb development: indeed, p63 has been identified as a crucial activator for the *DLX5* and *DLX6* gene during limb development (28) (Fig. 11).

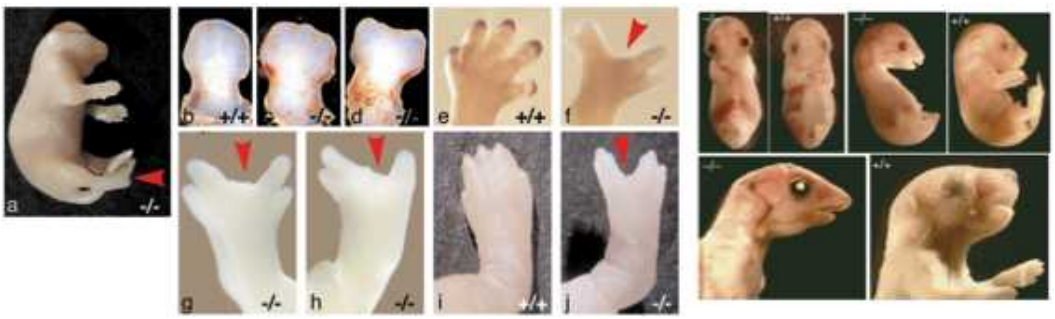

Figure 11. (left) Limb defects in *DLX5;DLX6* null mice. a: Homozygous mutants die around 18 dpc, showing severe craniofacial abnormalities and hindlimb defects. Hindlimbs of normal (b,e,i,j) and mutant (c,d,f,g,h) mice at 12.5 dpc (b–d), 14.5 dpc (e–h), and 18 dpc (i,j). At 12.5 dpc, the central part of the hindlimb palette appears flattened (c) or indented (d), at later stages reduced or absent digits and median clefts are observed. Note the variable phenotype between left (g) and right

(h) mutant hindlimb. In situ hybridization on normal (e) and mutant (f) 14.5 dpc hindlimbs with a *DLX5* probe showing absence of expression in the mutant. Abnormal or missing digits are indicated (red arrows). No obvious defect was been seen in the forelimbs. Genotypes of the *Dlx5–Dlx6* locus are indicated (61). **(right)** Characterization of the *p63* null mice phenotype (10).

2.4 Human syndromes and malformations associated to mutations in the *p63* gene

After the characterization of the *p63* null mice phenotype a lot of works focused on linking the genetic defects of several human syndromes characterized by skin and limb malformation to a region of chromosome 3q27 encopassing the *p63* gene (63-65). These works lead to the identification of at least 5 human syndromes caused by mutation in the *p63* gene showing defects resembling the p63-null mice phenotype. They include: Ectodactyl Ectodermal dysplasia Clefting (EEC), Ankyloblepharon-Ectodermal-displasia (AEC), Acro-Dermato-Ungual-Lacrimal-Tooth (ADULT), Limb-Mammary-Syndrome (LMS) and Split-Hand/Split-Footh Malformations (SHFM) type IV (65).

The pattern of mutations in the five human disorders linked to *p63* mutations reveals a remarkable specificity of the molecular defects in this gene and clinical consequences (Fig. 12).

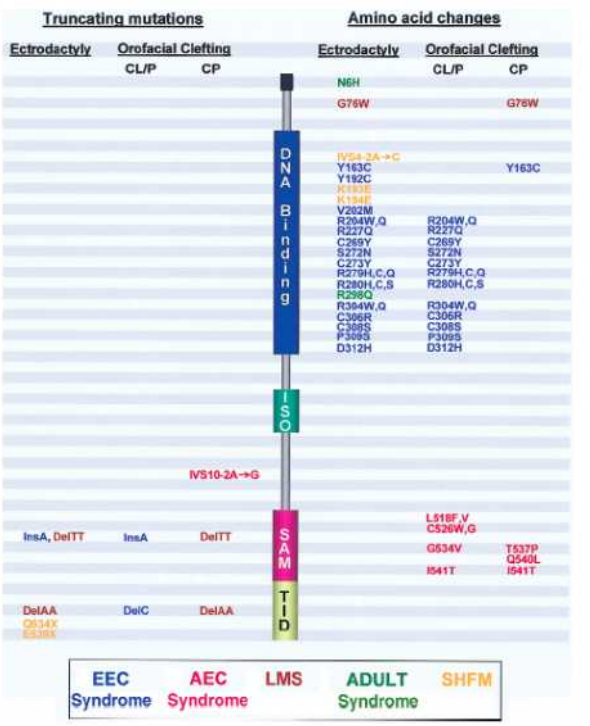

Figure 12. Distribution of mutations in p63 associated to human developmental related syndromes (65).

All of these disorders are the consequence of a mutation at a single *p63* allele: indeed mutations of the *p63* gene associated to these syndromes are heterozygous dominant mutations. Moreover, the p63 mutant proteins interfere with the normal activity of the wild-type protein thanks to their capacity to form omo and ethero-tetramers (65).

Phenotypic Characteristics of Human Syndromes

	Human Syndrome								
	EEC Syndrome	EE Syndrome	AEC Syndrome	RHS	LMS	SHFM	ADULT Syndrome	LADD Syndrome	ECP Syndrome
Limb:									
Split hand/split foot	++	+++	–	–	++	+++	++	–	+++
Fused digits, other	++	–	+/–	–	++	++	++	++	+
Ectodermal:									
Hair	+	++	+++	+++	–	–	+	–	–
Skin	+	–	+++	+	–	–	++	–	–
Nail	+	–	++	++	+/–	–	+	–	–
Teeth	+	++	++	++	+/–	–	++	++	–
Lacrimal ducts	+	–	+	++	+	–	+	++	–
Sweat glands	+	–	++	++	+	–	–	–	–
Breast/nipple hypoplasia	+/–	–	–	–	+++	–	+	–	–
Facial:									
CL/P	+++	–	+	+	–	–	–	–	–
CP	–	–	+	++	+	–	–	–	+++
Microretrognathia	+	–	+	–	–	–	–	++	–
Other:									
Fused eyelid	–	–	+++	–	–	–	–	–	–
Supernumerary nipple	+/–	–	+	–	–	–	–	–	–
Poor saliva production	–	–	–	–	–	–	–	++	–
Deformed ears/ear canals	+	–	–	+	–	–	–	+++	–
Hearing loss	+	–	+	–	–	–	–	+	–
Extensive freckling	–	–	–	–	–	–	+++	–	–
Kidney hypoplasia	+	–	+	–	–	–	–	+	–
Urethra/bladder	+	–	–	–	–	–	–	–	–
Hypospadias	–	–	–	+	–	–	–	+	–

NOTE.—+++ = Consistent feature; ++ = frequently observed; + = occasionally observed; +/– = rarely observed; – = never observed.

Figure 13. Table of phenotypic characteristic of p63 human syndromes (65).

2.4.1 Ectrodactyl-Ectodermal-dysplais Clefting (EEC)

The EEC syndrome is characterized by the triad of ectrodactily, ectodermal dysplasia and facial clefting. A number of associated anomalies are frequently found, among which there are lacrimal tract anomalies, urogenital anomalies, and conductive hearing loss. EEC patients occasionally have also mammary gland/nipple hypoplasia (14%), and hypohidrosis (11%). About two-thirds of these patients have ectrodactily and syndactyly is also frequent (43%). Cleft lip/palate (CL-CP) is present in about 40% of the EEC patients.

An extended analysis of EEC syndrome patients showed heterozygous mutations of *p63* gene in 40 of 43 unrelated families, indicating that *p63* is the gene mutated in EEC syndrome (63, 66).

Eighteen different mutations in the DBD have been reported in EEC syndrome patients. All of these mutations, apart from one, that is a frameshift mutations affecting only p63α isoforms, give rise to aminoacid substitutions in the DNA-Binding Domain common to all p63 isoforms. These aminoacids are crucially

important for the interaction with DNA target sequences, and their mutation seems to impair p63 binding to DNA (R204W/Q, R279C/H/S and R304W/Q).
In general, the missense mutations are predicted to affect all p63 isoforms, resulting in loss of transactivation of p63, while the frameshift mutation resulted in gain of transactivation potential for the truncated TAp63α isotype. So the missense and frameshift mutations appeared to exhibit divergent effects on the regulation of transcriptional activity of p63 as well, suggesting that both an increase and/or a decrease of transactivation by p63 lead to the developmental defects seen in EEC syndrome patients (65).

2.4.2 Ankyloblepharon-Ectodermal-displasia

The AEC syndrome, which is also known as "Hay-Wells syndrome," has little or no limb involvement but instead includes ankyloblepharon, which is a partial or complete fusion of the eyelids that is very rare in other p63 associated syndromes. Approximately 80% of the patients have severe skin erosion at birth, which usually recover in the first years of life. The eyelid fusion is present in about 45% of AEC patients. Nail and teeth defects are present in more than 80% of patients, while hair defects and/or alopecia are constant features (94%). Lacrimal duct obstruction is seen in 50% of patients, whereas mammary gland hypoplasia and hypohydrosis occur occasionally (both 13%). Interestingly, almost 40% of patients have hearing impairment and genital-urinary defects. Cleft lip is present in 44% and cleft palate in about 80%. Limb malformations are almost absent. Ectrodactyly has never been reported, but 25% of patients display mild syndactyly. Immunohistochemical examination of a skin sections from an AEC syndrome patient revealed an aberrant localization of p63.
Mutations in 12 unrelated patients with AEC have been detected, and 10 of these are missense mutations within the SAM domain of p63. These mutations are predicted to disrupt protein-protein interactions, by either destroying the compact globular structure of the SAM domain or substituting aminoacids that are crucial

for such interactions. Missense mutations in AEC syndrome affect only the α isotypes of p63.

The AEC mutations exert a selective dominant-negative function on wild-type p63 by affecting the expression of a number of p63 target genes, including Fgfr2 and Fgfr3. Impaired FGF signalling downstream of p63 is an important determinant of reduced ectodermal cell proliferation and defective self-renewing compartment in AEC syndrome (67).

2.4.3 Acro-Dermato-Ungual-Lacrimal-Tooth Syndrome

The ADULT syndrome is characterized by ectrodactyly and ectodermal abnormalities such as nail dysplasia, hypodontia, lacrimal duct obstruction, sparse hair and thin skin. These patients show neurodermitic signs (exfoliative dermatitis of the digits) and excessive freckling; on the other hand these patients do not manifest cleft-lip palate. Teeth, skin and nail defects are constantly present in ADULT syndrome; moreover hair (53%) and lacrimal duct defects (67%) are observed in ADULT patients. Four ADULT syndrome families and three sporadic cases have been reported. All the families and one of the sporadic cases have a point mutation in exon 8, changing R298 in the DNA binding domain into either a glutamine or a glycine. While EEC syndrome mutations in the DBD impair the binding of p63 protein to DNA, arginine 298 is not located close to the DNA-binding interface, and mutation of this arginine does not affect DNA binding. In particular, this mutation confers a novel transcriptional activation capacity to the ΔNp63γ isoform (68,69).

2.4.4 Limb Mammary Syndrome

The LMS syndrome is characterized by severe hand and/or foot anomalies, and hypoplasia/aplasia of the mammary gland and nipple. Less frequent findings include lacrimal-duct atresia, nail dysplasia, hypohydrosis, hypodontia, and cleft palate with or without bifid uvula. This syndrome differs from the EEC syndrome

in at least three aspects: mammary gland and nipple hypoplasia are consistent features of LMS but are only occasionally seen in EEC syndrome. Second, patients with LMS do not have hair and skin defects that are seen in EEC syndrome. Third, whereas patients with LMS have CP, those with EEC syndrome have CL/P but never have CP only. Phenotypically, LMS is most similar to ADULT syndrome (70).

Mutations in LMS are located in the N-and C-terminus of the *p63* gene. A large LMS family (29 affected members) has a point mutation in exon 4, causing a G76W substitution in the DNA-specific putative Trans-Activation Domain (TA2). One Other point mutation (S90W) is also located between the TA Domain and DBD. Other LMS mutations are reported in the C-terminus: these deletions in exon 13 (TT deletion) and exon 14 (ΔAA) cause a frameshift and a premature stop codon affecting only the p63α protein isoforms. Also a stop mutation in the TID domain (K632X) has been identified in a sporadic LMS patient. The latter mutation is predicted to impair the suppressive effect of the TID towards the TA domain, thus increasing the transactivation activity of p63 (70).

2.4.5 Split Hand/Split Foot Malformation type IV

The Split-Hand/Split-Foot Malformation (SHFM) syndrome, also known as ectrodactyly, is a congenital limb malformation, characterized by a deep median cleft of the hands and/or feet; however, the severity of SHFM is highly variable. In severe cases, the hands and feet have a lobster claw-like appearance, while in mildly affected patients, SHFM may be limited to syndactyly and several instances of non-penetrance have been documented. SHFM may occur as an isolated entity or as part of a syndrome: both forms are frequently found in association with chromosomal rearrangements such as deletions or translocations. The degree of phenotypic severity of an affected limb as well as the number of affected limbs differs from individual to individual (66, 70, 71) (Fig.14).

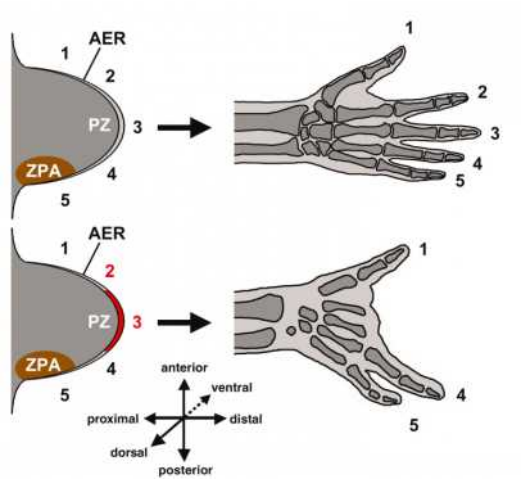

Figure 14. The ectrodactyly phenotype and underlying AER defect. (A) Clinical variability of ectrodactyly. (B) Normal development of the autopod (top) and ectrodactyly malformation (bottom). Ectrodactyly is caused by a failure to maintain median AER activity (red) in the developing limb bud (left), leading to the absence of the central rays (right). (71)

Five different phenocopies of SHFM were identified, and they are all characterized by specific abnormalities including: (1) monodactyly presenting as a single digital remnant derived either post-axially; (2) bidactyly (so called ‘‘lobster-claw’’) presenting as two digital elements separated by a deep median cleft; and (3) oligodactyly, by far the most common pattern, presenting as three or more digits in association with syndactyly and median cleft (71-73).

Disorder	Chromosomal location	Candidate gene (s)
SHFM-I	7q21	*Dlx5, Dlx6, DSS1*
SHFM-II	Xq26	*FGF13, TONDU*
SHFM-III	10q24	*DACTYLIN, LBX1,βTRCP*
SHFM-IV	sq27	*p63*
SHFM-V	2q31	*HOXD, Dlx1, Dlx2*

Figure 15. Human genetic disorders with ectrodactyly.

SHFM-I is linked to deletions, inversions and rearrangements affecting chromosome 7q21-22. The region of overlapping deletions encompasses several genes: *DYNC1l1, SLC25A13, DSS1, DLX5* and *DLX6*; of these genes only *DLX5* and *DLX6* have been shown to be specifically expressed in the AER of developing limb buds (28, 60, 61). Double inactivation of *DLX5* and *DLX6* in mouse caused ectrodactyly with severe defect of cental rays of HLs, a malformation typical of SHFM-I (61). Moreover, a *DLX5* mutation (DLX5 Q178P) has been identified in a family with an unusual SHFM, recessively linked to SHFM1 and associated with defects in both limb outgrowth as well as dorsal-ventral patterning (74). This represents one of the strongest evidence implicating DLX5 in the pathogenesis of SHFM.

SHFM-II has been mapped to chromosome Xq26.3; possible candidate genes in this region include *FGF13* and *TONDU*. Only a single SHFM family has been reported with X-chromosomal inheritance of isolated ectrodactyly; cytogenetic studies ruled out the possibility of translocations or X-chromosomal rearrangements in this family at the bases of pathogenesis (75).

SHFM-III is a limb malformation characterized by the absence of central digits, shown to be associated with tandem duplications of about 500 kb at 10q24. The genomic lesion involves the *DACTYLIN*, *LBX1* and *βTRCP* genes, but none of these genes is directly disrupted and no point mutations have been described (76,77). Instead, *FGF8* and *NFkB2*, two genes implicated in limb development, are located in the proximity of the re-arrangements breakpoints.

SHFM-IV is caused by several mutations dispersed throughout the *p63* gene. A point mutation (R58C) is located in the TA domain, a splice-site mutation maps in front of exon 4 (3'ss intron 4), four missense mutations are localized in the DBD (K193E, K194E, R280C, R280H), and two nonsense mutations were found in the TID domain (Q634X and E639X). Interestingly, these last mutations are known to disrupt the sumoylation site and therefore increase the stability and transcriptional

activity of the p63α protein. The R280C and R280H mutations have also been encountered many times in EEC syndrome (65).

Finally, **SHFM-V** is associated to deletions encompassing the chromosome region 2q24–q31. Patients with deletions in this region exhibit a number of abnormalities, including microcephaly, mental retardation, low-set ears, and limb abnormalities. Recent studies have revealed that the 2q24.3–q31 region can be subdivided into three distinct loci for limb abnormalities. This region encompasses the *HOXD* gene cluster, essential for the development of the extremities of limbs. Candidate genes located in the critical SHFM-V interval include *DLX1* and *DLX2*, two homeobox genes expressed in the AER and the PZ of limb buds (30, 78-80).

The generation of the KO mouse models phenocoping the defects showed by the SHFM patients contributed to the identification of the molecular pathways and the regulators controlling limb development. In particular, mouse model for SHFM-I have been generated by the combined deletions of *DLX5* and *DLX6* genes; the *Dactyplasia* mutant mice has been proposed as a good model of SHFM-III (60,61,77). Dactylin is one of the several factors involved in the regulation of cell proliferation in the AER (76,77). These KO mice exhibit defects in the stratification and development of AER structure and AER failure is at the bases of SHFM pathogenesis (30,71). These discoveries suggest that DLX5, DLX6, p63 and Dactylin could be link together in a same pathway essential for correct limb development.

2.4.6 Genotype-phenotype correlation of p63 mutations

The clustering of mutations in the DBD, for EEC syndrome, and in the SAM domain, for AEC syndrome establishes a clear genotype-phenotype correlation (Fig. 11). A number of observations can be made from more detailed analysis of the pattern of TP63 mutations. First, it is notable that the truncating mutations are all located in the C-terminal part of the protein. Hence, all p63 mutations known to

date leave the ISO domain intact. This allows the formation of tetrameric complexes between wild-type and mutant p63 proteins, which offers an explanation for the dominant effect of p63 mutations.

Ectrodactyly is only seen in combination with missense mutations in the DBD of p63 isoforms, or in combination with truncating mutations in the C-terminal part of the protein. In contrast, missense mutations in the C-terminal part are never associated with ectrodactyly. A second remarkable phenomenon involves the type of facial clefting. The facial clefts seen in conjunction with missense mutations in the conserved part of the DBD always involve the primary palate (CL/P), whereas mutations toward the C-terminal end of p63 may either involve the primary palate or the secondary palate (CP) (30, 64, 65) (Fig 16).

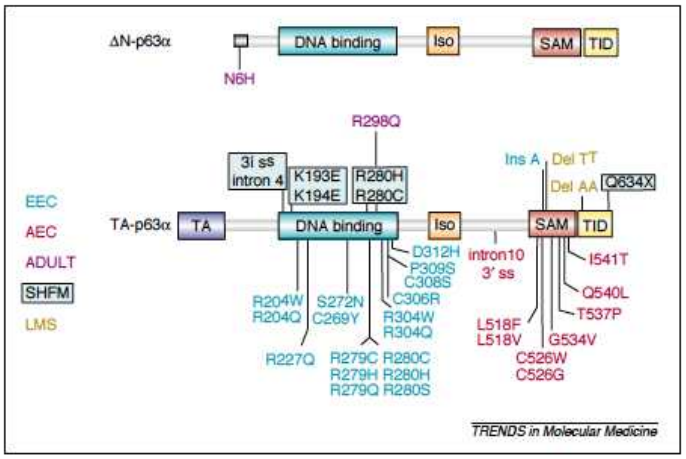

Figure 16. Illustration of p63 mutation in human syndromes establishing a clear genotype-phenotype correlation (64).

Some of *p63* mutations could also be ”gain of function” mutations: they caused an increase in the transcriptional activity of the mutant protein compared to the wild-type p63. For instance the R298Q, associated to the ADULT syndrome, conferes to the ΔNp63γ isoform an exceptional trans-activation capacity, which is absent in the wild-type ΔNp63γ protein. There could be two possible explanations for this gain of trans-activation activity: (1) the mutation creates a novel site for the binding of a

transcriptional coactivator; (2) the mutation releases a second TA domain (denoted as "TA2") that is normally kept in an inactive state. Simultaneously, some of these mutations are "loss of function" because they determine a reduction of the p63 transcriptional activity as it as been described for the missense EEC and SHFM-IV mutations in the regulation of the *DLX5* and *DLX6* genes (28).

It's important to consider that most of the mutations are located in the DBD domain and in the SAM domain. Often these mutations occur on residues that could be post-transcriptionally modified (like lysines, serine, threonine and tyrosines) possibly leading to an alteration of the normal signaling pathway regulating p63 isoforms activities.

2.5 Regulatory Post-Translational Modifications of the p53 family members

Post-Translational Modifications (PTMs) are series of covalent processing events that change the properties of a protein, by either proteolytic cleavage or by the addition of a modifying group to one or more aminoacids. PTMs have a great influence on the functions of a protein, as they can regulate its activity, localization, turnover and interaction with other proteins and molecules like nucleic acids, lipids and cofactors.

PTMs are often mediated by enzymes and occur at distinct aminoacid residues. These enzymes include kinases, transferases, phosphatases and ligases, which add or remove functional groups, proteins, lipids or sugars to or from aminoacid side chains. Conversely, proteases are enzymes that cleave peptide bonds to remove specific sequences or regulatory subunits.

PTMs are known to be essential mechanisms used by eukaryotic cells to diversify their protein functions and dynamically coordinate their signaling networks. Defects in PTMs have been linked to numerous developmental disorders and human diseases, highlighting the importance of PTMs in maintaining normal cellular functions and homeostasis.

The most important and studied PTMs for the p53 family members includes: phosphorylation, acetylation, and ubiquitilation. Indeed, these PTMs strongly modulate p53 family members function and stability in response to different stimuli and stress conditions.

Phosphorylation is the addition of a phosphate group to a serine, tyrosine or threonine residue in a peptide chain. The addition or removal of a phosphate group can alter protein conformation (and therefore function) by locally altering the charge and hydrophobicity.

Acetylation is a reversible process by which acetyl groups are placed onto the ε amino-group of lysine residues of a target protein and it is a well studied event occuring on histone tails during transcription. Indeed, acetylation of internal lysine residues of core histone has been found associated with transcriptional activation in eukaryotes. Histone acetylation has been shown to occur both globally throughout the genome as well as at specific promoters and it is an indicator of actively transcribed genes.

In the deacetylation reaction, histone deacetylases (HDACs) remove the acetyl groups re-establishing the positive charge of histones correlated with silencing of gene expression (81-85).

Acetylation was first discovered on histones, and the significance of histone acetylation in transcriptional regulation is well accepted. However, histones are not the only proteins that can be acetylated: a large number of transcription factors can be acetylated inducing their activation and stabilization (86-88).

Ubiquitilation is an enzymatic PTM process in which an ubiquitin protein is attached to a substrate protein. This process most commonly binds the last amino acid of ubiquitin (glycine 76) to a lysine residue on the substrate. Ubiquitination is carried out in three main steps: activation, conjugation, and ligation, performed by ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), and ubiquitin ligases (E3s), respectively. A protein can undergo monoubiquitilation or poly-ubiquitilation on different lysines. In general, monoubiquitilation is associated

with modification of cellular localization, affects protein activity, and promotes or prevents protein interactions. On the other hand, polyubiquitination, the formation of an ubiquitin chain on a single lysine residue on the substrate protein, is associated with protein degradation by the proteasome machinery.

In the last decade, studies focused on the identification and characterization of the PTMs controlling and regulating the members of the p53 family. Indeed p53 is subject to a huge number of post-translational modifications, which markedly influences the expression of p53 target genes. The most commonly reported post-translational modifications of p53 include phosphorylation, acetylation and ubiquitylation (88-91) (Fig. 17).

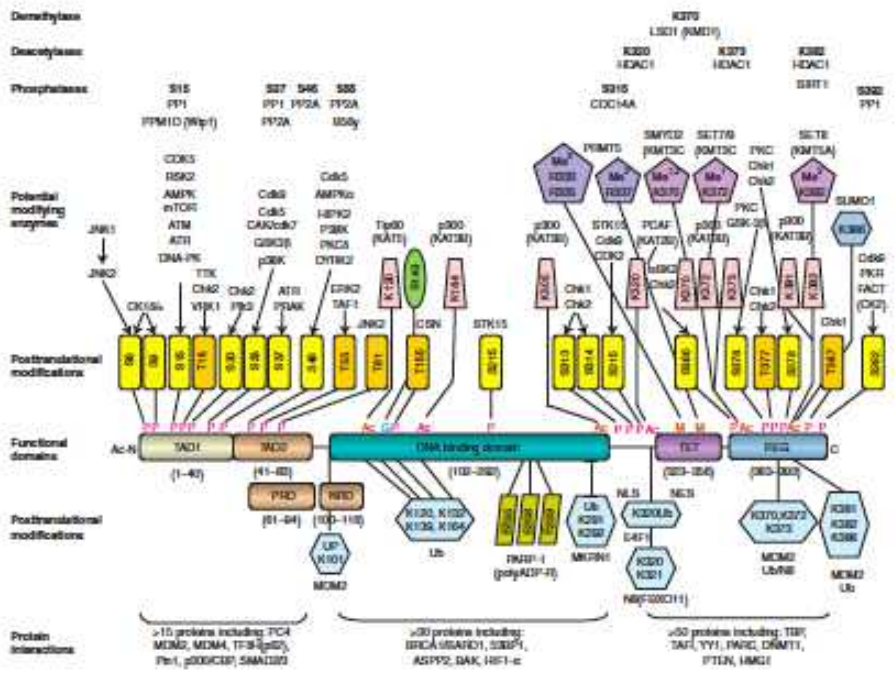

Figure 17. Post Translational modification of human p53 ([86,109])

p53 is a potent inhibitor of cell proliferation and survival, and it is therefore essential that p53 is kept inactive during normal growth. At homeostasis, the transcriptional activity of p53 is downregulated mainly in three ways: 1. By MDM2 ubiquitin–mediated proteasomal degradation; 2. by a decrease in nuclear p53 levels through nuclear export as a consequence on monoubiquitination

of p53 by MDM2; 3. by transcriptional repression of chromatin-associated p53 by formation of Mdm2-Mdmx-p53 complex. Upon DNA damage, degradation and nuclear export of p53 are suppressed, and nuclear import is concominantly enhanced, resulting in its nuclear accumulation leading to the activation of cell-cycle inhibitor and apoptotic related genes (91).

Among PTMs playing a crucial role in p53 activation and stabilization there are phosphorylation of serines, theronines and tyrosines located in the amino and carboxyl terminal region of p53 and acetylation of lysines located in the C-terminal region and in the DBD domain of the p53 protein. Phosphorylation of specific residues in p53 (Ser15, Thr18, and Ser20) increases p53 association with p300 and CBP acetyl-transferases and stimulates p53 transactivation functions. Specifically, acetylation of p53 is required for its activation and enhancement of sequence-specific DNA binding and target genes activation. Moreover, acetylation of p53 C-terminal residues is important as docking sites for the subsequent recruitment of acetyl-transferases and other transcription cofactors to promoter regions. In this context it's clear that PTMs of p53, resulting from the activation of different kinases/phosphatases and acetyl-transferases/deacetyl-transferases, play a critical role in the outcome of p53 activation (88, 92, 93).
In the following chapters I will concentrate on three crucial regulators of p53 and p73 that have been demonstrated to be essential for their activation in response to DNA damage. Moreover, these regulators have been shown to play a crucial role also during development suggesting that they could regulate also p63 functions during development.
These regulators are: the p300 acetyl-transferase, the c-Abl tyrosine kinase and the Pin1 prolyl isomerase.

2.5.1 p300 acetyl-transferase

Acetylation of p53 is an important reversible enzymatic process that occurs in response to DNA damage and genotoxic stress and is indispensible for p53 transcriptional activity (94).

One of the most important and well characterized acetyl-transferase for p53 is p300. p300 belongs from the p300/CBP family and the acetyl-transferase activity is located in the C-terminal fragment of p300 spanning aminoacids 1135-2414. In addition to the histone acetyl-transferase domain, p300 contains different domains, such as bromodomain and regions rich in Cys/His residues (C/H domains), conserved from Drosophila to mammals. These domains serve as binding sites for sequence specific transcription factors and other components regulating gene expression (83, 95). p300 is a transcriptional co-activator that operate in many transduction pathways controlling cell differentiation, growth control, and cellular homeostasis and its expression is essential to sustain a correct process of cell proliferation and development.

The major acetylation sites for p300 in p53 are located in the DBD domain and in the C-terminal region of p53; acetylation of these sites is important as docking sites for the subsequent recruitment of HAT and other transcription factor to promoter of p53 target genes (90, 92). One of the proposed mechanism by which acetylation promotes p53 stabilization and activation is that acetylation abrogates Mdm2 mediated repression by blocking its recruitment to p53 responsive promoters (94) (Fig. 18).

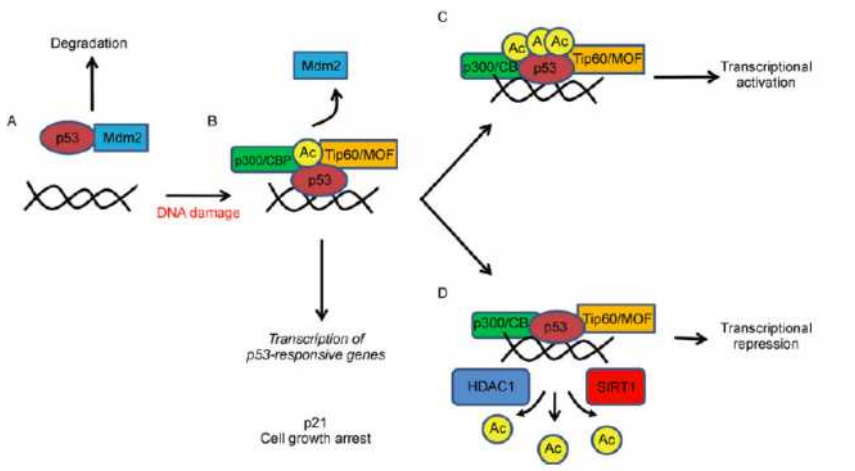

Figure 18. Role of p53 acetylation in gene regulation. (A) Unacetylated p53 is capable of activating genes that are involved in the negative regulation of p53, such as Mdm2, as a mechanism to keep p53 protein levels low during times of normal homeostasis. (B) Upon DNA damage, acetylation of p53 allows for the disruption of the Mdm2-p53 interaction and the recruitment of specific HATs to the promoters of genes involved in DNA repair and cell cycle control. (C) Full acetylation of p53 at all key acetylation sites promotes the activation of proapoptotic genes. (D) SIRT1 and HDAC1 are deacetylases that can reverse p53 acetylation and lead to transcriptional repression (94)

Recently, a previously undiscovered site acetylated by p300/CBP was described for p53 (K164). Deletion of K164 in combination with other known acetylation sites in p53 completely abrogated p53-dependent trans-activation of p21 and the ability to induce cell-growth arrest, confirming that p53 acetylation is a crucial PTM essential for p53 activation (94).

This residue is conserved in all the species known to encode for p53, including human, mouse, xenopus, and zebrafish, as well as in the p53-related proteins p63 and p73 (Fig. 19). In particular, lysine K164 in p53 corresponds to lysine K193 in p63; interestingly, this lysine is mutated in patients affected by SHFM-IV (K193E).

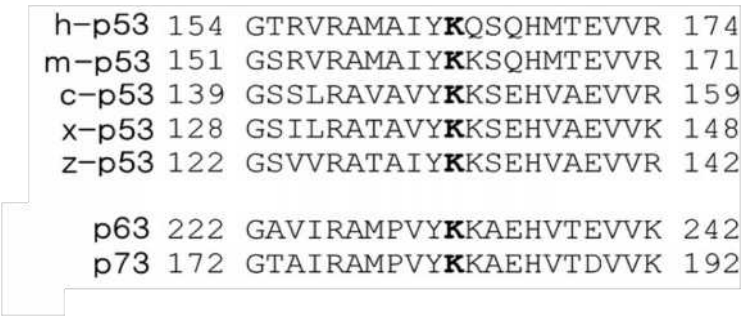

Figure 19. Alignment of the K164 flanking region of the human p53 protein with those of p53 from other species and of human p63 and p73. The conserved lysine residue is marked in bold; h: human; m: mouse; c: chicken; x: Xenopus; and z: zebrafish (94).

Acetylation has been largely demonstrated to be essential also for p73 regulation: the CH1 domain of the transcriptional co-activator p300 interacts with the N-terminus of p73 (96). p300 acetylates p73 on lysines 321, 327 and 331 and the trans-activation or induction of apoptosis by p73 is impaired in human p300 depleted cells, demonstrating an important role for p300 in p73 dependent apoptosis (97).

In this context, the recent identification of the K164 in p53, acetylated by p300, corresponding to K193 in ΔNp63α, found mutated in patients affected by SHFM-IV, suggest that acetylation could be a crucial PTMs occurring not only in response to DNA damage and cytotoxic stress but also during development.

First indications that p300 play an important role during development came from the characterization of *p300* null mice phenotype. These mice died between days 9 and 11.5 of gestation, exhibiting sever defects in neurolation, cell proliferation and heart development providing genetic evidence that p300 is essential for cell proliferation and development (98).

Furthermore, it's becoming clear that acetylation and deacetylation are crucial PTMs to guarantee correct development. Indeed, deletion of Histone Deacetyl-transferase *HDAC1* and *HDAC2,* in KO mice embryos results in dramatic failure of hair follicle specification and epidermal proliferation and stratification,

phenocopying loss of the key ectodermal transcription factor p63. HDAC1/2 proteins are present at the promoter regions of target genes repressed by ΔNp63 isoforms, suggesting that HDAC1 and HDAC2 are requested to repress those genes that need to be down-regulated by ΔNp63 in order to assure proper development (99).

2.5.2 c-Abl tyrosine kinase

It's well known that some PTMs are influenced or are promoted by an upstream modification, supporting the idea that there is a cross-talk between different types of modifications (100, 101). For instances, acetylation of p73 occurs after a phosphorylation event (102). The tyrosine kinase c-Abl regulates p73 in apoptotic response to cisplatin-induced DNA damage. The first kinase identified to phosphorylate p73, establishing an increase in p73 acetylation levels and consequent enhancement of its transcriptional activity, is c-Abl. In particular, DNA damage induces phosphorylation of p73 at tyrosine 99 (Y99) in a c-Abl dependent manner, and p73 proteins mutated at Y99 are impaired in mediating an apoptotic response upon ionizing radiations treatment (103, 104).

c-Abl (Abelson tyrosine kinase) is a member of a family of non-receptor tyrosine kinases and it is implicated in a large range of cellular processes including regulation of cell growth, oxidative stress and DNA damage. The c-Abl protein is localized at several subcellular sites, including the nucleus, cytoplasm, mitochondria, and the cell embrane, where c-Abl interacts with a large variety of cellular proteins, including signalling adaptors, kinases, phosphatases, cell-cycle regulators, transcription factors and cytoskeletal proteins (105-108).

For instances, c-Abl, upon DNA damage, physically interacts with p73 through the SH3 domain of c-Abl and the PXXP motif of p73 located in the linker region between the DBD and the OD domain, leading to p73 stabilization (102).

However, c-Abl is a critical regulator not only for p73 but also for p53: c-Abl is required for efficient accumulation of p53 upon DNA damage. Indeed, c-Abl

physically interacts with the C-terminal region of p53 and neutralize the ability of Mdm2 protein to promote p53 ubiquitination and degradation, leading to p53 transcriptional activation and induction of the apoptotic response (109-112).

It's well established that c-Abl is activated upon DNA damage (107,108); however, a crucial role in c-Abl activation is also played by cell to cell contact and stimulation with growth factor, like Platelet-Derived Growth Factor (PDGF), Epidermal Growth Factor (EGF), basic-Fybroblast Growth Factor (bFGF or Fgf2) (113). This finding support the idea that c-Abl could fulfill a crucial role during development. Indeed, c-Abl protein is involved in the development of many mammalian organ systems, including immune system and bones and exhibiting a pivotal role also in the regulation of hearth development. Indeed, *Abl* KO mice display dramatically enlarged hearts and die perinatally. Moreover, high levels of c-Abl are found in hyaline cartilage in the adult and *Abl* null mice are osteoporotic as a result of dysfunction in osteoblast functions (114,115).

2.5.3 PIN1 prolyl isomerase

As mentioned before, PTMs are known to act alone or in combinations to regulate protein functions. Moreover, it's also known that changes in protein conformation may facilitate specific post-translational modifications.

In literature, it's known that there are specific enzymes, called isomerases catalizing conformational change in substrate proteins. Among these enzymes a critical regulator of the p53 family members is PIN1 (116).

The human *PIN1* gene encodes an essential nuclear peptidyl-prolyl isomerase involved in the regulation of mitosis. PIN1 protein specifically catalyzes the cis/trans isomerization of phospho-Ser/Thr-Pro bonds and plays an important role in many cellular events through the effects of conformational change of its biological substrates (116). In particular, PIN1 is implicated in a large number of molecular processes related to human diseases, including cancer and Alzheimer's.

PIN1 contains an N-terminal WW domain and a C-terminal peptidyl-prolyl cis-trans isomerase (PPIase) domain connected by a flexible linker. The N-terminal WW domain of PIN1 is a small structural motif acting as a protein interaction module that binds to short proline-rich segments of target proteins. The WW domain of PIN1 mediates substrate recognition, interaction with anchoring proteins for subcellular localization and/or facilitation of nuclear import (117).

The C-terminal domain encodes an essential PPIase domain, which is an enzyme that catalyzes rotation around the peptide bond preceding a proline and may accelerate the folding and trafficking of some proteins.

It's well established that PIN1 plays a crucial role in the regulation of p53 and p73 stability.

Indeed, p53 physically interacts with PIN1 following DNA damage; this interaction requires a phosphorylation event on p53 at Ser33, Thr81, Pro82 mediated by different kinases including p38 MAPK, Cyclin Dependet Kinase 9 (CDK9) and Chk2 kinases. Thr81 and Pro82 are crucial sites through which PIN1 promotes Chk2-dependent phosphorylation of p53 on Ser 20, thereby stimulating dissociation of p53 from Mdm2 promoting p53 stabilization, activation of transcriptional activity triggering apoptotic function of p53 at mitochondria (118-123).

Moreover, PIN1 mediated isomerization of p53 promotes the phosphorylation of p53 on Ser20 by Chk2 essential for the p53-p300 interaction, required for p53 transcriptional activation. However, acetylation is not sufficient to activate the apoptotic function of p53 unless it dissociates from the apoptosis inhibitor iASPP. Several discoveries support a model in which PIN1 isomerase activity stimulates the release of iASSP inhibithor from p53. In this context, phosphorylation of Ser 46 is essential to allow PIN1 to trigger p53 dissociation from iASSP upon cytotoxic stress.Phosphorylation,of Ser46 upon DNA damage, could be mediated by Homeodomain Interacting Protein Kinase-2 (HIPK2) (Fig 20) (118) .

Ser46 phosphorylation is specifically induced by severe or persistent stress conditions and represents a major event in shifting the p53 response from cell-cycle arrest to apoptosis (123).

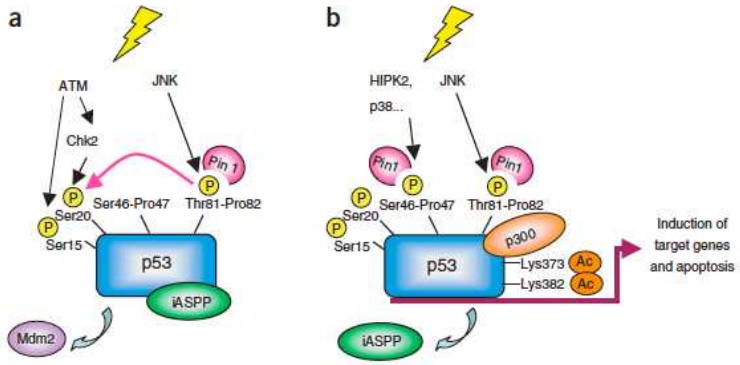

Figure 20. Model for regulation of p53 by Pin1 upon stress. (a) Upon DNA damage, Pin1-mediated prolyl isomerization of phospho-Ser/Thr-Pro sites (in particular Thr81-Pro82) in p53 favors Ser20 phosphorylation (P) by Chk2 and dissociation of p53 from Mdm2, promoting p53 stabilization. (b) In addition, Pin1 favors the binding of p53 to target promoters and the acetylation of DNA-bound p53 by p300. Upon phosphorylation of Ser46, Pin1 promotes dissociation of p53 from the apoptosis inhibitor iASPP (118).

PIN1 is a crucial regulator also for p73: PIN1 and p73 physical interact in a c-Abl-dependent manner: this interaction requires phosphorylation of p73 on tyrosine 99. Additional phosphorylation by the p38MAPK seems to further enhance p73–Pin1 interaction. Moreover, PIN1 increases the binding of p73 with p300 and stimulates subsequent acetylation by p300. PIN1 is an essential factor in determining p73 transactivation of genes encoding pro-apoptotic proteins. Specifically, siRNA-mediated downregulation of PIN1 abrogates the increases in the protein levels of PIG3 and BAX, crucial effectors for the apoptocic response, by p73 upon DNA damage, whereas trans-activation of the *p21* gene, involved in the regulation of cell-cycle, is unaffected (124).

All the knowledge on the regulation mediated by PIN1 on the p53 family members underlined that the presence of PIN1 is particularly important to induce an apoptotic response in response to DNA damage and stress conditions.

Despite lot of papers showed that PIN1 play a fundamental role in regulating pathway induced by DNA damage controlling the regulation of cell cycle progression and apoptosis, recently, it has been discovered that PIN1 could play an important role also during development. In particular, PIN1 controls the osteogenic activity of osteoblasts: *PIN1* -/- mice exhibited an age dependent decrease in bone mineral density and trabecular bone formation. Further analysis identified a defect in BMP signaling, essential for osteoblast commitment and differentiation, in *PIN1* -/- osteoblasts. These results suggest that PIN1 is also implicated in bone homeostasis suggesting that it might contribute to the pathogenesis of skeletal diseases (125).

This recent finding increases the number of proteins that could be substrates for PIN1, to proteins involved in limb and skeletal development.

So far, c-Abl, p300 and PIN1 have been linked in the same regulatory pathway, induced during DNA damage response, controlling p53 and p73 activity in order to guarantee a correct expression of pro-apoptotic genes and induction of the apoptotic response.

However, the characterization of the KO mice phenotypes for *p300*, *c-Abl* and *PIN1* suggests that these regulators play a crucial role also during development. In particular, the *c-Abl* and *p300* KO mice phenotypes are characterized by heart defects, resembling the defects observed in the *p63* KO mice phenotype. Moreover, the *c-Abl* and the *PIN1* KO phenotype indicate a crucial role for these proteins in the regulation of skeletal development.

All these evidences suggested that also the p63 protein activity could be influenced by these three regulators and, furthermore, that these factors could be linked together in the same pathway during development.

3. Aim of the work and main results

In the last few years a lot of studies have been centered on the identification of the p63 target genes in order to understand the complex networks of gene regulation governing the development of epithelia (18-20, 126, 127). However, little is known on the post-translational modification and the upstream signaling pathway regulating p63 function and stability.

As mentioned in the previous paragraphs, the p53 family members are largely regulated by a huge numbers of PTMs that finely tune their activation, stability and transcriptional activity in order to guarantee correct expression of target genes.

Despite the identification of some PTMs regulating p63 protein stability upon DNA damage and keratinocyte differentiation, little is known on the upstream pathway and the consequent PTMs controlling p63 stability and function during development (127-130).

In this context, my PhD thesis aimed at the identification of the upstream signaling pathway involved in the regulation of p63 activation and function that could be relevant during limb development. Indeed p63, and in particular the ΔNp63α isoform, is a crucial transcription factor required during development of epithelial tissues and limb: the discovery of the molecular mechanisms and post-translational modifications controlling p63 functions and activities are crucial to understand which are the cause and the alteration leading to malformation in syndromes where *p63* is the disease gene.

It's well known that some of the natural *p63* mutations, associated to developmental syndromes and malformations, occur on residues that could be post-translationally modified, like lysines and tyrosines. The recent identification of a new lysine acetylated in p53 (K164) (94) corresponding to ΔNp63αK193, mutated into glutamic acid (K193E) in patient affected by SHFM-IV, prompted us to verify

if acetylation could be a crucial PTM required for ΔNp63α activation during development.

My PhD thesis has been centered on uncovering which are the biological consequences of ΔNp63α acetylation and the identification of the upstream signaling pathway leading to this modification. Moreover, particular attention was placed on the analysis of the alteration caused by the ΔNp63αK193E mutation in human cells, with the aim to understand the molecular mechanisms at the basis of SHFM-IV pathogenesis.

Data collected during my PhD show that p300 acetylates a p63 peptide centered on lysine K193, *in vitro*. Furthermore, p300 physically interacts with ΔNp63α and contributes to its stabilization, by increasing its protein half-life, and transcriptional activation in U2OS and HaCat cells. The activity of the tyrosine kinase c-Abl is required for ΔNp63α-p300 physical interaction and the consequent stabilization mediated by p300.

Then, I tried to identify which could be the upstream signal that could activate this regulatory loop; in literature it's known that c-Abl is activated by bFGF treatment in human cells. First of all, I verified the effects of bFGF treatments, in U2OS and HaCat cells, on ΔNp63α protein stability. I found that the ΔNp63α isoform is stabilized upon bFGF treatment; then I focused my attention on FGF8, a crucial signaling molecule essential for limb outgrowth and correct development along the proximo-distal axis. Indeed, I found that treatments with FGF8 induced ΔNp63α stabilization in both U2OS and HaCat cells. Moreover, in collaboration with the laboratory of Professor G. Merlo (Università degli Studi di Torino), I confirmed that FGF8 treatments determine an increased in ΔNp63α protein levels also in mice limbs put in culture *ex vivo,* suggesting that this regulatory pathway could be acting during limb development.

Furthermore, FGF8 treatment in HaCat cells, stimulates c-Abl-ΔNp63α interaction, which in turns promotes the physical interaction between ΔNp63α and p300

leading to ΔNp63α acetylation. These data demonstrate that FGF8, c-Abl and p300 are linked together in a regulatory cascade controlling p63 stability.

Importantly, this regulatory cascade is not active on the ΔNp63αK193E mutant: indeed this mutant is not stabilized by FGF8 treatment or by p300 over-expression. Furthermore, this mutant has lost the ability to activate target genes involved in development and apoptotic response, while it behave as wild-type ΔNp63α on genes involved in cell-cycle control. This alteration is caused by a loss of DNA binding capacity of the ΔNp63α natural mutant to promoters of genes involved in the regulation of developmental processes or apoptotic response, while it retains its ability to bind promoters of genes connected to cell-cycle regulation.

These results suggest that the natural mutation ΔNp63αK193E alters the regulation induced by FGF8, which leads in normal conditions to the acetylation and transcriptional activation of wild-type ΔNp63α. It seems that the K193E mutations occurs on a crucial acetylation site for the ΔNp63α isoform required for the its efficient binding to genes controlling development.

Another project that I've exploited is focused on the molecular mechanisms regulating ΔNp63α stability and function during limb development. As described in the previous paragraphs ΔNp63α, together with the transcription factors DLX5 and DLX6, is expressed in the AER, an ectodermal structure required for correct limb outgrowth. Mutations in these genes, or alterations in the mechanisms regulating their expression or functions are at the bases of SHFM pathogenesis. In this context a tight regulation of ΔNp63α protein levels and activities is required to assure proper limb development.

We have found that FGF8 and PIN1 are important regulators of ΔNp63α protein stability. *In vitro*, PIN1 induces ΔNp63α degradation through the proteasome machinery while, *in vivo*, in KO *PIN1* mouse models, induces the accumulation of ΔNp63α in the embryonic limbs and ectoderm. On the other hand FGF8 treatment promotes ΔNp63α stabilization and activation by inhibiting the ΔNp63α-PIN1

interaction. Moreover, we found that *FG8* is a downstream target of the transcription factor Dlx5. Indeed, the limb buds of both *p63* and *DLX5;DLX6* KO mice, display poor stratification of the AER and *FGF8* expression is severely reduced. Therefore, DLX5, ΔNp63α, FGF8 and PIN1 participate in a time and location restricted regulatory loop essential for AER stratification, normal patterning and skeletal morphogenesis of the limb buds (1).

4. Conclusions and future perspectives

In conclusion, the work performed during my PhD contributes to a better understanding of the regulatory mechanisms controlling ΔNp63α function and stability in human osteosarcoma and keratynocyte cells. We have identified FGF8 as a crucial upstream signal required for ΔNp63α activation and stabilization during limb development: mutations or altered expression of regulators in this pathway leads to abnormal limb development and onset of pathogenesis. In particular we found that the signaling pathway induced by FGF8 treatments leading to ΔNp63α acetylation and transcriptional activation is not active on the ΔNp63αK193E mutant. Indeed, this mutation caused a deep change in the pattern of ΔNp63α target genes involved in limb development. These results shed new light on the molecular mechanism that could be at the bases of SHFM-IV pathogenesis.

It will be interesting study in more detail, if p300 is specifically required for the induction of genes required for limb development and in particular, if it is present together with ΔNp63α, on the promoter of target genes that need to be activated to guarantee correct limb development by performing Chromatin Immunoprecipitation (ChIP) experiments.

Intriguing and distinctly results on the role of p300 in limb development could be also obtained by *in vivo* experiments generating conditional *p300* KO mouse at the

time of limb bud induction and outgrowth in order to asses if p300 expression is essential together with p63 to guarantee correct limb outgrowth.

Indeed this aspect has never been studied carefully in literature. All the data on the implications of *p300* expression on development came from the analysis of the null mice phenotype, but since this protein plays crucial functions in a large number of cellular processes and it is widely expressed in embryos, the *p300* KO mice is embryonic lethal.

All these discoveries will contribute to understand and better characterize which are the molecular pathway necessary for the correct limb bud initiation and outgrowth. Indeed a clearer knowledge on the regulators and on the upstream pathway acting on p63 is essential to understand which are the molecular alterations eliciting pathogenesis.

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PART II

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DLX5, FGF8 and the *Pin1* isomerase control ΔNp63α protein stability during limb development: a regulatory loop at the basis of the SHFM and EEC congenital malformations

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Ectrodactyly, or Split-Hand/Foot Malformation (SHFM), is a congenital condition characterized by the loss of central rays of hands and feet. The p63 and the *DLX5;DLX6* transcription factors, expressed in the embryonic limb buds and ectoderm, are disease genes for these conditions. Mutations of *p63* also cause the ectodermal dysplasia–ectrodactyly–cleft lip/palate (EEC) syndrome, comprising SHFM. Ectrodactyly is linked to defects of the apical ectodermal ridge (AER) of the developing limb buds. FGF8 is the key signaling molecule in this process, able to direct proximo-distal growth and patterning of the skeletal primordial of the limbs. In the limb buds of both *p63* and *Dlx5*;*Dlx6* murine models of SHFM, the AER is poorly stratified and *FGF8* expression is severely reduced. We show here that the *FGF8* locus is a downstream target of DLX5 and that FGF8 counteracts Pin1–ΔNp63α interaction. *In vivo*, lack of *Pin1* leads to accumulation of the p63 protein in the embryonic limbs and ectoderm. We show also that ΔNp63α protein stability is negatively regulated by the interaction with the prolyl-isomerase Pin1, via proteasome-mediated degradation; p63 mutant proteins associated with SHFM or EEC syndromes are resistant to Pin1 action. Thus, DLX5, p63, Pin1 and FGF8 participate to the same time- and location-restricted regulatory loop essential for AER stratification, hence for normal patterning and skeletal morphogenesis of the limb buds. These results shed new light on the molecular mechanisms at the basis of the SHFM and EEC limb malformations.

INTRODUCTION

The *p63* gene codes for a transcription factor related to the *p53* and *p73* tumor suppressor genes, proposed as a master regulator of epidermal stem cell maintenance and proliferation, able to promote the epithelial stratification program typical of the mammalian skin. To date, several mutations in the *p63* gene have been identified associated with distinct human developmental syndromes, characterized by common features such as limb abnormalities, ectodermal dysplasia, and facial clefts (1–4). These syndromes are: the ectodermal dysplasia–ectrodactyly–cleft palate (EEC, MIM #129900), the ankyloblepharon–ectodermal

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dysplasia–clefting (AEC, MIM #106260), the limb–mammary syndrome (LMS, MIM #603543), the acro–dermato–ungual–lacrimal–tooth (ADULT, MIM #103285) and non-syndromic split-hand/foot malformation type-4 (SHFM-IV, MIM #605289) (1–4).

p63 homozygous mutant mice show severe defects affecting their limbs, skin and craniofacial skeleton (5–7). In $p63^{-/-}$ newborn animals, the hindlimbs (HLs) are absent whereas the forelimbs (FLs) are severely truncated in their distal segment. The limb defects of $p63^{-/-}$ mice have been associated with failure of stratification and signaling of the cells of the apical ectodermal ridge (AER), a transitory specialization of the ectoderm at the dorsal–ventral border of the limb bud, essential for proximo-distal growth of the limbs and patterning of the fingers (8–10). *p63* is expected to control AER function and maintenance via transcriptional regulation of AER-restricted target genes (2,11,12). Failure of AER stratification has also been associated with loss of expression of key morphogens for limb development, such as *FGF8* and *Dlx* genes (2).

Within the EEC disease phenotype, ectrodactyly (also known as SHFM, MIM #183600) is a recurrent finding and consists in the absence of the distal portion of the central rays of upper and lower limbs, resulting in a deep medial cleft, missing or hypoplastic central fingers and fusion of the lateral ones. In addition to be part of the EEC syndrome, SHFM comprises both sporadic and hereditary forms, syndromic or isolated, linked to six distinct loci (types I–VI) (2,13–15). The most common form, SHFM type-I, is associated with deletions of variable extent on chromosome 7q21, the minimal common deletion includes *DSS1* and the homeogenes *DLX5* and *DLX6* (16,17). 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 (18). In the mouse, the double knockout (DKO) of *Dlx5* and *Dlx6* leads to an ectrodactyly phenotype affecting the HLs (19,20), fully confirming that the human orthologs *DLX5* and *DLX6* are the disease genes for this malformation.

SHFM type-IV (MIM #605289) is caused by mutations in the *p63* gene. In 50 unrelated patients with isolated SHFM, 5 mutations in *p63* were found, suggesting that these may account for ~10% of sporadic cases of SHFM (1,4,21). Finally, SHFM type-III (MIM #246560) is linked to abnormalities of a genomic region comprising *dactylin* and several other genes, in mice and man; however, no disease gene has convincingly been demonstrated, as to date (22–24). Notably, the *FGF8* locus resides in the SHFM-III region; thus, this gene may represent a valid candidate for SHFM type-III (2).

Several studies have attempted to define p63-dependent transcription regulatory networks (25,26) with the hope to identify core genes and regulation at the basis of normal ectoderm development and differentiation, as well as to provide clues on the molecular bases of the ectodermal phenotypes in the EEC. Specifically, ectrodactyly has been linked to the ability of p63 to regulate transcription of *Dlx5* and *Dlx6*, both *in vitro* and in the developing embryonic limbs (7). This regulation takes place both at the proximal promoter level and via a conserved *cis*-acting genomic element, located ~250 kb centromeric to *DLX5*, that is deleted in a family with SHFM type-I (25). Thus, the *Dlx5* and *Dlx6* genes are true p63 transcriptional targets, whose regulation during limb development is presumably needed to maintain the specialization and stratification of the AER cells (2,7).

While the pathways downstream of p63 are beginning to be elucidated, our knowledge on the upstream regulations of p63 is minimal. The main questions that arise are: how is p63 expression maintained in (proliferating) ectodermal stem cells? How is p63 down-modulated in differentiating cells? How are changes in p63 level linked to loss of AER stratification and the onset of the SHFM phenotypes? Recently, one such regulation has been identified and consists in a loop-like regulation between p63 and IRF6 (27). Several biochemical observations suggest that the ΔN- and TA-p63 proteins are tightly regulated at post-translational level, via protein modification (phosphorylation, sumoylation and ubiquitination) and protein–protein interactions (28–30).

Here we show that the prolyl *cis/trans* isomerase Pin1 acts an additional regulator of p63 protein stability, inducing a phosphorylation-dependent, proteasome-mediated degradation of wild-type (WT) ΔNp63α, but not of a disease-causing p63 mutant. Conversely, FGF8 appears to counteract Pin1-induced p63 degradation, and thus to promote p63 stability by inhibiting the interaction of ΔNp63α with Pin1, *in vivo*. As the *FGF8* locus appears to be regulated by both p63 and DLX5, we propose a model in which these two SHFM-causing genes and *FGF8* take part in a regulatory loop that opposes Pin1-mediated degradation of p63, hence permitting stratification and specialization of ectoderm cells into the AER, in a time and region-restricted manner during limb development. In SHFM type-I, type-III and type-IV, such regulation is impaired leading to reduced AER stratification, limb malformation and skeletal defects.

RESULTS

The AER of *Dlx5;Dlx6* DKO embryos is poorly stratified

Mutations in *DLX5* or complex genomic alterations around the *DLX5;DLX6* locus cause, in human, the malformation known as SHFM type-I (16,18). In mice, the combined deletion of *Dlx5* and *Dlx6* (*Dlx5;Dlx6* DKO) causes a limb phenotype identical to human SHFM-I and is accompanied by reduced expression of *FGF8* in the AER cells (19,20). Thus, the *Dlx5;Dlx6* DKO mice represent a valid animal model for SHFM-I, and we set forth to use them to examine whether the loss of *Dlx5;Dlx6* may result in altered AER stratification.

We stained sections of WT and *Dlx5;Dlx6* DKO HL, at the ages E11.5 and E12.5, with anti-E-cadherin antibody and examined the stratification of the AER cells. We specifically focused on the central wedge of the AER, because previous publications have indicated that only this wedge loses expression of *FGF8*, *Bmp4*, *Msx2* and *Dlx2* (2,19,20,31 and our unpublished data). At earlier ages (E11.5), the organization of the *Dlx5;Dlx6* mutant AER appeared very similar to the WT (not shown), whereas at later ages (E12.5), the central wedge of the *Dlx5;Dlx6* mutant AER appeared less stratified compared with the equivalent region of the normal limbs (sectors 2 and 3, Fig. 1B). Notably, in more lateral regions of the AER (sector 1), the stratification was normal.

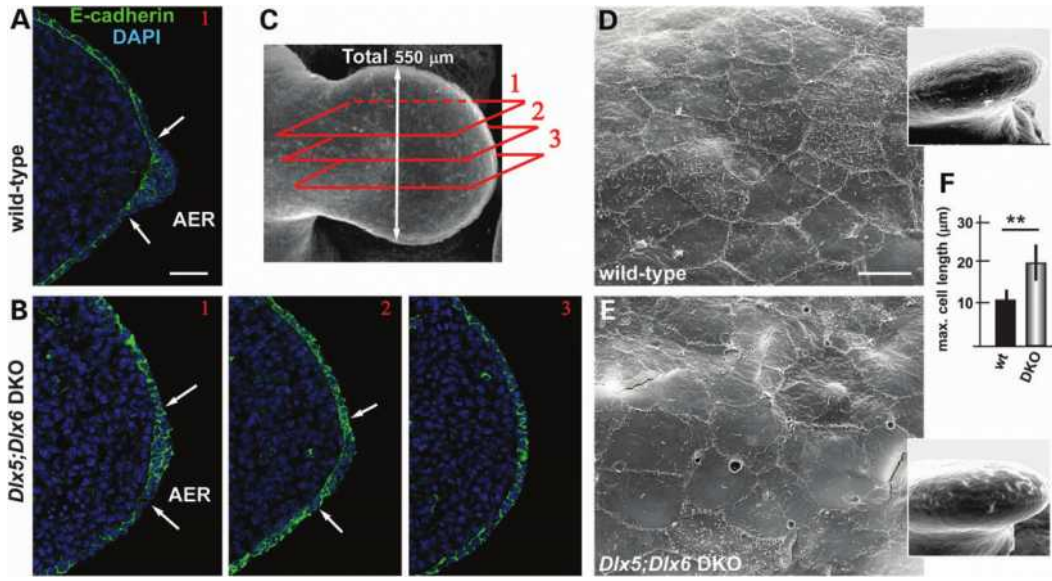

Figure 1. Impaired AER stratification in the limbs of *Dlx5;Dlx6* DKO mutants. (**A**–**C**) Immunofluorescent staining on WT and *Dlx5;Dlx6* DKO HLs, to detect E-cadherin (green), on serial transverse sections of the limbs, at E11.5. White arrows indicate the extension of the AER ectoderm. The position of the section planes of the micrographs (in A and B) along the anterior-to-posterior (1, 2, 3) are show in C. Scale bar in A = 20 μm. (**D**,**E**) SEM of the surface of the AER cells of WT (D) and *Dlx5;Dlx6* DKO (E) limbs. The mutant AER cells appear larger, show fuzzy borders and nearly lack microvilli. Scale bar in D = 10 μm. (**F**) Quantification of the size of AER cells (maximum cell length) comparing WT versus *Dlx5;Dlx6* DKO mutant specimens. The WT was used for normalization and made to 1. $P < 0.02$.

To further document this finding, we carried out scanning electron microscopy (SEM) on the AER of normal and *Dlx5;Dlx6* DKO limbs at the age E12.5 and observed that the cells of the central wedge of the *Dlx5;Dlx6* mutant AER appeared morphologically abnormal, with an increase in the length of the maximum diameter (10.8 ± 2 versus 19.7 ± 4.3; $P < 0.03$), more irregular borders and fewer microvilli on the apical surface (compare Fig. 1E with D).

Thus, between E11.5 and E12.5, in the absence of *Dlx5* and *Dlx6*, the central wedge of the AER fails to specialize into a pluristratified epithelium. Notably, we and others have previously shown that the AER of p63-null and of p63-R279H homozygous mutant limbs is poorly stratified, and this is accompanied by reduced *FGF8* expression and the appearance of severe limb defects (5–7).

The AER of *Dlx5;Dlx6* DKO limbs shows reduced levels of ΔNp63α

We decided to further investigate the molecular link connecting *Dlx5;Dlx6* and p63 in the embryonic limbs. We previously established that the expression of *ΔNp63* and *TAp63* mRNAs is not significantly changed in the *Dlx5;Dlx6* DKO limbs (7), and we also excluded changes in *Pin1* mRNA or protein levels (Supplementary Material, Fig. S1). Thus, we ruled out a direct transcriptional regulation for the observed reduction of p63 in the *Dlx5;Dlx6* DKO limb buds (Fig. 2) and opted for a post-transcriptional type of regulation.

Considering that p63 is essential for stratification of ectoderm-derived epithelia, we set forth to determine whether the absence of *Dlx5;Dlx6* may lead to altered levels of p63 protein in the AER cells of the developing limb buds. We carried out immunostaining for p63 on serial sections of the HLs, focusing on the central wedge of the AER, and semi-quantified the signal intensity along the anterior-to-posterior length of the limb bud (schemes in Fig. 2D and E). In the AER nuclei of the central wedge (sectors 2 and 3, Fig. 2B), p63 immunostaining is significantly reduced as compared with the same region of the normal limb (sector 2, −50%; sector 3, −65%), whereas no such difference was observed in lateral wedges of the AER (sector 1) or in the non-AER ectoderm (Fig. 2A and B, quantification in C).

Thus, p63 is down-regulated in the central AER cells by post-transcriptional mechanisms, at the same time as these cells fail to efficiently stratify. As p63 has been directly linked to ectodermal stratification (11), we can hypothesize that the mis-organization of the AER cells in *Dlx5;Dlx6* DKO limbs might be the consequence of altered p63 levels.

Pin1 interacts with ΔNp63α and promotes its destabilization

The stability of the p63 protein is tightly regulated by the action of several interacting or modifying proteins, including MDM2 and p53 (28–30). The enzyme peptidyl-prolyl *cis/trans* isomerase NIMA-interacting-1, Pin1, has been shown to modulate the activity of p53 and p73 by post-translational modifications (32–36). We therefore examined the possibility that Pin1 may

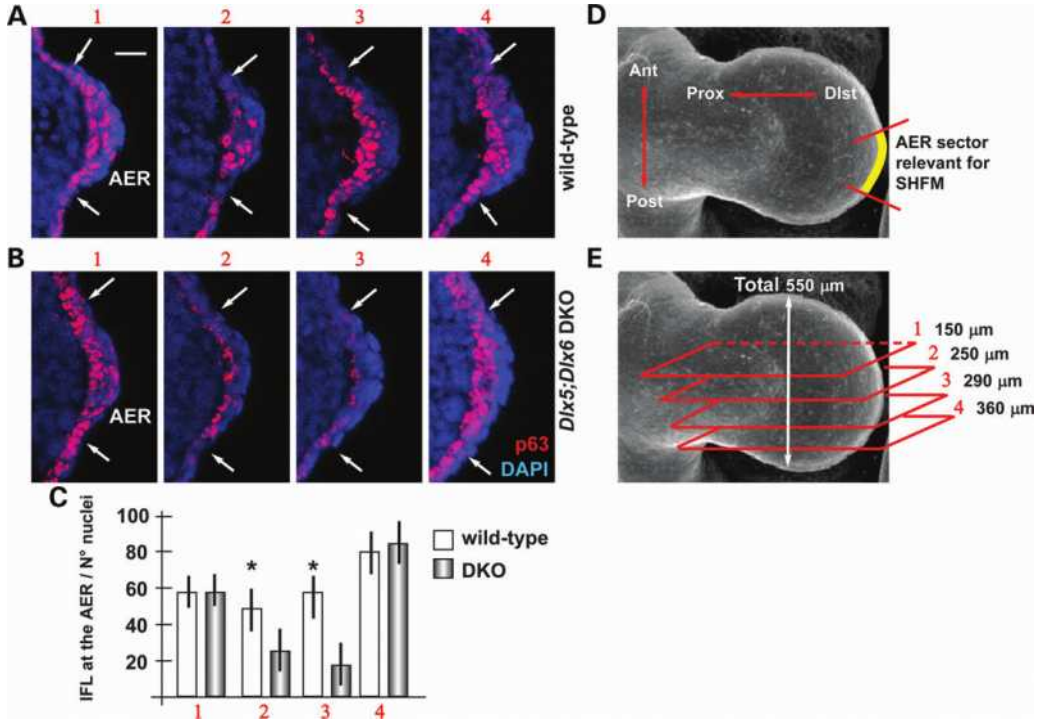

Figure 2. p63 protein level is reduced in *Dlx5;Dlx6* mutant AER cells. Immunofluorescent detection of p63 in the nuclei of the AER of WT and *Dlx5;Dlx6* DKO limbs, at the age E11.5. (**A**,**B**) Micrographs of WT (panels in A) and *Dlx5;Dlx6* mutant (panels in B) sections, corresponding to the positions (1, 2, 3 and 4) indicated in **E**. White arrows indicate the extension of the AER. Scale bar in A = 20 µm. (**C**) Semi-quantitative assessment of p63 signal in the AER of WT (open bars) and *Dlx5;Dlx6* mutant (gray bars), expressed as intensity per 100 nuclei, in arbitrary units. The results show that p63 staining is significantly reduced (−50%) in the positions 2 and 3 of the AER of the mutant limbs, relative to the WT. (**D**,E) Schemes illustrating the wedge of the AER relevant for the SHFM phenotype (yellow in D) and the position of the section planes (1, 2, 3 and 4) along the anterior-to-posterior axis (red rectangles in E). The numbers on the left of each section plane indicate the distance from the anterior margin.

also regulate ΔNp63α protein level and consequently modulate its activity, by a similar mechanism. Notably, previous experiments have shown a diminished transcription of two p63 targets in Pin1-overexpressing cells *in vitro* (37), suggesting that Pin1 could modulate p63 protein levels and/or activities.

To verify this point, we adopted an siRNA-based approach to down-regulate endogenous Pin1 expression in the U2OS human osteosarcoma cell line, which does not express *p63* endogenously. We transfected the U2OS cells with ΔNp63α and an anti-Pin1 siRNA. The depletion of Pin1 resulted in a significant stabilization of ΔNp63α, as compared with control-silenced cells (Fig. 3A). Conversely, overexpression of Pin1 resulted in a marked and dose-dependent reduction of ΔNp63α protein levels (Fig. 3B). The same experiment was performed on the A431 human epidermoid squamous carcinoma cell line, which expresses ΔNp63α endogenously and yielded similar results (data not shown).

We then tested whether disease-causing mutant p63 proteins are sensitive to the degrading action of Pin1, by transfecting expression vectors carrying the L518F (linked to AEC syndrome), the ΔAA (linked to LMS syndrome) or the E639X (linked to SHFM-IV syndrome) point-mutated variants. While the AEC mutant p63 protein was still sensitive to Pin1-induced degradation, the LMS and SHFM mutants were more resistant to such effect (Fig. 3C). Interestingly, while the AEC syndrome is not associated with limb developmental defects, the LMS and SHFM syndromes typically entail an ectrodactyly phenotype.

We then tested whether the effect of Pin1 on p63 might be mediated by the proteasome. U2OS cells were co-transfected with a Pin1 and a ΔNp63α expression vectors and then treated with the proteasome inhibitor MG132; we observed that the Pin1-induced depletion of ΔNp63α was partially reversed in MG132-treated cells, compared with controls (Fig. 3D), suggesting that Pin1-induced p63 protein destabilization is in part mediated by the proteasome.

We then examined whether Pin1 and p63 proteins physically interact *in vivo*. We carried out co-ImmunoPrecipitation (co-IP) experiments in HaCaT cells, which express both proteins endogenously, using anti-Pin1 and anti-p63 antibodies. co-IP with the anti-p63 antibody was able to pull down Pin1, and vice versa, indicating that these two proteins interact, either directly or indirectly via complex formation (Fig. 3E). Pin1 is known to interact with its partner proteins in a phosphorylation-dependent manner and to catalyze *cis/trans* isomerization of

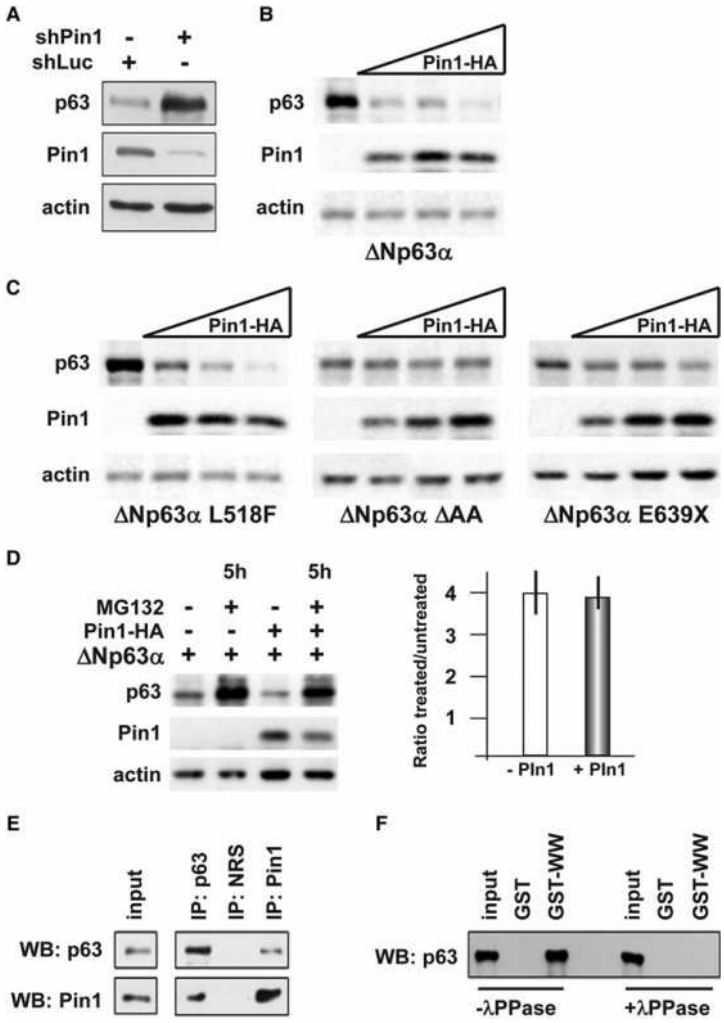

Figure 3. The Pin1 isomerase promotes ΔNp63α degradation. (**A**) Western blot analyses of whole protein extracts of U2OS cells transiently co-transfected with ΔNp63α (50 ng) and an anti-Pin1 siRNA (10 pmol/cm^2), or an anti-LacZ siRNA (siCtr 10 pmol/cm^2) as negative control. Actin is used for loading control. (**B**) Western blot analyses of whole protein extracts from U2OS cells transiently co-transfected with increasing amounts (20, 40 and 80 ng) of Pin1–HA-tagged vector (indicated on top), and WT ΔNp63α (30 ng). The α-isoform is efficiently degraded by Pin1 expression, whereas the β- and the γ-isoforms are not (Supplementary Material, Fig. S1). (**C**) Western blot analyses of whole protein extracts from U2OS cells transiently co-transfected with increasing amounts (20, 40 and 80 ng) of Pin1–HA-tagged vector (indicated on top), and the disease-relevant mutated versions of ΔNp63α L518F (AEC associated), ΔAA (LMS associated) and E639X (SHFM associated) (30 ng each) (indicated at the bottom). Actin is used for loading control. The mutant p63 proteins linked to congenital limb malformations (LMS and SHFM) are relatively resistant against Pin1-induced degradation, compared with WT p63, whereas the mutant p63 linked to AEC, with no limb defects, is sensitive to Pin1. (**D**) Western blot analyses of whole protein extracts from U2OS cells, transiently co-transfected with ΔN-p63α and Pin1–HA-tagged expression vectors, and 20 h later either treated with 5 μM of the proteasome inhibitor MG132 or left untreated (DMSO only). Proteins were extracted after 5 h of treatment and assayed by western analysis; actin is used for loading control. On the right, quantification of the p63 protein level, expressed as the ratio between the treated and the untreated sample, in cell transfected (gray bar) or not transfected (open bar) with Pin1. The results show that Pin1-mediated destabilization of p63 is mainly mediated by the proteasome. (**E**) Western blot analyses of proteins immunoprecipitated with anti-p63 (IP p63) or anti-Pin1 (IP Pin1) antibodies, revealed using, respectively, anti-Pin1 (bottom, lanes) and anti-p63 (top panel). Input control is shown on the left. (**F**) Western blot analysis of GST-pull down assay done with anti-Pin1, revealed with anti-p63 antibody, in the presence (+λPPase, on the right) or absence (−λPPase, on the left) of protein phosphatase during the pull-down. The input sample is also loaded as control. While untreated samples contained p63, in the presence of IPP the p63 protein is absent.

selected peptide bonds (38). Therefore, we tested whether the Pin1–p63 interaction might depend on phosphorylation. We transfected the U2OS cells with ΔNp63α and then detected the interacting proteins by using GST-WW (or GST as a control), in the presence or absence of λ-phosphatase, as previously described (39). Treatment of the cell lysates with λ-phosphatase resulted in a loss of the interaction between Pin1 and p63 (Fig. 3F), suggesting that this interaction requires a phosphorylation event.

Absence of *Pin1* results in increased levels of ΔNp63α protein *in vivo*

We next sought evidence that Pin1 regulates p63 protein levels during embryonic development, and specifically in the embryonic ectoderm. We collected samples of ectoderm and limb buds from E11.5 WT and *Pin1*$^{-/-}$ embryos (40,41) and stained sections with an anti-p63 antibody that recognizes all p63 isoforms. Of note, the ΔNp63α isoform is the most abundantly expressed in the limb buds at this age (7). The results show that the intensity of p63 immunostaining in the nuclei was increased both in the AER (Fig. 4B) and the non-AER (Fig. 4D) ectoderm of *Pin1*$^{-/-}$ embryos, 4- and 3-folds, respectively, relative to the WT (Fig. 4A and C).

As p63 IFL signal was found to be reduced in the *Dlx5;Dlx6* DKO limbs (Fig. 2), we asked whether this was due to increased Pin1 expression, either mRNA or proteins. Thus, we stained adjacent sections of WT and *Dlx5;Dlx6* DKO limbs with anti-Pin1 antibody, but no significant difference in the Pin1 signal was detected in the mutant limbs (Supplementary Material, Fig. S2). Antibody specificity was confirmed by complete lack of staining in *Pin1* KO embryonic limbs. We also determined the mRNA abundance of *Pin1* mRNA in RNA extracted from WT or *Dlx5;Dlx6* DKO limbs, by Real-Time qPCR, but again could not detect any significant differences (Supplementary Material, Fig. S2). Thus, we concluded that the reduced p63 levels observed in the *Dlx5;Dlx6* DKO AER cells are unlikely to be due to changes in Pin1 level.

FGF8 is downstream of *DLX5* and counteracts Pin1-dependent degradation of p63

We decided to further investigate the molecular link connecting *Dlx5;Dlx6* and p63 in the embryonic limbs. We previously established that the expression of *ΔNp63* and *TAp63* mRNAs is not significantly changed in the *Dlx5;Dlx6* DKO limbs (7), and we also excluded changes in *Pin1* mRNA or protein levels (Supplementary Material, Fig. S1). Thus, we excluded a direct transcriptional regulation for the observed reduction of p63 in the *Dlx5;Dlx6* DKO limb buds (Fig. 2) and opted for a post-transcriptional type of regulation.

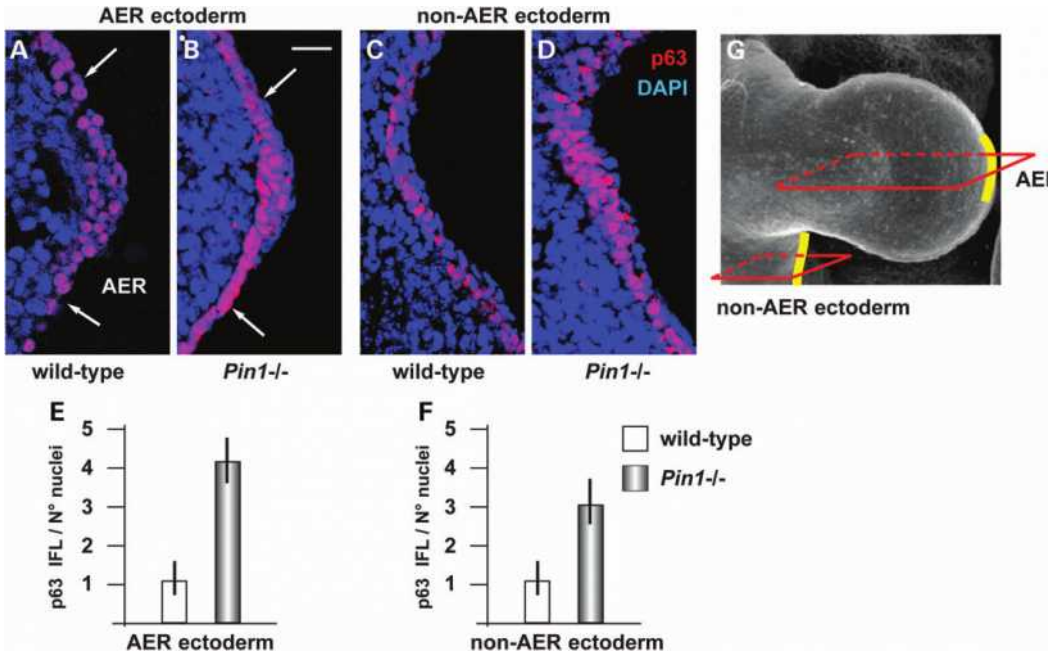

Figure 4. Loss of Pin1 causes stabilization of p63 in the embryonic ectoderm. (**A**–**D**) Immunofluorescent detection of p63 on the AER ectoderm (A,B) and the non-AER ectoderm (C,D) of WT (A,C) and Pin1 KO (B,D) embryonic limbs, at the age E11.5. White arrows in A–C indicate the extension of the AER ectoderm. IFL signal is specifically detected in the nuclei. (E,F) Semi-quantitative assessment of p63 signal in the AER (**E**) and the non-AER ectoderm (**F**), expressed as intensity per 100 nuclei, in arbitrary units. Wild-type was normalized and made to 1. In the absence of Pin1, the p63 signal is increased ∼4-folds in the AER, and ∼3-folds in the general ectoderm. (**G**) A scheme to illustrate the approximate position of the section planes in A–D, along the anterior-to-posterior axis. The AER and non-AER ectoderm are highlighted in yellow. Scale bar in B = 20 μm.

In the AER of both *Dlx5;Dlx6* DKO and *p63* mutant limbs, expression of *FGF8* is reduced, as revealed by *in situ* hybridization (5,6,19). FGF8 is a well-known limb morphogenetic diffusible factor, essential for the maintenance of AER stratification, for limb growth and morphogenesis (42–44), and the partial or complete absence of members of the FGF family causes a set of developmental limb defects (42,45). Thus, we focused on FGF8 and raised the hypothesis that Dlx5 and/or p63 may concur to positively regulate *FGF8* transcription (2).

First, we quantified the expression of *FGF8*, *Pin1* and *ΔNp63* in the HL and FL of WT and *Dlx5;Dlx6* mutant embryos, by Real-Time qPCR, and observed that *FGF8* expression in the mutant limbs is reduced by 40%, compared with the normal limbs (Fig. 5A), whereas expression of *Pin1* and of *p63* did not significantly change. This result confirms previous *in situ* hybridization data showing reduced expression of *FGF8* in the central wedge of the AER of *Dlx5;Dlx6* DKO HLs (19) (our unpublished data). Notably, a reduction of *FGF8* mRNA abundance was also seen in the embryonic FLs of *Dlx5;Dlx6* DKO embryos, showing no evident developmental defects, suggesting that the down-modulation of *FGF8* is not the mere consequence of cell suffering, but more likely a transcriptional misregulation occurring in both the HLs and the FLs.

The *FGF8* locus lies in a genomic region that has previously been implicated in the SHFM type-III malformation in human, and to an ectrodactyly phenotype in the *dactylaplasia* mouse strain (22,23). Currently, the disease gene causing this malformation is uncertain, but there is evidence suggesting that the underlying genetic mechanism is a genomic position effect. p63 binding sites have been detected in this region, via ChIP-seq experiments on human keratinocytes (25). We searched the *FGF8* locus and flanking regions for the presence of predicted Dlx5 binding sites, conserved between mouse and human, using a position–weight matrix (PWM) approach (31,46), and detected four such sites (Fig. 5B, and Supplementary Material, Fig. S3). To verify whether DLX5 physically interacts with these genomic elements, we carried out ChIP analyses on two of these regions, named DBE-1 and DBE-2, located, respectively, ∼1 kb downstream of *FGF8* (DBE1) and within the first intron of *dactylyn* (DBE2) (Fig. 5B). We transfected U2OS cells with the *DLX5-myc-tag* expression vector, and with an empty vector as control, and then immunoprecipitated the chromatin with anti-myc-tag.

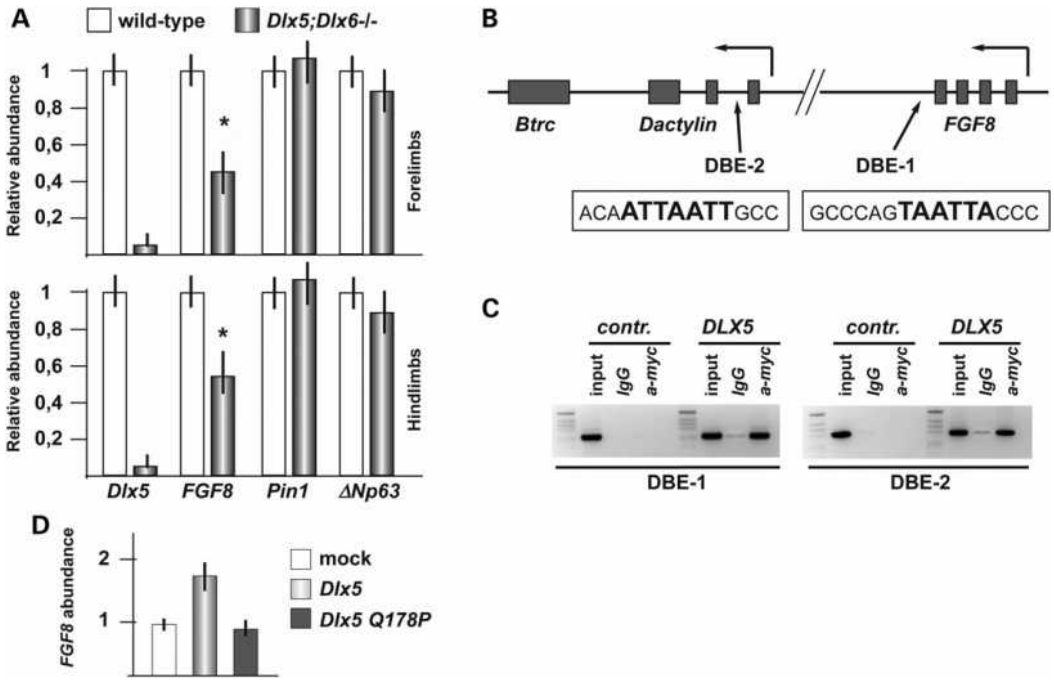

Figure 5. FGF8 is regulated by Dlx5. (**A**) Relative abundance of FGF8, Pin1 and ΔN-p63 mRNAs in samples from WT (gray bars) and *Dlx5;Dlx6* DKO (black bars) HLs and FLs. Values are expressed relative to the WT specimen, which was set to 1. The Dlx5 mRNA level is used to confirm the mutant genotype. Experiments were done on independent biological duplicates. (**B**) Scheme of the FGF8 and Dactylyn loci, showing the SHFM-III critical region and the approximate position of the two best predicted Dlx5 sites DBE-1 and DBE-2, indicated with black arrows (31). The exact (human or mouse) sequence corresponding to the DBE-1 and -2 sites is reported in open boxes; the bold characters represent the Dlx5 PWM (46). Solid boxes represent the exons. The position of p63 binding sites [from (25)] is reported in Supplementary Material, Fig. S3. (**C**) ChIP analysis on the chromatin of U2OS cells, transfected with DLX5-myc-tagged and immunoprecipitated with anti-myc. The DBE-1 (left) and DBE-2 (right) elements were amplified by PCR. Enrichment is detected in cells transfected with DLX5, compared with mock-transfected cells, or with chromatin precipitated with an irrelevant antibody (IgG). Input chromatin is shown of the left of each blot. (**D**) Relative abundance of endogenous FGF8 mRNA upon transfection of U2OS cells with the WT (light gray bar) or the Q178P mutant (dark gray bar) DLX5-HA expression vectors. Values are expressed relative to sample from control transfected cells (open bar), set to 1.

The results show an enrichment of the DBE-1 and DBE-2 elements in the presence of DLX5-myc protein, compared with the negative controls (Fig. 5C).

Next we asked whether overexpression of *DLX5* in U2OS cells (not expressing *DLX5* endogenously) indeed resulted in higher *FGF8* mRNA levels. We carried out Real-Time qPCR on RNA extracted from U2OS cells transfected with either the WT *DLX5* or the Q178 mutant *DLX5* expression vectors and measured the abundance of endogenous *FGF8* mRNA. Expression of WT DLX5 resulted in a 1.7-fold increase in *FGF8* mRNA, whereas the mutant DLX5 protein had a minimal effect on FGF8 expression levels (Fig. 5D). Together, all these data indicate that *FGF8* is a transcriptional target of DLX5 and that the SHFM-linked DLX5-Q178P mutant, linked to SHFM-I, looses the capacity to efficiently activate *FGF8* expression.

Next we investigated by which mechanism FGF8 participates in Dlx5-Pin1-p63 regulatory loop, by investigating the effect of FGF8 on the stability/degradation of p63. We transfected U2OS cells with the *Pin1-HA-tag* expression vector and then treated the cells with either purified FGF8 or with DMSO as negative control. While in the absence of exogenous FGF8, Pin1 could efficiently down-modulate ΔNp63α protein levels, in the presence of FGF8 this effect was reduced (Fig. 6A). Similar results were obtained by treating U2OS cells with FGF2 (data not shown).

Interestingly, treatment with FGF8 alone resulted in increased levels of ΔNp63α. These results indicate that FGF8 counteracts the ability of Pin1 to induce ΔNp63α, degradation.

In order to reveal by which molecular mechanism FGF8 prevents Pin1-mediated degradation of ΔNp63α, we tested by co-IP whether FGF8 could modulate Pin1-ΔNp63α protein–protein interaction, *in vivo*. In the presence of recombinant FGF8, ΔNp63α–Pin1 interaction was significantly reduced compared with the interaction detected in the absence of FGF8 (Fig. 6B). Finally, as binding of Pin1 to its target protein is known to be dependent on phosphorylation of serine/threonine residues, we verified the phosphorylation status of ΔNp63α in untreated versus FGF8-treated cells by using anti-phospho serine and threonine antibodies on immunoprecipitated ΔNp63α from HaCaT cells. A reduction of basal serine phosphorylation levels of ΔNp63α was evident upon FGF8 treatment (Fig. 6C). Similar results were obtained with anti-phospho threonine antibodies (data not shown).

These results suggest that FGF8 protects ΔNp63α from Pin1-dependent degradation by interfering with the ability of Pin1 to physically interact with ΔNp63α.

DISCUSSION

p63 is emerging as the master transcriptional regulator of expansion, development and differentiation of ectoderm-derived cells and tissues. Great attention has been placed on the identification of its downstream transcriptional targets (25,26,47); however, an equal complex set of regulations controls the p63 level, stability, activity, and degradation (28–30). The increasingly complex regulation upstream and downstream of p63 reflects the peculiar and critical activity of p63 to finely orchestrate the timing of exit from the cell cycle and the dynamic of stratification of mammalian ectoderm (27,48).

Ectodermal dysplasias are often accompanied by limb malformations, and specifically the p63-linked EEC comprises the ectrodactyly (SHFM) phenotype, with varying degrees of penetrance and severity. Six loci have been identified in hereditary forms of SHFM, and additional SHFM loci might exist to account for sporadic cases. For type-I and –IV, the transcription factors *DLX5-DLX6* and *p63*, respectively, are the recognized disease genes (18,49). For SHFM-III, the F-box/WD40 gene *Dactylin* has been proposed (22–24). In the recessive form SHFM type-VI, the *WNT10b* gene has recently been found mutated (15). The existence of several phenocopies of ectrodactyly has long suggested the possibility that the corresponding disease genes might participate in a regulatory cascade; however, the only established link is the transcriptional regulation of p63 on *Dlx5;Dlx6* (7,25,50). By examining the murine models of SHFM available to date, namely the $p63^{null}$, $p63^{EEC}$ (for SHFM-IV and EEC), the *Dlx5;Dlx6* DKO (for SHFM-I) and the spontaneous mutant strain *Dactylaplasia* (*Dac*, for SHFM-III), the striking observation is that in all these models the AER shows reduced *FGF8* expression and lack or has impaired stratification, with accompanying limb developmental defects of varying severity (5–7,19,20,51).

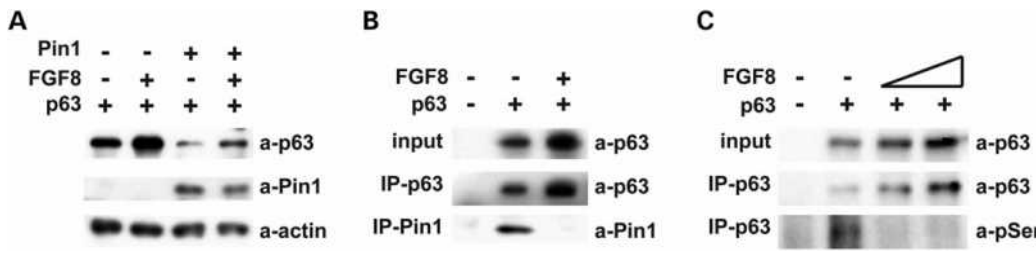

Figure 6. FGF8 counteracts Pin1-induced p63 degradation. (**A**) Western blot analysis of total protein extracts from U2OS cells co-transfected with the ΔNp63 expression vector (30 ng) and the Pin1–HA-tagged expression vector (40 ng) and either treated for 3 h with 1 ng/ml of recombinant FGF8 or left untreated (DMSO only). In the presence of FGF8, Pin1-induced p63 degradation is significantly less efficient. (**B**) Extract from HaCaT cells, either treated with 1 ng/ml of FGF8 or left untreated (DMSO only), immunoprecipitated with anti-p63 polyclonal antibody (p63 H-129, SantaCruz) and analyzed by western blot with either p63 monoclonal antibody or Pin1 monoclonal antibody. Input (no IP) is shown on the top. U2OS cells not expressing endogenous p63 were used as negative control. In the presence of FGF8, Pin1 is no longer able to interact and co-immunoprecipitate p63. (**C**) Extract from HaCaT cells, either treated with 1 ng/ml of FGF8 or left untreated (DMSO only), immunoprecipitated with anti-p63 and analyzed by western blot with α-phospho-ser. In the presence of FGF8, the amount of serine phosphorylation is clearly diminished.

Here we show that the Pin1 *cis/trans* isomerase is a regulator of p63 protein stability, inducing proteasome-mediated degradation of ΔNp63α. We also show that FGF8 counteracts this function and thus promotes p63 stability. The *FGF8* locus appears to be regulated by DLX5; thus, we propose a model (see Fig. 7) in which these two SHFM disease genes, together with *FGF8* and *FGFR1*, take part in a regulatory loop that tightly controls p63 protein level. According to our model, the activation of this loop permits stratification and specialization of ectoderm cells into the AER, in a time and region-restricted manner during limb development. In SHFM type-I, type-III and type-IV, such regulation is impaired leading to reduced AER stratification, limb malformation and skeletal defects. Although some molecular details still remain to be fully clarified, our novel findings together with previous work from our team (7) provide a developmental and molecular explanation for a set of congenital limb malformations.

Our model helps to explain other observations and findings. The *FGF8* locus is located close (∼50 kb) to the chromosomal region implicated in SHFM type-III and in the *Dac* mice. Although *Dactylyn* has been proposed as the disease gene for these malformations (22–24), no clear evidence is available on its role in limb development. Conversely, there are reasons to implicate FGF8 in the molecular pathogenesis of this disorder. First, the SHFM-III/Dac rearrangement does not interrupt any gene, and therefore, it is likely to act by perturbing the chromosomal organization and affecting expression of nearby genes; second, the *FGF8* expression is reduced in the limb buds of *Dlx5;Dlx6*, $p63^{null}$, $p63^{EEC}$ and *Dac* mutant embryos (5–7,19,22) (and this report); third, the presence of Dlx5 (this report) and p63 (25) binding sites in conserved genomic regions near the *FGF8* and the *Dactylyn* loci; fourth, FGF8 plays a critical role for limb bud growth, patterning, morphogenesis as well as AER maintenance (9,42,51–53). Thus, it is tempting to propose that misregulation of *FGF8* expression is the molecular lesion at the basis of SHFM-III/Dac; however, direct evidence for this is lacking and should be explored in future works.

The role of FGF8 in the signaling from the AER for the proximal limb development is well known (45,52), however not fully comprehended in cellular terms. The AER-specific conditional disruption of *FGF8* does not lead to altered AER induction and stratification, *per se* (54); however, this could be explained by the fact that during limb development, FGF4, FGF9 and FGF17 have been shown to compensate for the loss of *FGF8* (45). Conversely, the AER-specific conditional disruption of *Fgfr2* leads to altered AER stratification and function and limb defects (55). AER-derived FGFs have been shown to promote non-directional mesenchymal cell movements during limb bud morphogenesis (44), and consistently, the conditional inactivation of *Fgfr1* in the limb mesoderm disrupts the relative proportions of the limb elements and leads to profound limb malformations (56). Most relevant, mutations in *FGFR1* have recently been found in patients with Hartsfield syndrome (OMIM 615465), a congenital condition comprising ectrodactyly (57). This finding clearly supports the notion that impairment of the FGF signaling is directly involved in the molecular pathogenesis of ectrodactyly.

While FGFs promote mesenchymal cell movements, in the same article the authors show that Wnt5a promotes oriented cell divisions/movements during limb development (44), and Wnt5a is a known target of Dlx5 (58). We believe that these findings are highly relevant for the comprehension of the SHFM malformation, and we are tempted to speculate that the reduced expression of *FGF8* and *Wnt5a* (our unpublished data) in the central AER of *Dlx5;Dlx6* DKO limbs may induce mis-oriented divisions/movements of mesenchymal cells in this sector, hence altered morphogenesis/loss of central digits. This possibility warrants future experimental work. When a more complete model will be available, the hope is to be able to exploit this knowledge to restore normal levels of these soluble signaling factors, toward correcting the SHFM defects.

Altered Pin1-dependent p63 regulation may impact on several cellular processes, in addition to ectoderm stratification. Cells from *Pin1* knock-out mice have difficulties in exiting the G0 and entering the S phase, and *Pin1* null animals have meiotic defects and are hypofertile (40,41). In these animals, an altered phosphorylation levels of RB correlate with tumor growth (59). However, as Pin1 interacts with p53 and p73, the contribution of p63 is uncertain. Likewise, a Pin1/mutant p53 axis has been identified that promotes aggressiveness of breast cancer cells; however, the relevance of p63 in this context is not well defined (37). A role for p63 has been established in cancer types of ectodermal or endodermal origin, in particular lung and skin carcinomas. Indeed, ΔNp63α regulates keratinocyte proliferation by controlling PTEN expression and localization (60). Notably, a mis-activation of p63 in squamous cell carcinomas has been functionally linked with the activation of the FGFR2 receptor (61), further supporting the view that FGFs participate in Pin1-dependent p63 stability.

It would be important to define whether this regulatory pathway participates in skin carcinogenesis.

MATERIALS AND METHODS

Mouse strains

The *Dlx5;Dlx6* DKO mouse strain (20) was maintained in a mixed C57/BL6:DBA genetic background. The *Pin1* null mouse strain was originally generated by Fujimori and coworkers (40), then transferred onto a C57/BL6 pure background by Atchison and coworkers (41) and maintained in this background. The day of the vaginal plug was considered as embryonic age 0.5. Embryos were collected at the indicated ages in cold PBS, fixed in cold 4% PFA for 8–12 h and processed for cryopreservation and sectioning according to standard protocols. Extra-embryonic tissues were used for genotyping by PCR.

Immunofluorescence on embryonic limbs

Longitudinal sections of 12–15 μm were collected on glass slides, blocked with PBS with 1% BSA for 1 h at RT and incubated with the following primary antibodies, diluted from 1 : 250 to 1 : 50 in PBS + 1% BSA, ON at 4°C: anti-Pin1 (G8 sc-46660, Santa Cruz), with anti-p63 (4A4 sc-8431, Santa Cruz) and with anti-E-cadherin (36/E 610182, BD) and then incubated with secondary antibodies anti-mouse-Cy2 and anti-rabbit-Cy3 (Jackson ImmunoResearch) diluted 1 : 200, 1 h at RT, washed, stained with DAPI for the nuclei detection and examined with a Zeiss Observer-Z1 fluorescent microscope, equipped with Apotome system. Raw images were digitally

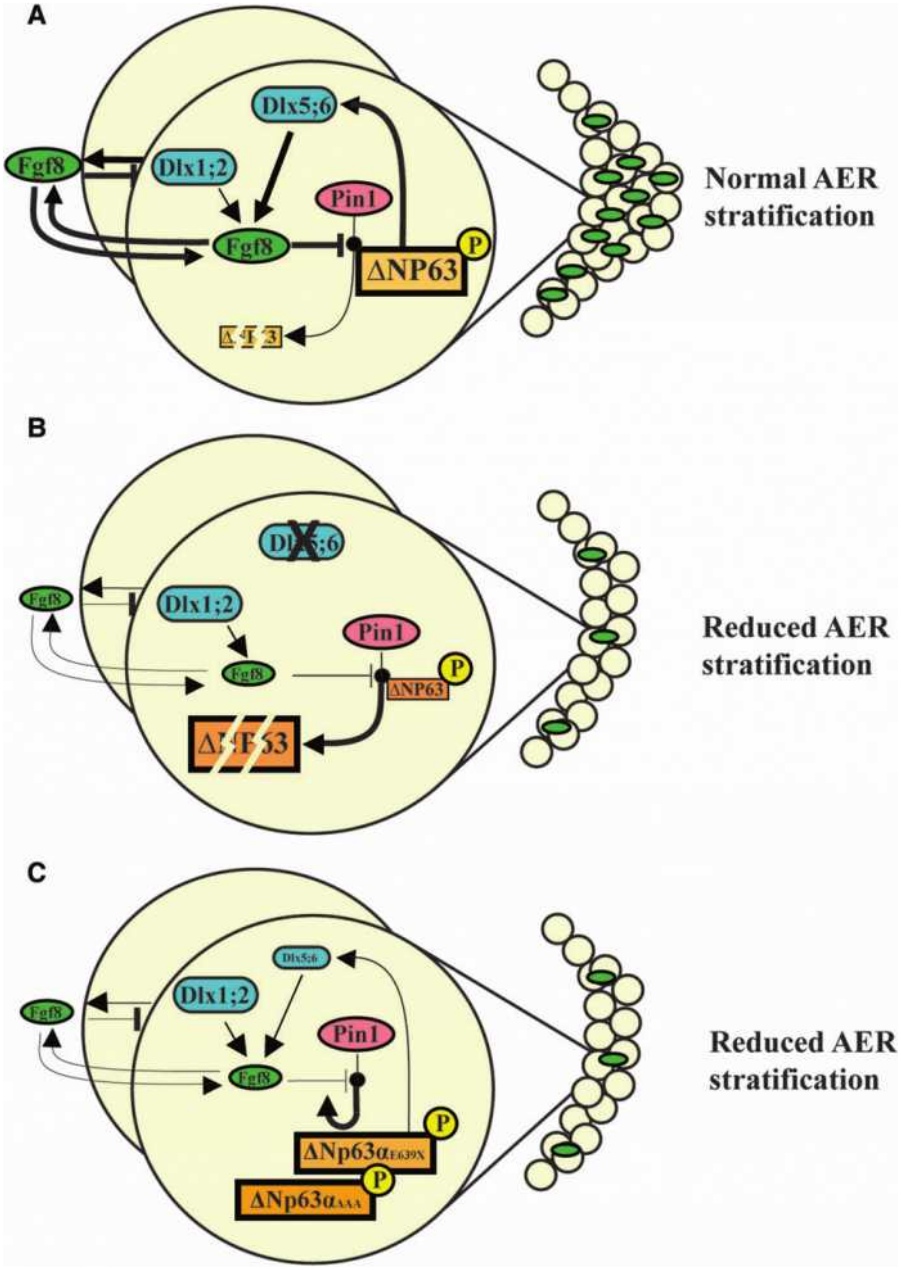

Figure 7. Model of the molecular loop between Dlx5 and p63 during AER stratification. Our proposed model of the regulatory loop linking the Dlx5 and the p63 SHFM-genes, via the activity of Pin1. (**A**) In WT AER cells, *Dlx5;Dlx6* positively control FGF8 transcription, a function most likely shared with Dlx1 and Dlx2 [see ref. (2)]. Likewise, p63 also activates FGF8 transcription, acting on independent genomic elements. AER-expressed FGF8 acts on the AER cells and prevent Pin1-induced ΔNp63 protein degradation: such fine mechanism dynamically maintains a control over the level of p63 in the AER cells, to assure their time- and region-restricted ability of these cells to stratify. (**B**) Mutation or loss of Dlx5 yield to a reduced FGF8 expression and an augmented ability of Pin1 to induce p63 degradation. Consequently, ΔNp63 tends to be depleted in the AER nuclei, which may result in a further down-modulation of FGF8. Reduced FGF8 and p63 cause impairment in the ability of the AER cells to stratify. (**C**) Mutant p63 associated with congenital limb malformations in human (LMS and SHFM) are relatively resistant to Pin1-induced degradation, although they appear to be transcriptionally inactive. Solid arrows indicate activation; solid stamps indicate repression. Line widths are proportional to the intensity and/or efficiency. Transcription factors are frames in squares; the other genes are framed in ovals.

processed to normalize the background and optimize the contrast, with Photoshop (Adobe), and mounted with QuarkXpress (Pantone).

Semi-quantitative immunofluorescence analysis was performed with ImageJ-64 (v1.45) software. Images were first converted to grayscale, and the DAPI channel was used to count nuclei. p63 intensity was quantified after background correction and normalized respect to the number of nuclei in the region of interest. Data are presented as mean and s.d. of ~4/5 different sections of three different embryos. A significant *T*-test score is indicated by asterisks: *indicates $P < 0.05$, **indicates $P < 0.01$.

Plasmids

Vectors expressing the WT ΔNp63α isoform of, or the disease-linked mutant *p63*, were previously described (62,63). The *DLX5*-myc-tagged expression vectors were obtained from OriGene, and previously used (58). The Q178P *DLX5-myc* point mutation [based on the sequence in NM_005221.5 (18)] was generated by site-directed mutagenesis in the *DLX5-myc* expression plasmid and sequence-verified (Bio-Fab Research, Rome, Italy). The *Pin1* si-RNA was previously described (37).

Cell cultures and transfections

The U2OS human osteosarcoma and the A431 human epidermoid squamous carcinoma cell lines were maintained in Dulbecco's modified Eagle's medium (D-MEM) and 10% fetal bovine serum. For transfection, 50,000 cells were seeded into 24-well multi-plates and the next day transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The total amount of transfected DNA (500 ng) was kept constant using empty vector when necessary. After 24 h, cells were lysed and assayed for western blot analysis (29). MG132 treatment was initiated the day after transfection with 5 μM MG132 (Sigma) for 5 h. FGFs treatments were initiated 20 h after transfection with 1 ng/ml of FGF8 or FGF2 for 3 h.

Western blot analyses

Twenty-four hours after transfection, cells were lysed in 100 μl of loading buffer (2% sodium dodecyl sulfate, 30% glycerol, 144 mM β-mercaptoethanol, 100 mM Tris–HCl pH 6.8 and 0.1% Bromo-Phenol Blue): extracts were separated on SDS–10% polyacrylamide gel, transferred on nitrocellulose membrane (Protran, Millipore) and incubated with the relative antibodies and developed according to the manufacturer's instructions (GeneSpin). The following primary antibodies were used: α-p63 (4A4, sc- 8431, Santa Cruz), α-actin mouse monoclonal (A2066, Sigma), α-Pin1 mouse monoclonal (G9, sc-46660, Santa Cruz), α-Pin1 rabbit polyclonal, α-phospho-ser polyclonal (Invitrogen, 618100) and α phospho-thr polyclonal (Cell Signaling, # 9381). As secondary antibodies, we used the following: α-mouse secondary (sc-2005, Santa Cruz) and α-rabbit secondary (sc-2030, Santa Cruz).

Co-immunoprecipitation

HaCaT cells (4×10^6/150 mm plate) were treated with FGF8 (1 ng/ml) for 3 h and then harvested for the preparation of whole-cell lysates using RIPA buffer [10 mM Tris–HCl pH 8, 2 mM EDTA, 0.1% SDS, 0.1% sodium deoxycholate, 140 mM NaCl, 1 × Triton, supplemented with 1 mM phenylmethylsulfonyl fluoride and protease inhibitors (all from Sigma)]. Cell lysates were incubated on ice for 20 min., vortexed and centrifuged at $6600 \times g$ for 10 min to remove cell debris. Protein concentration was determined with Bradford Reagent (Sigma). Three milligrams of cell lysate was incubated overnight at 4°C with 3 μg of anti-p63 (H-129, sc-8344, SantaCruz). The immunocomplexes were collected by incubating with a mix of Protein A–Agarose and Protein G–Sepharose (Sigma) overnight at 4°C. The beads were washed three times: the first wash with RIPA buffer and the others with PBS. The beads were then resuspended in 2× Lysis buffer, loaded directly on a 10% SDS–polyacrylamide gel and subjected to western blot with the indicated antibodies.

mRNA quantification by Real-Time qPCR

Embryonic FLs and HLs were dissected from embryos at the indicated age, in cold RNase-free PBS, under microscopic examination. A minimum of three limbs were pooled, according to the genotype and collected in Trizol (Invitrogen). Total RNA was extracted with the TRI reagent (Sigma) and treated with DNAse I (Ambion). Reverse-transcription and cDNA synthesis were done using kits (Invitrogen), as previously reported (7,64). Three nanograms of each cDNA sample were used in Real-Time qPCR analyses using SYBR green IQ reagent (Biorad) on T900 HT Fast Real Time PCR Sistem (Applied Biosystems). The *TATA-binding protein* (*TBP*) and the *GAPDH* mRNAs were used for normalization. Primer sequences are provided in Supplementary Material, Table S1. Experiments were repeated twice on independent samples; every point was done on biological duplicates. Analyses were performed with ABI 2.1 software (Applied Biosystems).

Total RNA from U2OS cells was extracted using the TRI reagent and treated with DNase-I (Ambion). One micrograms of RNA was retrotranscribed with SuperScriptII (Invitrogen). qPCR was performed using SYBR green IQ reagent (Biorad) on the Rotor Gene machine. Primers were designed to amplify regions of 80–120 bp in size. *Tubulin* and *GAPDH* mRNAs were used for normalization. Experiments were repeated twice on independent samples. Primer sequences are provided in Supplementary Material, Table S1.

Chromatin immunoprecipitation

ChIP analyses were performed on sheared genomic DNA from 1×10^6 U2OS cells transfected with 12 μg of *DLX5-myc* or *DLX5-Q178P-myc* vectors, or with the empty pcDNA3 vector as control and immunoprecipitated with 5 μg of anti-myc-TAG mouse monoclonal antibody (SantaCruz, sc-40) or 5 μg of anti-Flag antibody (Sigma, F3165) as previously described (7). For negative control, an irrelevant antibody was used. The sequences of the oligonucleotides used for this analysis are provided in Supplementary Material, Table S1).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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DLX5, FGF8 and the *Pin1* isomerase control ΔNp63α protein stability during limb development: a regulatory loop at the basis of the SHFM and EEC congenital malformations

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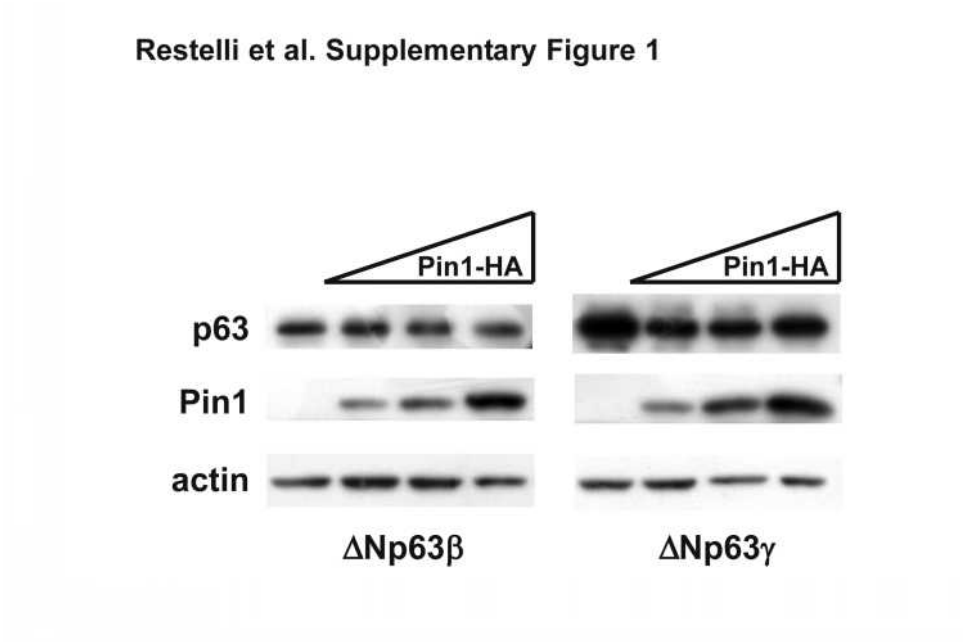
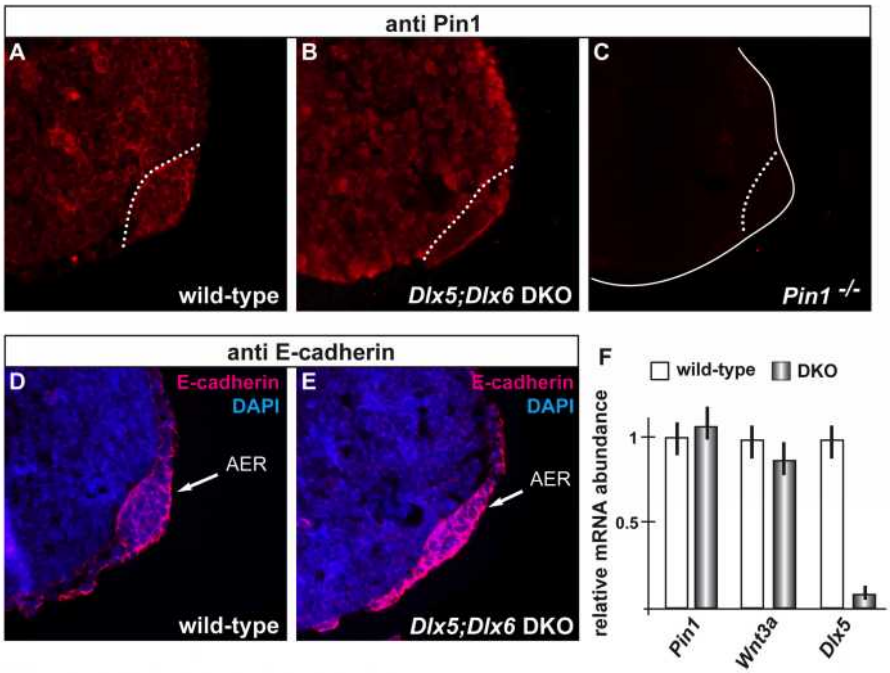
Supplementary Figures and Table



Legend to Supplementary Figure 2

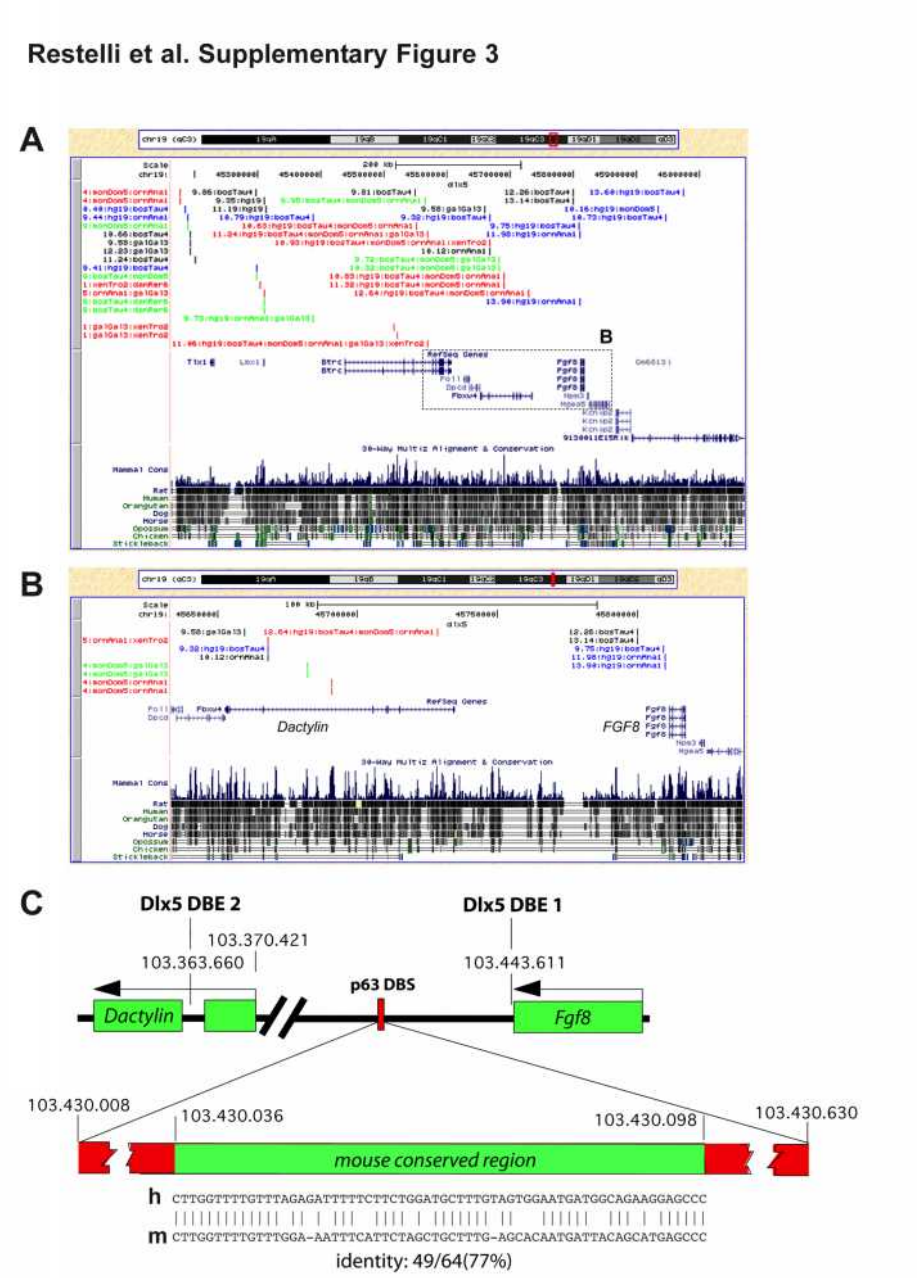
Western blot analysis of whole protein extracts from U2OS cells transiently co-transfected with increasing amounts (20, 40 and 80 ng) of *Pin1-HA*-tagged vector (indicated on top), and wild-type ΔNp63β (on the left) or ΔNp63γ (on the right) (30 ng each). As opposed to the α isoform, the β and γ isoforms are not degraded by *Pin1* expression.



Legend to Supplementary Figure 2

A,B. Immunofluorescent staining with anti-Pin1 on sections of the embryonic HLs of wild-type (A) or *Dlx5;Dlx6*-/- (B) embryos, at the age E11. Scale bar in A=20 μm. **C.** As control, the same staining was done on sections of HLs from *Pin1* KO embryos, at the age E11. The lack of staining in the *Pin1*-/- specimen indicates that the antibody is highly specific. **D,E.** Staining with anti-E-cadherin on adjacent sections, counterstained with DAPI. The AER is outlined with a dotted line and indicated with white arrows. **F.** Real-Time quantitative determination of the relative abundance of the mRNA of *Pin1, Wnt3a* and *Dlx5* in RNA samples from the HLs of wild-type and *Dlx5;Dlx6*-/- embryos, normalized against *GAPDH* and *TBP* mRNA. The abundance of the wild-type is set=1. As expected, the *Dlx5* mRNA is nearly absent. The *Pin1*

mRNA is slightly increased while the *Wnt3a* mRNA is slightly decreased, however these differences are not significant.



A. Location of predicted conserved Dlx5 binding elements (DBE) around the around the murine *Dactylyn* and the *FGF8* loci on chromosome 19q (10q in human), based on the UCSC mouse genome browser. Dlx5 sites were bio-informatically predicted

using the published PWM, as described (ref. (31)). Sites are indicated with coloured vertical bars (asterisk) and annotated with the species conservation. A color code is used to indicate the number of species in which the site is conserved. Red indicated the most conserved ones. **B.** Enlargement of the area indicated with a solid box in A, centred around the genomic region comprising *FGF8* and *Dactylyn*. The chromosomal position and coordinates are reported on the top, the mammalian genomic conservation is reported on the bottom. **C. (top)** Location of the DLX5 DBE-1 and DBE-2 sites, corresponding to the sites tested by ChIP analysis (see Fig. 5 and corresponding text), in the human genomic region around *FGF8* and *DACTYLYN* (chrom. 10q). **(bottom)** Location of a p63-binding site (p63 DBS) within a conserved region of the human genome, between *FGF8* and *DACTYLYN*, as reported (ref. (25)).

Supplementary Table I

A. Sequences of the primers used for Real-Time qPCR on mouse embryonic tissues.

mGAPDH	F	5' TGTCAGCAATGCATCCTGCA
mGAPDH	R	5' TGTATGCAGGGATGATGTTC
mTBP	F	5' GGGTTATCTTCACACACCATGA
mTBP	R	5' CGGTCGCGTCATTTTCTC
mRps9 F		5' GACCAGGAGCTAAAGTTGATTGGA
mRps9 R		5' TCTTGGCCAGGGTAAACTTGA
mDlx5	F	5' TCTTATGGCAAAGCGCTCAA
mDlx5	R	5' CGTTCACGCCGTGGTACTG
mDlx6	F	5' TCCAGTGTGGGACGTTTCTG
mDlx6	R	5' CTGTTGGGAGGCATACTGACG
mFGF8	F	5' TGAGCTGATCCGTCACCA
mFGF8	R	5' TCCTGCCTAAAGTCACACAGC
mPin1	F	5' GTCCCTTCAGCAGAGGTCAG
mPin1	R	5' ACAGTAGCAGGAAGGGCATC
mWnt3a	F	5' GAGTGCTCAGAGAGGAGTACTGG
mWnt3a	R	5' CTTAGTGCTCTGCAGCCTGA
mΔNp63	F	5' ATGTTGTACCTGGAAAACAATG
mΔNp63	R	5' GATGGAGAGAGGGCATCAAA

B. Sequences of the oligonucleotides used for ChIP analysis on Dlx5 Binding Elements (DBE) near the *FGF8* locus

DBE-1 (FGF8) For	CCTCTGAAGACTCGGATGTTCC
DBE-1 (FGF8) Rev	AGGAAACGCTTTCATCTGCAC
DBE-2 (FGF8) For	GCCCGAGGCAGCTTGTCTA
DBE-2 (FGF8) Rev	GAGCCCTCACTAATGGGGTTTTA

PART III

FGF8, c-Abl and p300 cooperate in the regulation of ΔNp63α protein stability

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Abstract

The p63 transcription factor, homolog to the p53 tumor suppressor, plays a crucial role in epidermal and limb development. Dominant mutations in the *p63* gene give rise to several human congenital syndromes characterized by skin, craniofacial and limb defects. However, little is known on the post-translational modifications controlling ΔNp63α functions. Here we report that, FGF8 is a crucial signalling molecule inducing ΔNp63α stabilization and activation in both human cells and in mice embryonic limb buds *ex vivo*. FGF8 treatments determined an increase in the binding of ΔNp63α with the tyrosine kinase c-Abl and in the level of p300 driven acetylation. *In vitro*, p300 catalizes acetylation of lysine K193: triggering ΔNp63α stabilization and transcriptional activation. A prerequisite for the p300-ΔNp63α is interaction is the ΔNp63α phosphorylation by the tyrosine kinase c-Abl. Notably, this regulatory pathway induced by FGF8 is not active on the ΔNp63αK193E natural mutant, associated to the Split-Hand/split Foot Malformation IV (SHFM-IV) syndrome: that displays promoter specific altered DNA binding activity that results in altered expression of ΔNp63α target genes involved in limb development. Our results link together FGF8, c-Abl and p300 in a regulatory loop essential for the activation and stabilization of ΔNp63α: mutations or altered expression of regulators in this pathway leads to abnormal limb development and possible onset of SHFM-IV pathogenesis.

Introduction

The p63 transcription factor is highly homologous to the *p53* tumor suppressor gene in the DNA binding and in the oligomerization domains (1-4). The *p63* gene encodes for at least ten different protein isoforms differing in their amino and carbossi-terminal regions as a consequence of alternative transcription start site and alternative splicing respectively (5,6). ΔNp63α is the most expressed isoform, largely present in the embryonic ectoderm and in the proliferating adult cells, including the basal layer of the epidermis, breast and oral epithelium (7,8). p63 plays a pivotal role during development: indeed, p63 null mice show severe defects in limb, skin and craniofacial development and die after birth due to dehydration caused by absence of epidermal stratification.

In particular, limb defects of p63 -/- mice have been associated with developmental failure of the Apical Ectodermal Ridge (AER), an essential structure required for limb outgrowth, and loss of expression of key morphogens (9, 10). The DLX5 and DLX6 transcription factors, playing key roles in the development and morphogenesis of the head and limb skeleton, co-localize with ΔNp63α in the AER and are direct ΔNp63α target genes (11-13). It's well established that also FGFs signaling molecules are essential for AER development. Notably, FGF8 is essential for the correct functions of the AER and the consequent limb bud initiation and *FGF8* knock-out mice display severe defects in skeletal and limb developmet (15-19). Recently, we linked together p63, DLX5, FGF8 and the prolyl isomerase PIN1 in a regulatory pathway important for correct stratification and proper AER development in mouse limb buds. FGF8 induced the expression of the *DLX5* gene and, on the other hand, counteracts the degradation of ΔNp63α protein induced by Pin1 expression in both human osteosarcoma cell lines and *in vivo* (20).

Ectrodactily, also knows as Split-Hand-split-Foot Malformation (SHFM), is characterized by the absence of the limb central rays, resulting in a deep medial cleft, missing or hypoplastic central fingers and fusion of the other ones (21). There are several phenocopies of SHFM: in particular SHFM-I is linked to deletions,

inversions and re-rearrangements affecting chromosome 7q21. This region comprises the DLX5, and DLX6 transcription factors while SHFM-IV is caused by mutations in the *p63* gene, localized in the DBD domain (K193E and K194E) and in the C-terminal region (Q634X and E639X) of p63 (22-26).

Several p63 target genes have been identified and p63 activity is finely regulated by different post-translational modifications like sumoylation, phosphorylation and ubiquitylation (27-32).

However the upstream regulatory pathway controlling p63 functions and activities are not fully understood and the understanding of these molecular mechanisms is crucial for understanding p63 function during development.

A huge number of Post-Translational Modifications (PTM) occurs on p53 with a critical role in the regulation of its stability and function (33-35); among these modifications, acetylation seems to play a pivotal role (36-39). Acetylation is a reversible reaction catalyzed by histone acetyl-transferases that occurs on lysines of a target protein and its functions in transcriptional activation is well accepted (40). For instances, p73 is acetylated by p300 on lysines located in the DBD and oligomerization domain in response to DNA damage (41). Acetylation of p73 enhances its ability to bind and activate proapototic target genes. Furthermore, p73-p300 interaction requires the activity of the prolyl-isomerase Pin1 that induces conformational changes of p73 when it is phosphorylated by the tyrosine kinase c-Abl (42, 43). Acetylation of p53 is enhanced in response to stress and well correlate with p53 stabilization and activation: indeed, acetylation of p53 antagonize the activity of the principal negative regulator of p53, Mdm2, an ubiquitin-ligase that keep the p53 protein at low levels in normal conditions. Moreover, acetylation of p53 by p300 was found to promote its sequence specific DNA binding (36-39). Recently, a new lysine (K164) acetylated by p300 was identified in p53 (39). This lysine is well conserved in p63 and p73 and it corresponds to K193 in ΔNp63α. Interestingly, natural mutations of ΔNp63α K193 into glutamic acid (K193E) are associated to the SHFM--IV syndrome, characterized by limb abnormalities.

Here we report that ΔNp63α is acetylated by p300 acetyl-transferase on K193 *in vitro*, and mutations of this lysine alter the transcriptional capacity of p63 specifically on p63 target genes involved in the regulation of limb development. Furthermore, for the first time we found that FGF8 is the first component of a regulatory pathway promoting the physical interaction of ΔNp63α with c-Abl and p300 and the consequent acetylation and transcriptional activation of ΔNp63α.

RESULTS

p63 is acetylated *in vivo*

In order to assess whether p63 is acetylated in *vivo*, HaCat cells, a human keratynocyte cell line expressing ΔNp63α protein endogenously, were treated with the deacetylase inhibitors Trichostatin-A (TSA), inhibiting deacetylases of class I and II and Valproic-Acid (VPA), inhibiting only class I deacetylases. The treatments determined an increase in ΔNp63α abundance (Fig. 1A). Same results were obtained also in conditions of ΔNp63α overexpression in U2OS cells, a human osteosarcoma cell line not expressing endogenous p63 (Fig. 1B). By immunoprecipitation of ΔNp63α protein from HaCat total cell extracts, we observed a strong increase in the levels of ΔNp63α acetylation upon TSA treatment (detected by using an antibody against acetylated lysines) (Fig. 1C)).

These results show that ΔNp63α protein is acetylated in human cells and that one or more histone acetyl-transferases could be responsible for ΔNp63α acetylation.

p300 acetyl-transferase positively regulates ΔNp63α protein stability

Acetylation of p53 and p73 is a crucial post-translational modification required for their stabilization and activation in response to DNA damage and during the apoptotic response. (36, 39, 41, 42, 44). One of the most important acetyl-transferase for the p53 family members is p300 (36, 39, 44, 45).

To determine if p300 was acting also on ΔNp63α, we silenced endogenous p300 in HaCat cells by transfecting increasing amounts of shRNA plasmid against the p300

transcript. p300 depletion induced ΔNp63α destabilization (Fig. 2A). Accordingly when U2OS and HaCat cells were co-transfected with increasing amounts of a p300 and ΔNp63α encoding plasmids, ΔNp63α was stabilized in a dose dependent manner (Fig. 2B). Interestingly, a p300 construct mutated in the HAT domain, failed to stabilized endogenous ΔNp63α in HaCat cells (FIG. 2B). Finally, to prove that p300 overexpression was positively altering ΔNp63α stability, we co-transfected ΔNp63α and p300 plasmids in U2OS cells and measured ΔNp63α protein half-life by treating cells with the protein synthesis inhibitor Cycloheximide (CHX). As shown in Fig. 2C, the half-life of ΔNp63α was greatly increased by p300 co-transfection from 8 to 10 hours. In order to verify whether p300 was directly interacting with ΔNp63α we performed co-immunoprecipitation experiment in U2OS cells, by transfecting ΔNp63α alone or together with p300. Equal amounts of cell extracts were immunoprecipitated with an antibody against the p300 protein. As shown in Fig. 2D, ΔNp63α physically interacts with p300 *in vivo*.

Taken together these results demonstrate that p300 interacts and positively regulates ΔNp63α stability.

The natural K193E mutation impairs p300 acetylation of ΔNp63α

Recently, a new p300 acetylation site was identified in p53 (lysine K164) (39). This lysine is well conserved in p63 and p73 and corresponds to K193 in ΔNp63α (Fig. 3A). Interestingly, natural mutations of ΔNp63α K193 into glutamic acid (K193E) are associated to the SHFM-IV syndrome, characterized by limb abnormalities. In order to verify whether p300 acetylates ΔNp63α K193, we perform an *in vitro* acetylation assay by using p300 recombinant protein and different p63 peptides centred on lysine K193. Moreover, a p53 centred on lysine K164, acetylated by p300 (39), was used as a positive control for the reaction. Interestingly, we found that the p63 peptide was acetylated *in vitro* (Fig. 3B) and

acetylation levels were higher than those one obtained with the p53 peptide. The p63 peptide used in the reaction contains two lysines (K193 and K194) that were mutated one at a time, or simultaneously to verify whether both were acetylated by p300. As shown in figure 3B, p300 preferentially acetylates lysine K193.
Accordingly, p300 overexpression in U2OS cells did not induce ΔNp63αK193E stabilization (Fig. 3C) suggesting that the integrity of K193 is required to induce p300 mediated stabilization of ΔNp63α.

The natural ΔNp63αK193E mutation alters p63 transactivational potential in a promoter specific manner

In order to verify whether p300 could act as a co-activator for p63 we performed luciferase assays with the *DLX5* promoter, a known ΔNp63α target gene expressed in the AER of developing limbs (13). Interestingly, we observed that p300 co-transfection greatly enhanced the transcriptional activity of ΔNp63α; on the other hand, the ΔNp63α K193E mutant had a reduced transcriptional activity on this promoter that could not be further enhanced by p300 over-expression (Fig. 4A). Same results were also obtained with the *DLX6* promoter, another ΔNp63α target gene expressed in the AER of developing limb (data not shown) (13). We then investigated whether the K193E mutation could alter the ΔNp63α transcriptional activity on other p63 target genes involved in different cellular processes; for this aim we used the *p57kip2* promoter, a p63 target gene involved in cell-cycle regulation (46). Interestingly, we found that the ΔNp63αK193E mutant behaved as the wild type on this promoter (Fig. 4B), suggesting that the K193E mutant alters the p63 transcriptional activity in a promoter specific manner.
This finding prompted us to verify whether this natural mutation alters the normal pattern of gene expression: we performed a colony assay and compared the growth capacity of cell over-expressing ΔNp63α and ΔNp63αK193E. As expected, over-expression of ΔNp63α protein led to a decrease in the colony forming capacity respect to the control empty vector (pCDNA3) as previously reported (47).

Notably, the expression of the ΔNp63αK193E mutant caused an increase in the number of colony compared to ΔNp63α wild type protein, suggesting a perturbation in the normal expression of ΔNp63α target genes (Fig. S1).

In order to further characterize the altered transcriptional activity of the ΔNp63αK193E mutant, we performed real-time qPCR analysis in U2OS cells stably transfected with the ΔNp63α and ΔNp63αK193E expressing vectors. Interestingly, we confirmed that the ΔNp63αK193E mutant has an impaired transcriptional activity on genes involved in limb development and apoptosis like *PERP*, Ikkα, *CASP10, EGFR* but it behaves as the wild-type ΔNp63α on genes involved in different cellular processes like *p53* (Fig.5A). These set of data clearly show that the K193E mutation alters the transcriptional activity of p63 in a gene specific manner.

In order to understand the molecular basis of differential gene activation, we decided to verify whether the natural mutant ΔNp63αK193E display an altered DNA binding activity by Chromatin Immunoprecipitation assay in U2OS cells stably transfected with the ΔNp63α and ΔNp63αK193E expressing vectors. We observed that the ΔNp63αK193E mutant is not recruited on genes involved in the regulation of developmental and apoptotic processes (*Perp, Casp10* and *EGFR*) while it is recruited on promoter of genes involved in cell cycle regulation (*p21* and *p53*) (Fig. 5B). In conclusion, the ΔNp63αK193E mutant displays altered DNA binding activity and transcriptional activity restricted to genes involved in the regulation of developmental processes.

FGF8, c- Abl and p300 act together to stabilize ΔNp63α

The natural mutation ΔNp63αK193E is associated to the SHFM-IV syndrome characterized by ectrodactily and limb defects (21, 24, 25). Many reports have demonstrated the essential role of FGF8 in the patterning and the development along the dorsal-ventral axis during limb formation (15, 18, 19). In order to verify

whether FGF8 could be an upstream signal for ΔNp63α activation, we treated HaCat cells with increasing amounts of FGF8 and obtained a strong stabilization of the ΔNp63α isoform (Fig. 6A). We performed the same experiments in U2OS cells transiently transfected with ΔNp63α and ΔNp63αK193E plasmids. Interestingly, while the wild type ΔNp63α is stabilized by this treatment the ΔNp63αK193E mutant was not influenced by FGF8 treatment (Fig. 6B).

Treatments with bFGF (FGF2) induce the activation of the tyrosine kinase c-Abl (48) and c-Abl is a crucial regulator of the p53 family members activity (43, 49-51). In particular, c-Abl phosphorylation of p73 is required to promote the interaction of p73 with p300, leading to p73 acetylation and transcriptional activation (43). In order to verify whether c-Abl is required to induce ΔNp63α stabilization mediated by FGF8 and bFGF tratments, we stably silenced c-Abl expression in HaCat cells and then treated these cells with FGF8 and FGF2. Interestingly, c-Abl silencing abolished ΔNp63α stabilization by FGF8 (Fig. 6C) and FGF2 (data not shown), suggesting that FGFs stabilization of ΔNp63α requires c-Abl tyrosine kinase activity. To verify whether c-Abl, was promoting the interaction between p63 and p300, we performed a co-immunoprecipitation experiments of p300 with wild-type ΔNp63α and a ΔNp63α mutant (ΔNp63α3Y) that has the three tyrosine known to be phosphorylated by c-Abl (52, 53), mutated into phenylalanin. Interestingly, we observed that there is a significant reduction in the binding of ΔNp63α3Y mutant to p300 (FIG. 6D), suggesting that a phosphorylation event catalized by c-Abl on ΔNp63α is required for the interaction between ΔNp63α and p300. Furthermore, over-expression of p300 on the ΔNp63α3Y mutant has no effect on its stability (Fig. 6E): confirming that phosphorylation of ΔNp63α by c-Abl is crucial for the ΔNp63α-p300 interaction and ΔNp63α stabilization upon acetylation by p300.

To further confirm that FGF8, c-Abl and p300 are linked together in the same regulatory cascade, promoting p63 stabilization and activation, we treated HaCat cells with FGF8 and performed co-immunoprecipitation of p63 and c-Abl. Notably,

upon FGF8 treatment we observed a great increase in p63 c-Abl interaction and in the levels of ΔNp63α acetylation (Fig. 6F).

In conclusion, all these data demonstrate that FGF8, c-Abl and p300 act in a signalling pathway that leads to p63 acetylation, stabilization and activation. The integrity of this regulatory pathway is required to guarantee a correct development: indeed, the presence of ΔNp63αK193E mutation leads to limb malformations.

In order to verify whether FGF8 is a crucial upstream regulator for ΔNp63α stability during limb development *in vivo*, we treated mice limb buds put in culture *ex vivo* at E10.5 with FGF8. These treatments determined a great increase of ΔNp63α protein abudance (Fig. 7A*)*, demostrating that FGF8 is a crucial activator of ΔNp63α also *in vivo*.

Discussion

The p63 transcription factor is arising as a master regulator of development and differentiation of ectoderm derived cells and tissues. In the last years close attention has been paid to the analysis and identification of p63 downstream target genes, and to the characterization of p63 post-translational modification upon DNA damage and differentiation processes (27-32). However, the upstream pathway regulating and activating p63 protein, especially during development, are not fully understood. Here we report that FGF8, c-Abl, p300 and ΔNp63α are linked together in a regulatory pathway aimed at regulating ΔNp63α activity during limb development. Indeed, we found that treatments with FGF8, a signalling molecule essential for limb outgrowth and correct development, determine an increase in ΔNp63α protein stability in both in human cell lines and in embryonic mice limb buds put in culture *ex vivo* at E10.5. In particular, our data support a model in which FGF8 promotes the interaction of c-Abl and ΔNp63α that is indispensible for the interaction between p300 and ΔNp63α proteins (Fig7B).

Acetylation of p53 and p73 transcription factors occurs after DNA damage stress and is essential for transcriptional activation of their target genes (36, 37, 39, 42, 44). However, the importance of acetylation during developmental processes is not completely clear; nevertheless, acetylation and deacetylation processes are fundamental to assure correct limb development. Indeed, double Knock-Out mice for Histone Deacetylase 1 and 2 (HDAC1/HDAC2) displayed malformations in the embryo similar to those observed in the *p63* null mice. Infact HDAC1 and HDAC2 are required to mediate the repressive function of p63 on its target genes that need to be down-regulated to assure correct development (54). Similarly, it's possible to speculate that Histone acetyl-transferases, like p300, are needed to activate p63 target gene expression once they need to be correctly expressed to assure proper development. Indeed, Luciferase Assay experiments in human osteosarcoma cell lines demonstrated that p300 is required to enhance ΔNp63α transcriptional activation on the promoter of genes related to limb development, like *DLX5* and *DLX6* (13) (Fig 7B). Interestingly, we demonstrated *in vitro* that a p63 peptide is acetylated by p300 on lysine K193, homolog to lysine K164 acetylated by p300 in p53.

K193 in ΔNp63α is found mutated to glutamic acid in patients affected by the SHFM-IV syndrome characterized by severe limb defects. Interestingly, the natural ΔNp63α mutant is not able to induce the activation of genes required for development (like *Perp, Ikkα, Egfr*) and apoptotic process (*Casp10*). Apoptosis is considered an important process required for correct limb development because it allow the correct shaping of limbs and digits (). On the other hand the ΔNp63αK193E mutant maintained its ability to correctly induce the expression of genes connected to cell-cycle regulation (like *p53* and *p57kip2*). We found that the altered expression of ΔNp63α target genes linked to development (29, 55-57) was due to lack in the capacity of ΔNp63αK193E natural mutant to bind the promoters of these genes, while the natural mutant is normally bound to the promoters of target genes involved in cell-cycle. However, it is not clear how this mutation could

alter the binding of p63 in a promoter specific manner: our data introduce a model in which p300 appears to be an important regulator of ΔNp63α function during development. It is possible to speculate that p300 is required to selectively induce and activate, together with ΔNp63α, genes required to assess a correct process of limb development. Anyway it remains to be characterized the contribution of p300 in limb development and the effect of p300 depletion on limb outgrowth. Indeed, the depletion of p300 in KO mouse model is embryonic lethal and p300 -/- mice stops to develop before the limb buds are formed (58).

However, the work presented here run to the identification of an important regulatory loop activated by FGF8 signalling molecule that is essential for ΔNp63α activation and stabilization in both human osteosarcoma and keratynocite cell lines and in mice limb buds put in culture ex vivo. Importantly, this regulatory cascade triggered by exposure to FGF8 signalling molecule is not active on the ΔNp63αK193E mutant. Indeed, this mutation caused a deep change in the pattern of ΔNp63α target genes involved in development. These results shed new light on the molecular mechanism that could be at the bases of SHFM-IV pathogenesis and contribute to a better understanding of the molecular mechanism governing ΔNp63α function in order to guarantee proper expression of target genes essential for correct limb development.

Materials and Methods

Plasmids

All expression vectors encoding p63 cDNAs, p300 cDNAs, c-Abl have been previously described (42, 43, 47, 59). The shRNA against p300 (shp300) and control shRNA (shLuc) were purchased from Origene company.

Cell Culture and transfection

U2OS, HaCaT cells were manteined in DMEM supplemented with 10% FBS (Euroclone) at 37°C in a humified atmosphere of 5% (v/v) CO2 in air.

For transient transfection, 50,000 cells were seeded into 24-multiwell plates and on the next day transfected with Lipofectamine 2000 (Invitrogen) or Lipofectamine LTX (Invitrogen) for HaCaT cell, under the conditions suggested by the manufacturer. Transfection efficiency was always checked by transfection of a β-gal or GFP expressing vectors. The total amount of transfected DNA (500 ng for 50,000 cells) was kept constant using empty vector as necessary.

For stable transfection 300,000 HaCat or U2OS cells were plated and on the next day, HaCat cells were transfected with 3 μg of shAbl and 3 μg of shLuc using Lipofectamine LTX (Invitrogen). After 24 hours, cells were trypsinized and plated in a medium containing puromycin (0.8 μg/ml; Sigma). After 8 days of selection, clones were pooled and kept in puromycin. U2OS cells were transfected with 3 μg ΔNp63α or 3 μg ΔNp63αK193E and 3 μg ΔNp63αK193R using Lipofectamine LTX (Invitrogen). After 24 hours, cells were trypsinized and plated in a medium containing Neomicin (G-418, 600 μg/ml). After 3 weeks of selection, clones were pooled and kept in Neomicin.

U2OS and HaCaT cells were treated with 0.5 or 1 mM Valproic Acid (VPA), 5ng/ml or 10 ng/ml Trichostatin (TSA), 1 or 3 ng/ml FGF8 or FGF2, 10 μM cycloheximide.

Western Blot and antibodies

24 hours after transfection, cells were lysed in 100 µl of Loading Buffer 2X (2% sodium dodecyl sulfate, 30% glycerol, 144 mM β-mercaptoethanol, 100 mM Tris–HCl pH 6.8 and 0.1% Bromo-Phenol Blue). Samples were incubated at 98°C for 10 minutes and resolved by SDS-PAGE. Proteins were transferred to a nitrocellulose membrane (Protran, Millipore). The blots were incubated with the following antibodies (p63 4A4 sc-8431, Santa Cruz Biotechnology), p300 (Santa Cruz Biotechnology), c-Abl (Sigma), acetylated lysine (Cell-signalling) and actin (M2, Sigma). As secondary antibodies, we used the following: a-mouse secondary (sc-2005, Santa Cruz) and-rabbit secondary (sc-2030, Santa Cruz) Proteins were visualized by an enhanced chemi-luminescence method (Genespin) according to manufacture's instructions.

Luciferase activity assay

For reporter gene assays, cells were transiently co-transfected with the *Dlx5*, *Dlx6* and *p57kip2* luciferase reporter plasmids and expression plasmids encoding for ΔNp63α, ΔNp63αK193E and p300. Cells were seeded in 24-well plates and transfected using Lipofectamine 2000 (Invitrogen, Life Sciences) At 24hours post-transfection, cell extracts were prepared with Luciferase lysis buffer (1% Triton, 25 mM Gly-Gly pH 7.8, 15 mM $MgSO_4$, 4 mM EDTA), and the luciferase activity was measured using the Beetle Luciferin Kit (Promega Inc.) on a TD 20/20 luminometer (Turner design). Results from experiments, performed three times in triplicate wells, are expressed as relative luciferase activity after normalization with the beta-Galactosidase plasmid as internal control. Basal activity of the reporter was set to 1. Each histogram bar represents the mean of three independent transfection experiments performed in triplicate. Standard deviations are indicated.

Co-Immunoprecipitation

U2OS and HaCaT cells (1.25 x 106/100 mm plate) were transfected with the indicated vectors. 24 hours after transfection cells were harvested for the preparation of whole-cell lysates using RIPA buffer (10 mM Tris–HCl pH 8, 2 mM EDTA, 0.1% SDS, 0.1% sodium deoxycholate, 140 mM NaCl, 1X Triton,supplemented with 1 mM phenylmethylsulfonylfluoride and protease inhibitors (all from Sigma)). Cell lysates were incubated on ice for 20 minutes and vortexed, then were centrifuged at 6600 g for 20 minutes to remove cell debris. Protein concentration was determined with the Bradford Reagent (Sigma). 2mg/ml of cell lysates were incubated overnight at 4°C with 2 μg of anti-p63 (H-129 sc-8344, Santa Cruz), anti–p300 (Santa Cruz) and anti acetyl-lysine (Cell Signaling). The immuno-complexes were collected by incubating with a mix of Protein A Agarose and Protein G Sepharose (Sigma) overnight at 4°C. The beads were washed three times: the first wash with RIPA buffer and the others with PBS. The beads were then resuspended in 2X Loading buffer loaded directly on a 10% SDS polyacrylamide gel and subjected to western blot with the indicated antibodies.

ChIP assay

For ChIP assays, U2OS cells were used. ChIP assays were performed as described previously (42). Briefly, after fixing in 1% formaldehyde, cells were lysed for 5 minutes in 50 mM Tris, pH 8.0, 2 mM EDTA, 0.1% NP-40, and 10% glycerol supplemented with protease inhibitors (all from Sigma). Nuclei were re-suspended in 50 mM Tris, pH 8.0, 1% SDS, and 5 mM EDTA. Chromatin was sheared by sonication, centrifuged, and diluted 10-fold in 50mM Tris, pH 8.0, 0.5% NP-40, 0.2 M NaCl, and 0.5 mM EDTA. After pre-clearing with a 50% suspension of salmon sperm-saturated protein A, lysates were incubated at 4C overnight with anti-p63 (H137, Santa-Cruz). Immune complexes were collected with sperm-saturated protein A, washed three times with high salt buffer (20mM Tris, pH 8.0, 0.1% SDS, 1% NP-40, 2mM EDTA, and 500mM NaCl), and three times with

1Tris/EDTA (TE). Immune complexes were extracted in TE containing 1% SDS, and protein–DNA cross-links were reverted by heating at 65C overnight. DNA was extracted by phenol–chloroform, and 1/20th of the immunoprecipitated DNA was used in each PCR reaction. PCR reactions were performed for 25–35 cycles of denaturation at 95 C for 45seconds, annealing at 55–57 C for 45seconds, and extension at 72 C for 45seconds. Primer sequences are reported in Table S3.

RNA extraction and RealTime qPCR

For quantitative Real-time qPCR total RNA was extracted from U2OS cells with the TRI Reagent (Sigma). 1 μg of total RNA was reverse-transcribed using SuperScriptIII cDNA Preparation Kit (Life-Technology). RealTime quantitative PCR (qPCR) was performed with SybrGreen supermix (BIORAD). *Tubulin* mRNA was used for normalization. For Real-Time qPCR reaction the sequence of the primer pairs are described in Table S2.

***In vitro* acetylation assay**

In vitro acetylation assay was performed following instructions provided by Fluorescent HAT Assay Kit (Active Motif, 56100). The purified recombinant p300 catalitic domain was incubated with acetyl-CoA and specific synthetic substrate peptides. The developer solution used reacts with the free sulfhydryl groups in the CoA-SH producing fluorescence, which is red by a fluorometer. Peptides containing the sequence of interest of p63 protein, along with positive and negative controls were designed and purchased from GeneScript. Sequence are reported in FIG 3. For fluorescence reading, a BF10000 Fluorocount was used.

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Figure legends

Figure 1. ΔNp63α protein is acetylated in human keratynocytes and osteosarcoma cells

A. Western Blot (WB) analyses of whole HaCat cell extracts treated with increasing amounts of Trichostatin (TSA) (5 ng/ml and 10 ng/ml) for 5 hours or Valproic acid (VPA) (0,5 mM and 1 mM) for 3 hours. **B**. WB analysis of U2OS cell extracts transfected with an equal amount of ΔNp63α ex pressing vector and then treated with increasing amounts of Trichostatin (TSA) (5 ng/ml and 10 ng/ml) for 5 hours or Valproic acid (VPA) (0,5 mM and 1 mM) for 3 hours. **C**. Whole cell extracts from HaCat cells treated with 5ng/ml of TSA for 5 hours were analyzed by immunoprecipitation of endogenous ΔNp63α with an anti p63 antibody follone by WB analysis with an anti- acetylated lysines.

Figure 2. p300 positively regulates ΔNp63α protein stability and half-life

A. WB analysis of whole HaCat cell extracts transiently transfected with increasing amounts of shRNA ex pressing vector (shp300: 20ng and 40 ng or shLuc as a control). **B**. (left) WB analysis of whole HaCat cell extracts transiently transfected with increasing amounts of p300 expressing vectors (10 and 20 ng) (right). WB analysis of U2OS whole cell extracts transiently co-transfected with equal amounts of ΔNp63 expressing vectors and increasing quantity of p300 encoding plasmids. **C**. WB analysis of U2OS whole cell extracts transiently co-transfected with ΔNp63α and p300 expressing vectors. Protein hal-life was measured by treating the cells with 10 μM Cycloheximide (CHX) either in the absence or presence of p300 for the indicated times. **D.** Whole U2OS cell extracts transiently co-transfected with ΔNp63α expressing vectors either in the absence or in the presence of p300 were analyzed by immuno-precipitation with an anti p300 antibody followed by WB analysis with an anti-p63 antibody.

Figure 3. p300 acetylates p63K193 *in vitro*

A. Alignment of the p53 K164 flanking region of the human p53 protein with those of p53 from other specie and of human p63 and p73. The conserved lysine is marked in bold (h: human; m: mouse). **B**. *In vitro* acetylation assay performed according to the HAT assay kit protocol (Active Motif) with an H4 peptide and p53 peptide (positive controls), H4 plus anacardic acid 15 μM (HAT inhibitor) and p63 peptides (peptide sequences are reported in the figure). **C**. WB analysis of U2OS whole cell extracts transiently co-transfected with ΔNp63α and ΔNp63αK193E expressing vectors (30 ng) and increasing amounts of p300 encoding plasmid (10 and 20 ng).

Figure 4. ΔNp63αK193E mutation alters p63 transcriptional activity in a promoter specific manner.

A. Luciferase assay performed in U2OS cells transiently co-transfected with the -1200 bp Dlx5 reporter promoter (200 ng) in the presence of ΔNp63 or ΔNp63K193E (50 ng) together with increasing amounts of p300 (5, 10 and 20 ng) expressing vectors. Cells were lysed 24 hours after transfection and luciferase activity was determined. The basal activity of the reporter plasmid was set to 1. Data are presented as fold activation/repression relative to the sample without effector. Each histogram bar represents the mean of three indipendent transfection duplicates. Standard deviation are indicated. **B**. Luciferase assay performed in U2OS cells transiently co-transfected with the -1200 bp *Dlx5* and *p57kip2* reporter promoters (200 ng) in the presence of increasing amounts of ΔNp63 or ΔNp63K193E (50 ng) plasmids. Cells were lysed 24 hours after transfection and luciferase activity was determined. The basal activity of the reporter plasmid was set to 1. Data are presented as fold activation/repression relative to the sample without effector. Each histogram bar represents the mean of three indipendent transfection duplicates. Standard deviations are indicated.

Figure 5. The ΔNp63αK193E mutant displays an altered DNA binding activity and transcriptional activity on developmental related genes

A. Expression of *Casp10, Ikkα, Perp, Egfr* and p53 analyzed by Real-Time qPCR in U2OS cells stably transfected with pCDNA3 (empty vector), ΔNp63α and ΔNp63αK193E. ΔNp63αp63 wt and mutant proteins expression was confirmed by WB analysis. **B**. U2OS cells were stably transfected with the pCDNA3 (empty vector), ΔNp63α and ΔNp63αK193E. Cells were subjected to ChIP, and the recovered chromatin was amplified with *Perp, EgfR, p53, p21* and *Casp10* promoter-specific primers.

Figure 6. FGF8 positively regulates ΔNp63α protein stability inducing its interaction with c-Abl and promoting p63 acetylation

A. WB analysis of whole cell extracts of HaCat cells treated with increasing amounts of FGF8 (0,5 ng/ml and 1 ng/ml) for 3 hours. **B**. WB analysis of U2OS whole cell extracts transiently transfected with ΔNp63α and ΔNp63αK193E encoding plasmids. 24 hours after transfection U2OS cells were treated with increasing amounts of FGF8 for 2 hours (0,5 ng/ml and 1 ng/ml). **C**. WB analysis of HaCat cells whole cell extracts stably transfected with an shRNA against c-Abl or shLuc and treated with increasing amounts of FGF8 (0,5 ng/ml and 1 ng/ml) for 3 hours. **D**. Whole U2OS cell extracts transiently co-transfected with ΔNp63α and ΔNp63α3Y encoding vectors in the presence of p300 overexpression and analyzed by co-IP with an antibody against p300 followed by WB analysis with an anti-p63 antibody. **E**. WB analysis of U2OS whole cell extracts transiently co-transfected with ΔNp63α and ΔNp63α3y expressing vectors and increasing amounts of p300 encoding plasmid (10 and 20 ng). **F**. Whole HaCat cell extracts treated with FGF8 (0,5 ng/ml) or DMSO (control) for 3 hours were analyzed by immunoprecipitation of endogenous p63 followed by WB analyses with the indicated antibody (anti-c-Abl antibody and anti ac-lys antibody).

Figure 7. FGF8 positively regulates ΔNp63α protein stability in mice embryonic limb buds.

A. Forelimbs (FLs) were isolated at E10.5, put in cultured and treated with increasing amounts of FGF8 (0,5 μg/ml and 1μg/ml) for 24 hours. **B.** Our model link together FGF8, c-Abl and p300 in a regulatory pathway leading to ΔNp63α stabilization and transcriptional activation in emvbryonic limb buds. Exposure of AER cells to FGF8 induces a signalling intracellular cascade leading to c-Abl activation: once activated c-Abl phosphorylates ΔNp63α on tyrosine residue. This phosphorylation event is indispensible for the interaction of ΔNp63α with the p300 acetyl-transferases and consequent acetylation: essential for ΔNp63α stabilization and transcriptional activation. In the absence of FGF8, ΔNp63α interacts with the prolyl isomerase PIN1, determing ΔNp63α degradation through the proteasome. This pathway induced by FGF8 treatments could be indispensible for the regulation of the ΔNp63α protein stability and the consequent expression of ΔNp63α target genes regulating limb development. The activation of this pathway could be essential for AER stratification and limb outgrowth.

Figure S1. Growth suppression by expression of ΔNp63α and ΔNp63αK193E. p53, ΔNp63α, ΔNp63αK193E and pcDNA expression vectors (0.3 g each) were transfected into U2OS cells. After 2 weeks of selection with G-418 (Neomicin, Formedium), colonies were fixed and stained to demonstrate suppression of colony formation.

Figures

Fig.1

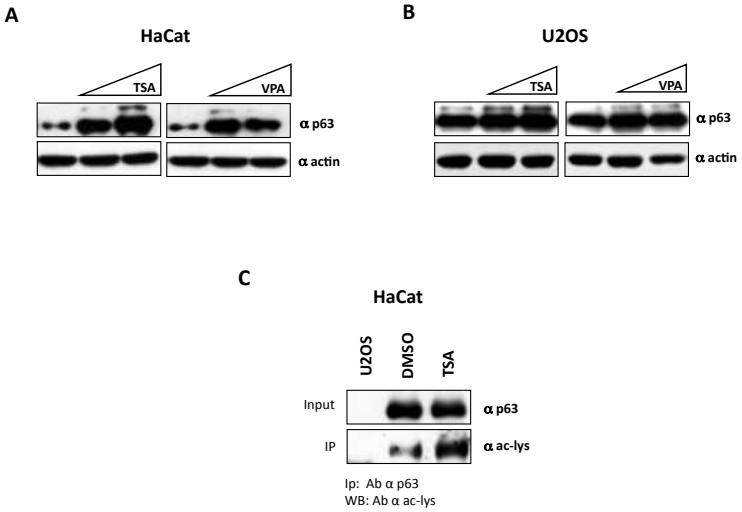

Fig.2

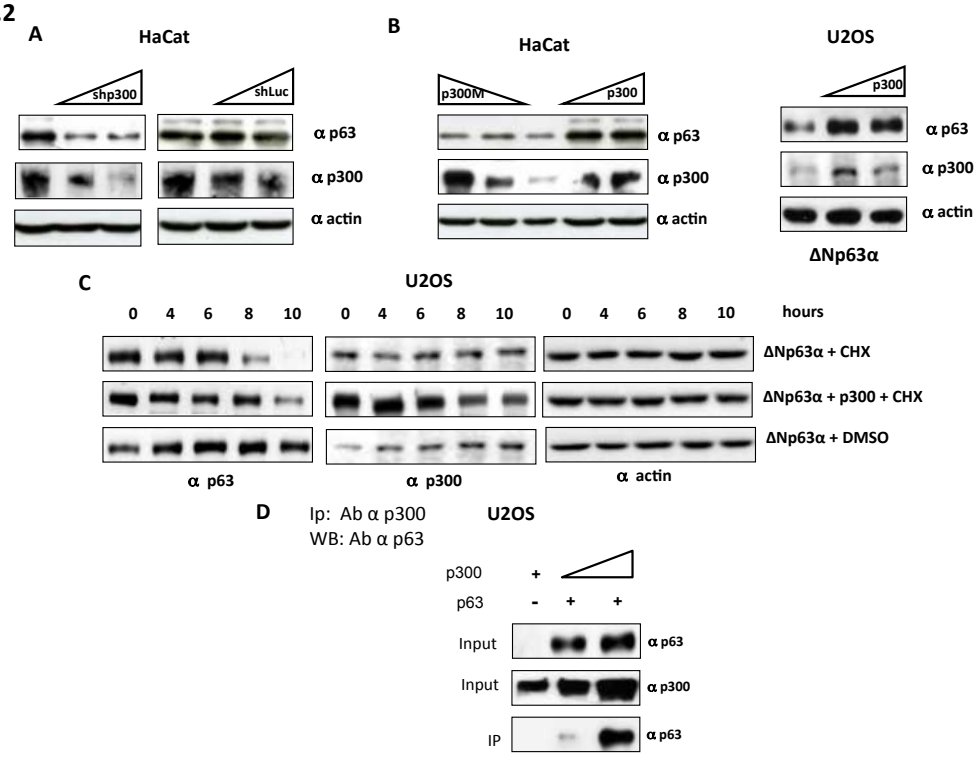

Fig.3

A

h- p53 154 GTRVRAMAIYKQSQHMTEVVR 174
m- p53 151 GSRVRAMAIYKKSQHMTEVVR 171

p63 181 GAVIRAMPVYKKAEHVTDVVK 203
p73 172 GTAIRAMPVYKKAEHVTDVVK 192

B **Peptide sequences**

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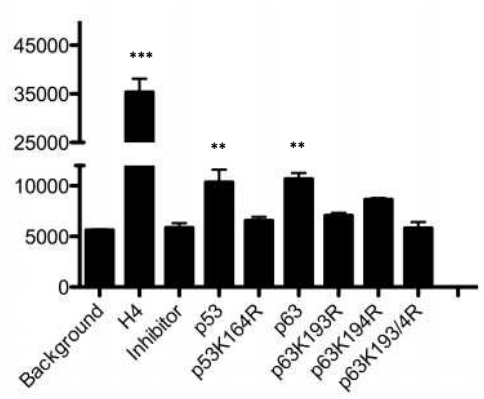
p53 VRAMAIYKQSQHMT
p63 IRAMPVYKKAEHVTE



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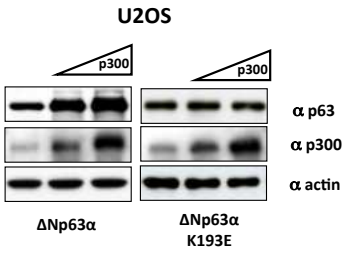

Fig.4

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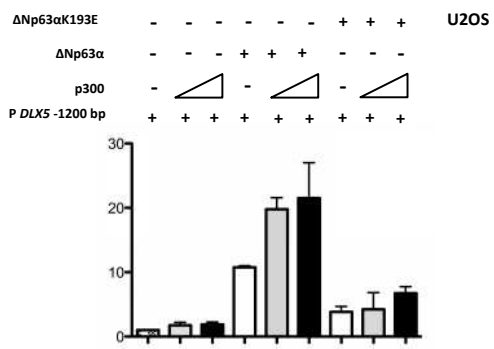

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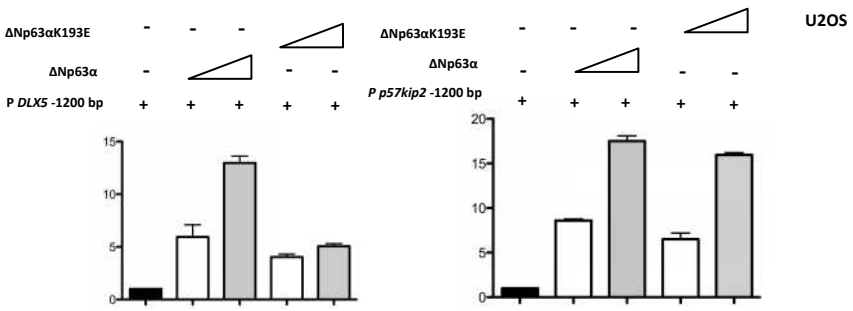
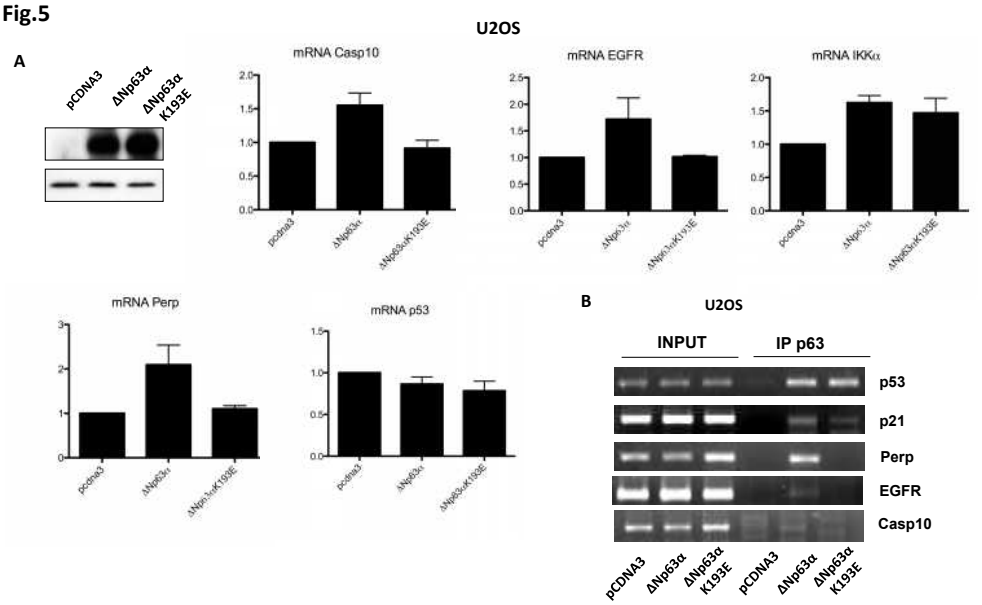


Fig.5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mRNA Perp
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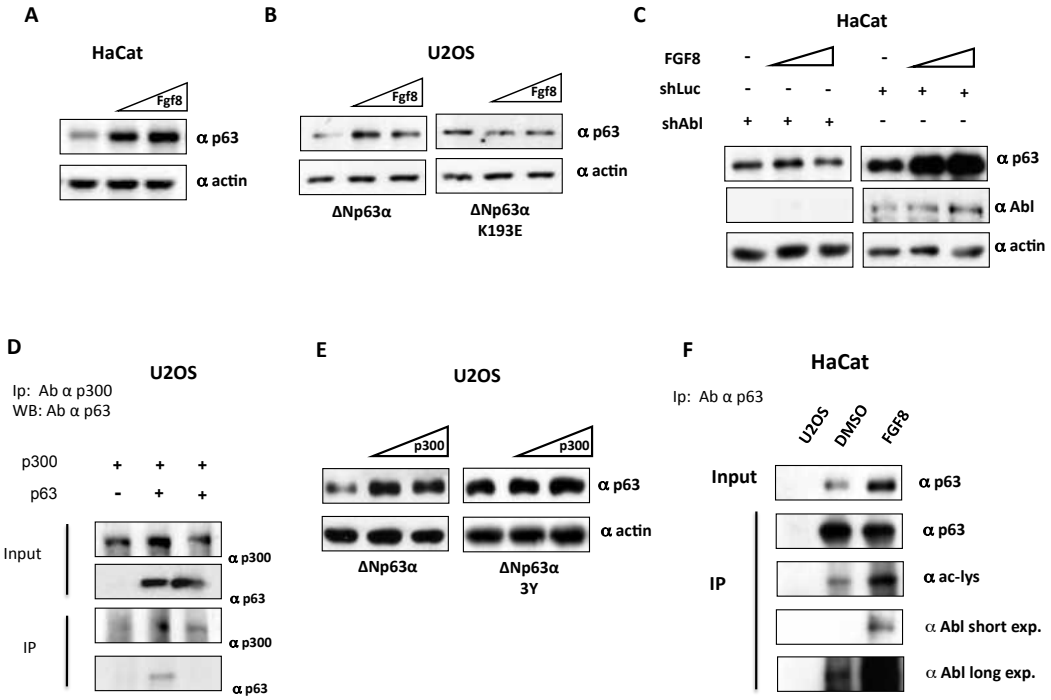
Fig.6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α actin
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ΔNp63α
ΔNp63α K193E
C
HaCat
FGF8
shLuc
shAbl
α p63
α Abl
α actin
D
U2OS
Ip: Ab α p300
WB: Ab α p63
p300
p63
Input
IP
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α p63
E
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α p63
α actin
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Fig.7

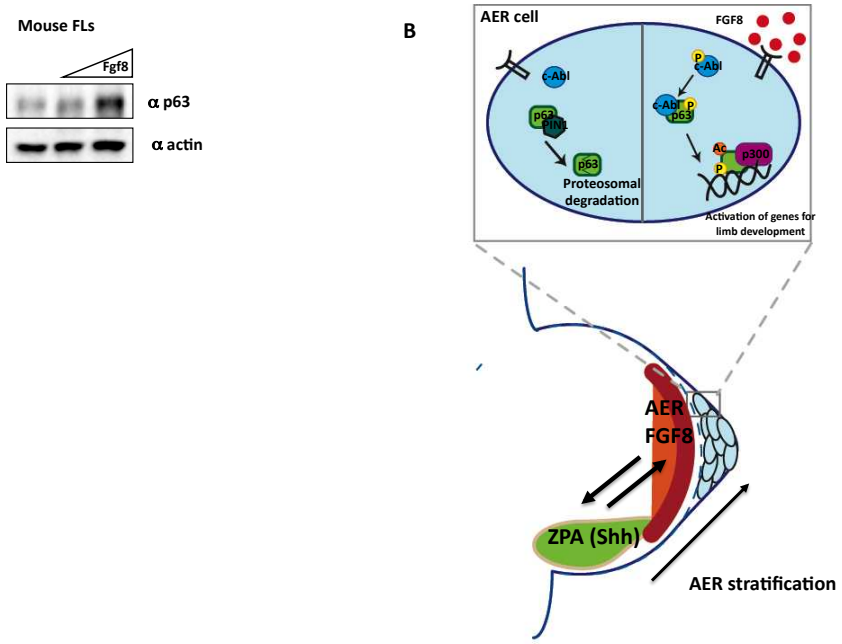

Fig. S1

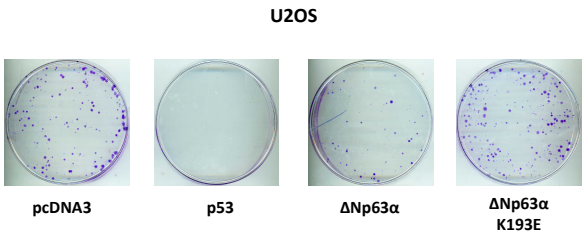

Table S2

PRIMERS FOR REAL-TIME qPCR

Gene (human)	Sequence (5' to 3')
Tubulin FOR	CTTCAGTGAGACGGGCGCTGGCAAGCAC
Tubulin REV	TGATGAGCTGCTCAGGGTGGAA
Ikka FOR	TTCGGGAACGTCTGTCTGTACC
Ikka REV	GGCACCATCGTTCTCTGTTT
Perp FOR	GACCCCAGATGCTTGTCTTC
Perp REV	AAAGCCGTAGGCCCAGTTAT
p53 FOR	ACATGACGGAGGTTGTGAGG
p53 REV	CCAAATACTCCACACGCAAA
Casp10 FOR	ACCCGACAAAGGGTTTCTCT
Casp10 REV	GCCAGGAAACTTAGGGAGGT
EgfR FOR	CCCAGTACCTGCTCAACTGGT
Egfr REV	TGCCAGGTCGCGGTG

Table S3

PRIMERS FOR ChIP

Gene (human)	Sequence (5' to 3')
Ikka FOR	(see ref. 57)
Ikka REV	(see ref. 57)
Perp FOR	AGGTGGAACACCACACCCTA
Perp REV	CGGGGATATTGGCAGAACTA
p53 FOR	AGAGTGTGGGATTCGTGAGC
p53 REV	CCAGGGACGAGTGTGGATAC
Casp10 FOR	GAACAGGCAAAGAAGGTGGT
Casp10 REV	AGGTTGCAGTGAGCCAAAAT
EgfR FOR	AATGGAAGAATCGGGTGTTG
Egfr REV	AAAGAGGAGCTGCCCTAACC