UNIVERSITY OF NAPOLI FEDERICO II

Doctorate School in Molecular Medicine

Doctorate Program in Genetics and Molecular Medicine
Coordinator: Prof. Lucio Nitsch
XXVII Cycle

"Molecular mechanisms at the basis of epidermal defects and skin fragility in AEC syndrome"

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Napoli 2015

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LIST OF PUBLICATIONS

Epidermal cell junctions and their regulation by p63 in health and disease, Giustina Ferone, **Maria Rosaria Mollo** and Caterina Missero, Cell and Tissue Research- Special Issue Review, 2015 Feb 3. PubMed PMID: 25645146.

p63-dependent and independent mechanisms of nectin-1 and nectin-4 regulation in the epidermis, **Maria Rosaria Mollo**, Dario Antonini, Karen Mitchell, Paola Fortugno, Antonio Costanzo, Jill Dixon, Francesco Brancati and Caterina Missero, Experimental Dermatology, 2015, 24, 114–119. doi: 10.1111/exd.12593. PubMed PMID: 25387952; PubMed Central PMCID: PMC4329386.

p63 control of desmosome gene expression and adhesion is compromised in AEC syndrome, Giustina Ferone, **Maria Rosaria Mollo**, Helen A. Thomason, Dario Antonini, Huiqing Zhou, Raffaele Ambrosio, Laura De Rosa, Domenico Salvatore, Spiro Getsios, Hans van Bokhoven, Jill Dixon and Caterina Missero, Hum Mol Genet. 2013 Feb 1;22(3):531-43. doi: 10.1093/hmg/dds464. Epub 2012 Oct 29. PubMed PMID: 23108156; PubMed Central PMCID: PMC3542863.

LIST OF ABBREVIATIONS

AEC syndrome Ankyloblepharon- Ectodermal defects- Cleft lip/palate syndrome
+/L K14-Cre;p63$^{+/floxL514F}$
L/L K14-Cre;p63$^{floxL514F/floxL514F}$
Krt5 Keratin 5
Krt14 Keratin 14
Krt1 Keratin 1
Krt10 Keratin 10
Krt6 Keratin 6
Krt16 Keratin 16
Krt17 Keratin 17
Lor Loricrin
Ivl Involucrin
Flg Filaggrin
Cdh1 E-cadherin
H&E Haematoxylin and Eosin
Krt15 Keratin 15
Dsg1 Desmoglein1
Dsp Desmoplakin
Dsc3 Desmocollin 3
IF Intermediate Filaments
AJ Adherens Junctions
TEM Tissue-electron mycroscopy
NICD Notch intracellular domain
NEXT Notch Extracellular Truncation
qRT-PCR quantitative real time RT-PCR
Tslp Thymic stromal lymphopoietin

ABSTRACT

AEC syndrome is a rare autosomal dominant disorder, characterized by skin erosions, ectodermal dysplasia and cleft lip and/or palate. Missense mutations in the p63 gene, a key regulator of stratified epithelia, are causative of the disorder. The pathogenesis and the biological mechanisms underlying the skin erosions of AEC syndrome have been elusive. We generated a conditional knock-in mouse model carrying an inducible L514F mutation, found in AEC patients. $p63^{+/floxL514F}$ mice were crossed with the K14-Cre knock-in mouse line to obtain $p63^{L514F}$ expression soon before birth. Newborn mutant mice were indistinguishable from their wild-type littermates, however few days after birth focal skin blistering and scaling was observed accompanied by weight loss and often by death. Histological analysis and dye penetration assays revealed focal disruption of the epidermal barrier, followed by severe skin inflammation and epidermal hyperplasia. Consistently with the focal gaps in the epidermis of mutant mice, we found a strong reduction of desmosomal component Dsg1, Dsc3 and Dsp and a downregulation of two components of adherens junctions, Pvrl1 and Pvrl4 in AEC mutant keratinocytes. Similar results were obtained in human keratinocytes derived from AEC patients, indicating impaired cell-cell adhesion is AEC syndrome. In addition, we found an unbalance in the basal keratins. The intermediate filaments keratin5 (Krt5) and keratin14 (Krt14) are essential to withstand mechanical stress in the epidermis and are known p63 target genes. Both Krt5 and Krt14 expression was strongly downregulated in AEC mutant keratinocytes and in p63 knockdown, whereas in AEC mutant epidermis only Krt5 was significantly affected indicating that in vivo p63 plays a crucial function in Krt5 regulation. Importantly, we found a strong reduction in KRT5 expression also in human AEC patients, thus indicating impaired intermediate filaments network in AEC syndrome. Consistent with a reduction in Krt5, a reduced number of keratin bundles were observed by tissue-electron microscopy (TEM) in the basal compartment of the epidermis.

Skin fragility lead to focal failure of epidermal barrier, causing a progressive inflammation characterized by hyperplasia and hyperkeratosis in the skin of mutant mice. Clear signs of severe inflammation were preceded by strongly elevated levels of Thymic stromal lymphopoietin (Tslp), an IL-7 like cytokine, known to cause systemic defects which were observed in AEC mice and in at least one AEC patient. Strong induction of Tslp was associated with reduced Notch expression. Interestingly, reactivation of the Notch pathway could reduce Tslp expression in mutant keratinocytes.

Together these data support the hypothesis that the basal cell fragility and blistering observed in p63 mutant mice and AEC patients are due to alteration in adhesion molecules belonging to different categories. These results together with reduced Notch signaling lead to excessive production of the proinflammatory molecule Tslp.

1. BACKGROUND

1.1 The epidermis

The epidermis is the uppermost, multilayered compartment of the skin that serves as a protective barrier against external environmental insults, mechanical trauma and dehydration. The skin epidermis rests on the basement membrane rich in extracellular matrix, which separates the epidermis and its appendages from the underlying dermis. It is composed of 4-5 layers depending on the region of skin being considered. Those layers in descending order are the cornified layer (stratum corneum), granular layer, spinous layer and basal layer (Fig. 1). The stratified squamous epithelium of the epidermis is maintained by cell division within the basal layer. During the epidermal maturation the cells undergo a complex series of morphological and biochemical changes that result in the production of the stratum corneum, the highly cornified outermost layer of the tissue composed by cornified cell envelopes assembled by cross-linking of structural proteins and lipids. More in detail, the cells of the basal layer remain attached to an underlying matrix through a series of adhesion molecules such as hemidesmosome, laminin and anchoring fibrils, and proliferate. Some of their daughter keratinocytes enter the spinous layer through asymmetric mitoses, where they exit the cell cycle, grow larger and establish robust intercellular connections (Simpson et al., 2011). The cells switch expression of basal layer specific keratins, Keratin5 and Keratin 14, to Keratin1 and Keratin10 (Krt1; Krt10) typical of the spinous layer (Fuchs and Green, 1980). In addition other keratins are also expressed suprabasally, as Keratin6, Keratin16 and Krt17 (Krt6; Krt16 and Krt17), but only in hyperproliferative condition such as wound healing. The spinous layer also contains Langerhans cells, which are derived from a precursor in bone marrow and are involved in immune response. Above the spinous layer, keratinocytes contain numerous electrondense keratohyalin granules packed with the protein profilaggrin in their cytoplasm, hence the name “stratum granulosum”. Cells in the granular layer flatten and assemble a water-impermeable cornified envelope underlying the plasma membrane and express filaggrin (Flg) and loricrin (Lor) as markers. In addition, cornified envelope proteins, which are rich in glutamine and lysine residues, are synthesized and deposited under the plasma membrane of the granular cells. As response to the increased permeability to the calcium, the cells activate transglutaminase, generating □-glutamyl □-lysine crosslinks to create an indestructible barrier. Finally, stratum corneum keratinocytes release lysosomal enzymes to degrade major organelles, including the nucleus. The final steps of terminal differentiation involve also the extrusion of lipid bilayers, packaged in lamellar granules, onto the scaffold of the cornified envelope. The dead stratum corneum cells create an impenetrable layer that is continually replaced (Fuchs, 2007).

Keratinocytes move from the proliferative basal layer through the granular layer and continually replaced the cells of the outward layers. Indeed, as the body's outer frontier, the epidermis is subject to repeated trauma that must be repaired after wounding. (Shen et al., 2013). Within this layer, epidermal stem cells divide to self-renew and produce transient amplifying (TA) cells, which possess a more limited proliferative capacity. Transit amplifying cells constitute the major cell type in the basal layer of the developing and mature epidermis and after few rounds of cell division they exit from the cell cycle, and initiate a terminal differentiation program, as they migrate outward toward the tissue surface.

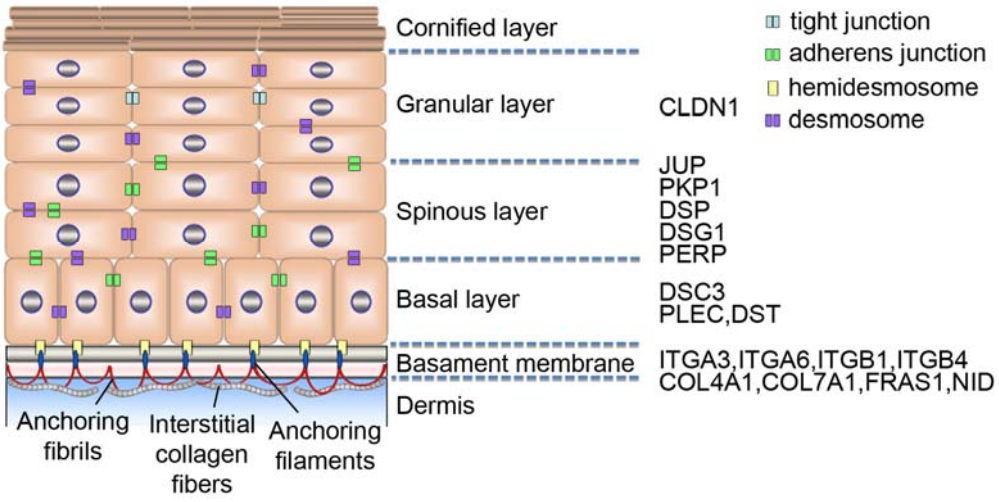


Figure 1. Epidermal architecture. The epidermis is a stratified epithelia composed by the indicated layers and is separated from the dermis by the basement membrane that is functionally organized in the lamina lucida (gray) and the lamina densa (pink). Each layer of the epidermis is characterized by specific cell–matrix and cell– cell adhesion molecules. Here, we represent adherens junction (green), desmosome (violet) and tight junction (blue) that are required for cell–cell contacts and hemidesmosomes (red) that connect basal cells to the basement membrane. Anchoring filaments (blue) span the basement membrane contacting anchoring fibrils (red) that connect the lamina densa to the anchoring plaques within the dermis (not represented). p63 direct target genes involved in cell adhesion and the localization of their protein products are shown (from Ferone et al., 2015).

1.2 Structure and function of p63

p63 is a tetrameric transcription factor belonging to the p53 gene family consisting of three genes, p53, p63, and p73 (Yang et al., 2002). They share three functional domains: an N-terminal transactivation domain which shares 25% homology with N-terminal part of p53, a central DNA binding domain

which shares 65% of homology with the corresponding p53 domain and C-terminal tetramerization domain, which shares 35% of homology with the oligomerization domain of p53 (Yang et al., 2002). p63, p53 and the third member p73, constitute a family of key transcriptional regulators in cell growth, differentiation and apoptosis. Indeed, while p53 is a major player in tumorigenesis (Kemp et al., 1994), p63 and p73 encompass pivotal roles in embryonic development (Mills et al., 1999; Yang et al., 2000). More in detail, p63 has a crucial role in embryonic development of stratified epithelia where it can function either as an activator or a repressor (Koster and Roop, 2004; LeBoeuf et al., 2010). In particular, p63 is a master regulator of epidermal gene expression and it is critical for cell proliferation (Truong et al., 2006; Senoo et al., 2007; Antonini et al., 2010), cell adhesion (Carroll et al., 2006; Koster et al., 2007) and stratification (Koster and Roop, 2004; Truong et al., 2006) while suppressing terminal differentiation (Nguyen et al., 2006).

p63 consists of 16 exons located on chromosome 3q28. It has two independent transcriptional start sites that give rise to two different N-termini isoforms: TA and □N that in turn produce □□□□ or □□ends as the result of alternative splicing events towards the C-terminal region, therefore at least six different protein isoforms can be produced (Yang et al., 1998). In particular, TAp63 contains an acidic N-terminal transactivation domain similar to the one found in the canonical full-length p53. TAp63 is expressed at very low levels in most tissues, whereas it is constitutively expressed in female germ cells where it is activated by phosphorylation upon DNA damage, inducing oocyte death (Suh et al., 2006; Gonfloni et al., 2009; Kerr et al., 2012). In contrast ΔNp63 is abundant in the basal regenerative layer of the epidermis and other stratified epithelia, and it is virtually absent in other tissues (Yang et al., 1998). ΔNp63 is by far the most abundant protein in keratinocytes and in the epidermis whereas the TAp63 is barely detectable by RNA-seq (Rizzo et al., 2014). The first 26 amino acids of the N-terminal region in ΔNp63 are required for its transcriptional activity, together with the proline-rich domain (PR) and a domain (TA2) located between the OD and the SAM (sterile-α-motif) domain (Dohn et al., 2001; Ghioni et al., 2002; Helton et al., 2006). The main isoform expressed in stratified epithelia is the p63α that contains at the C-terminal end a SAM domain and a post-SAM domain (PS) absent in p63ß, p63γ, or in p53 (Yang et al., 1998). SAM domains have diverse functions and can interact with themselves, bind to other SAM domains, bind to non–SAM domain proteins, or to RNA (Qiao and Bowie, 2005). The SAM domain in p63 is a five helical bundle domain, is unable to form homodimers (Chi et al., 1999; McGrath et al., 2001) and its function remains uncovered. The PS domain is also called transcriptional inhibitory domain (TID) and it is composed of two elements. The first interacts with the TA domain of another TAp63 molecule, forming a closed and inactive dimer that can be activated by phosphorylation, resulting in an active tetramer (Su et al., 2010; Deutsch et al., 2011). The other element controls p63 protein concentration via a sumoylation-dependent mechanism

(Straub et al., 2010). However, the functional role of the PS domain in the context of the ΔNp63α that lacks the TA domain is poorly understood.

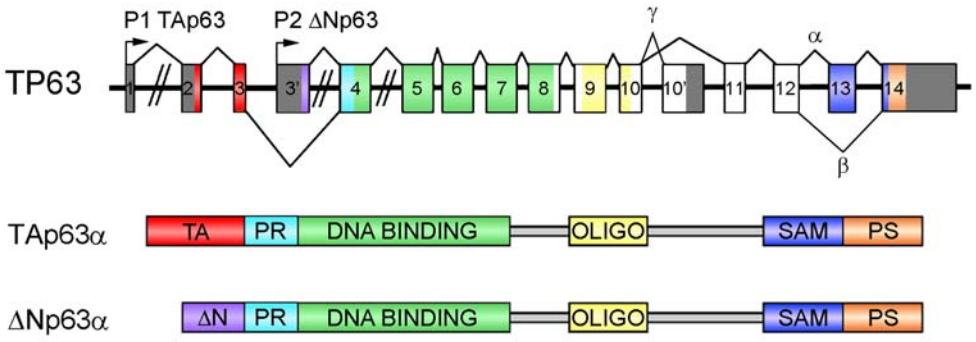


Figure 2. ***p63*** **gene and protein structure.** a) *p63* gene structure showing the two transcription start sites (P1 and P2) which give rise to TAp63 and ΔNp63 transcripts, and the splicing variants at the 3' end, which lead to the □□□□ and □□isoforms. In the lower panel, the TAp63□ and ΔNp63□□□□□□□□□□ isoforms are represented. Conserved protein domains are: Transactivation domain (TA); proline rich domain (PR); DNA-binding domain (DBD); Oligomerization domain (OD); SAM (Sterile Alpha Motif) domain; post-SAM domain (PS) (from Ferone et al., 2015).

1.3 Phenotype of p63 deficient mice

The generation of p63-deficient mouse models by two independent groups shed light on the crucial role of p63 in the development of ectodermal derived tissues. Indeed, mice lacking the p63 gene die for dehydration soon after birth and display severe defects of all stratified epithelia and their derivatives, facial clefting and impaired limb formation, suggesting that p63 plays a non-redundant role in these tissues (Mills et al., 1999; Yang et al., 1999). Defects in the surface epithelium of p63-null mice have been ascribed to loss of proliferative potential of keratinocyte stem cells (Yang et al., 1999; Senoo et al., 2007), and/or altered epidermal stratification and cell differentiation associated with reduced expression levels of Krt5/Krt14 and Krt1/Krt10 (Mills et al., 1999; Koster and Roop, 2004; Romano et al., 2009).

The knockout mouse models are born alive but have striking developmental defects. Their limbs are absent or truncated and also structures dependent upon epidermal mesenchymal interactions during embryonic development, such as hair follicles, teeth and mammary glands, are absent. Phalanges and carpals were absent in both the p63-homozygous mutant, whereas more proximal forelimb structures were slightly heterogeneous in the extent of the truncation in the two models. The femur and all distal skeletal elements were also absent. These defects are caused by a failure of the apical ectodermal ridge (AER) to

differentiate. The lack of a proper AER limb buds in p63 null mice results from a failure of the ectoderm to undergo growth and differentiation that give rise to this stratified epithelium. Indeed, several genes that are important in limb-bud outgrowth are not expressed, such as Fgfr8 (a marker of the AER) and Msx-1 (which expression in the mesenchyme depends on an ectodermal signal), or abnormally expressed, such as Lmx-1 (a marker of the dorsal limb mesenchyme) (Mills et al., 1999; Yang et al., 1999).

The skin in the knockout mouse model generated by McKeon's group lacks expression of the basal layer markers as Krt5 and Krt14 and also spinous layer markers Krt1 and Krt10. Neverthless, isolated patch of the epidermis showed the expression of late differentiation markers such as Loricrin, Involucrin, Filaggrin. The authors argue that p63 is required for the initial development and continued regeneration of the epidermis and that the loss of p63 in the tissues failed to maintain the proliferative potential of stem cells in skin (Fig. 3)(Yang et al., 1999). In addition, to reinforce this hypothesis the same authors in another study strongly showed that p63 is dispensable to maintain the proliferative potential of epithelia stem cells of the thymus and epidermis (Senoo et al., 2007).

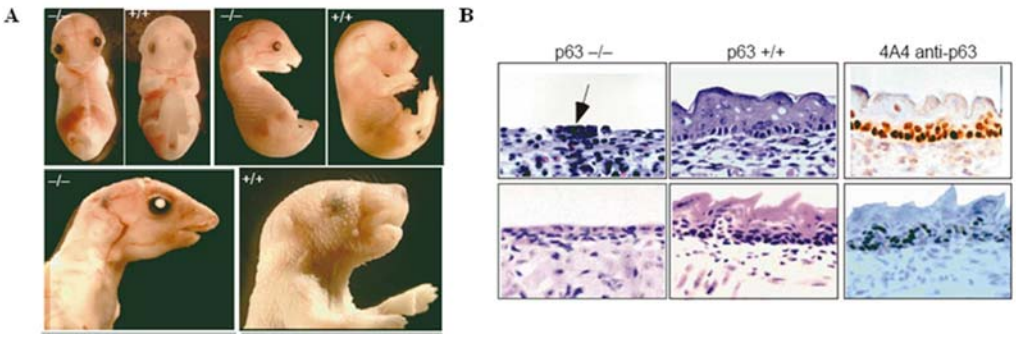


Figure 3. Phenotype of p63 knockout mice. A) The newborn p63 null mice show defects in limb formation and craniofacial defects associated with skin and appendages aberrant development for lack of stratification and differentiation. B) H&E staining of the epidermis at E17 display p63-/- mice lacking squamous stratification in the epidermis. Middle, wilde-type H&E control mice showing extensive stratification. In the right the basal staining with anti-p63 antibody to show the endogenus expression of p63 in the epidermis (adapted from Yang et al., 1999).

The knockout mice generated by Allan Bradley developped aberrant skin and appendages due to lack of stratification and differentiation (Fig. 4)(Mills et al., 1999). The authors showed that in p63 null mice all structures that required the ectodermal mesenchymal signal were compromised because the ectoderm failed to receive the signal. They showed that the skin of p63 null mice were

covered by a single disorganized layers of ectodermal cells or flattened epidermal cells in which they did not detect the expression of any early or late differentiation markers. Their results suggested that p63 is the determining factor of stratification, and supported the hypothesis that p63 is required for simple epithelial cells to commit to a stratified epithelial lineage during development (Mills et al., 1999).

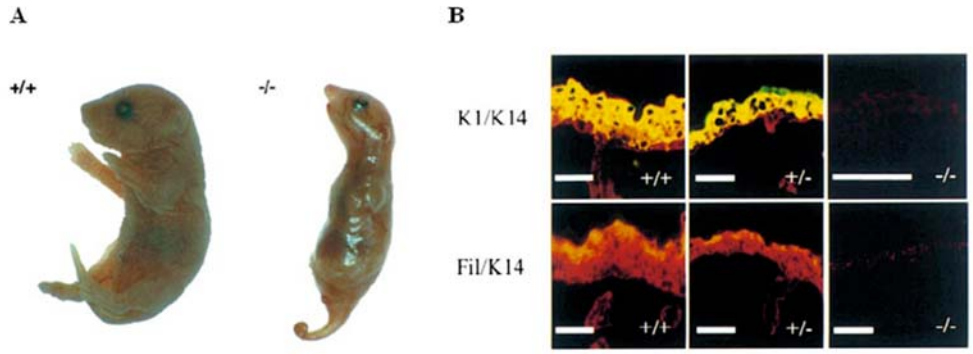


Figure 4: The phenotype of p63-deficent newborn mice. A) The p63 null mice show severe limb and skin defects. B) The expression of different markers in the epidermis of p63 null mice show the staining for Krt14 in red and Krt1 and Filaggrin (Fil) in green. Krt14 is weakly express in p63 null mice, whereas Krt1 and Fil are not detectable in the epidermis (adapted from Mills et al., 1999).

1.4 p63-associated syndromes

p63 plays an essential role in the regulation of ectodermal, orofacial and limb development. In particular, ΔNp63α is thought to play multiple essential roles in epidermal development and stratification, keratinocyte proliferation and differentiation, controlling expression of several epidermal genes including itself and other transcription factors (Yang et al., 1998; Antonini et al., 2006; Laurikkala et al., 2006; Nguyen et al., 2006; Truong et al., 2006; Koster et al., 2007; Moretti et al., 2010; Thomason et al., 2010; Romano et al., 2012). Not surprisingly, mice lacking the p63 gene die soon after birth with severe defects of skin and all stratified epithelia and their derivatives, craniofacial abnormalities and impaired limb formation (Mills et al., 1999; Yang et al., 1999). Consistently, heterozygous mutations in the human p63 gene are responsible for several ectodermal dysplasia (ED) syndromes, which are congenital disorders characterized by abnormalities of two or more ectodermal structures, such as hair, nails, sweat glands and digits. In particular, mutations in the p63 gene can cause at least five different syndromes: Ectrodactyly, Ectodermal defects-Cleft lip/palate syndrome (EEC, OMIM 604292),

Ankyloblepharon-Ectodermal defects-Cleft lip/palate syndrome (AEC, OMIM 106260), Limb Mammary Syndrome (LMS, OMIM 603543), Acro-Dermato-Ungual-Lacrimal-Tooth syndrome (ADULT, OMIM 103285) and Rapp-Hodgkin Syndrome (RHS, OMIM 129400). Furthermore, two non-syndromic human disorders are caused by p63 mutations: isolated split hand/foot malformation (SHFM4, OMIM 605289) and recently non-syndromic cleft lip (Brunner et al., 2002; Rinne et al., 2009) (Fig. 5).

EEC syndrome is the most common p63-linked ED and is mainly caused by point mutation in the DNA binding domain (DBD) of the p63 gene and most likely alter the DNA-binding properties of the protein. EEC syndrome comprises limb malformation, ectodermal dysplasia (abnormal teeth, skin, hair, nails and sweat glands) and orofacial clefting (Rinne et al., 2007). Representative limb malformations are ectrodactyly and syndactyly. Ectodermal dysplasia is seen as lightly colored, sparse hair and absence of eyelashes, eyebrows and alopecia can be observed. Skin is thin and dry, sometimes resembling dermatitis.

LMS was the first p63 syndrome linked to chromosome region 3q27. Mutations in LMS are located in the N- and C-terminus of the p63 gene. The LMS phenotype comprises malformations of the hands and/or feet and hypoplastic nipples and/or mammary glands. Ectodermal defects are much less prominent than in EEC syndrome but mammary gland hypoplasia or aplasia is more frequent in LMS than in EEC. Lacrimal duct obstruction and dystrophic nails are frequently observed (59 and 46% respectively), hypohydrosis and teeth defects are detected in about 30%, but other ectodermal defects such as hair and skin defects are rarely detected if at all. About 70% of LMS patients have similar limb malformations as in EEC syndrome, and about 30% orofacial clefting, notably always in form of cleft palate(van Bokhoven and Brunner, 2002).

ADULT syndrome phenotype is most similar to LMS syndrome, although clear differences can be seen when observing larger families or patient population. The main difference with the other p63-associated syndromes is the absence of orofacial clefting and the presence of hair and skin defects. Neverthless, teeth, skin, nail, hair and lacrimal duct defects are constantly present in ADULT syndrome (100, 91, 100%, 53% and 67%, respectively). A point mutation in exon 8, changing R298 in the DNA binding domain into either a glutamine or a glycine has been found. While EEC syndrome mutations in the DNA binding domain impair the binding of p63 protein to DNA (Celli et al., 1999), arginine 298 is not located close to the DNA-binding interface, and mutation of this arginine does not affect DNA binding (Duijf et al., 2002). Two other mutations are located in the N-terminus.

RHS share most features with **AEC**, but is differentially classified because of the lack of skin erosions and the absence of ankyloblepharon in Rapp-Hodgkin syndrome. Other ED symptoms, such as orofacial clefting and the absence of limb malformations are similar to AEC. In both syndromes clefting in lip and/or palate is equally frequent. The strong overlap between AEC and RHS

suggest that they are variable manifestations of the same clinical entity (Bertola et al., 2004; Rinne et al., 2007). AEC and RHS mutations are located in the C-terminus of the p63 protein. They are either point mutations in the SAM domain or deletions in the SAM or PS domains (Celli et al., 1999; McGrath et al., 2001; Barrow et al., 2002; van Bokhoven and Brunner, 2002; Kantaputra et al., 2003).

SHFM is a pure limb malformation (ectrodactyly and syndactyly) condition, without orofacial clefting or ectodermal dysplasia. The non-syndromic SHFM4 is caused by several mutations, which are dispersed throughout the p63 gene Possibly, SHFM is caused by altered protein degradation, even though different degradation routes are involved (Rinne et al., 2007). A non-syndromic orofacial clefting type was also linked to p63 gene, R313G is the first mutation discovered (Leoyklang et al., 2006).

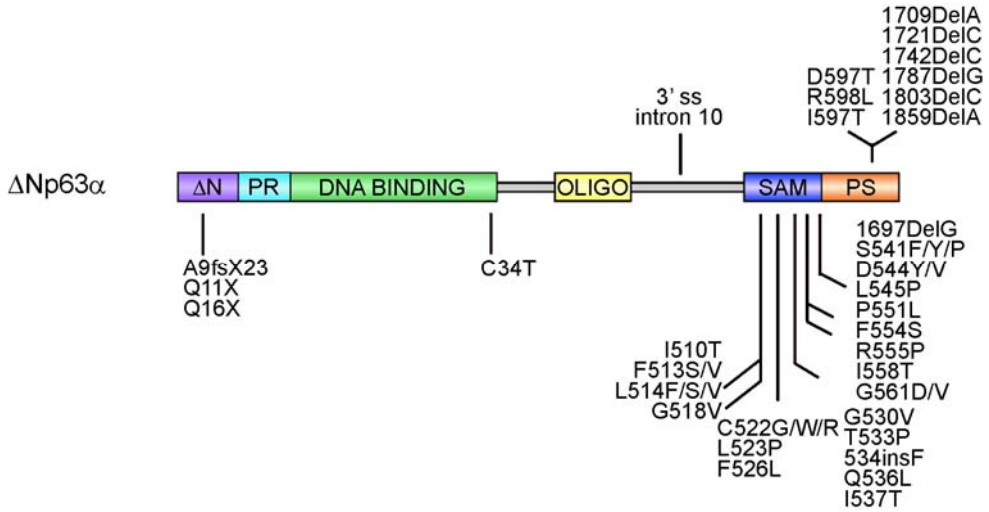


Figure 5. p63 and its mutations. Spectrum of p63 mutations causative of AEC syndrome. Most AEC mutations cluster preferentially in the SAM domain and PS domain; however some missense mutations were also identified at the 5' of the ΔNp63 isoform leading to premature termination codons, translation re-initiation, and truncation of a non-canonical transactivation domain in the DN-specific isoforms (from Ferone et al., 2015).

1.5 AEC syndrome

Ankyloblepharon-Ectodermal defects Cleft lip/palate syndrome (AEC), also known as Hay–Wells syndrome, was first reported by Hay and Wells in 1976(Hay and Wells, 1976). AEC syndrome is a rare autosomal dominant disorder, characterized by congenital fusion of the eyelids (ankyloblepharon), ectodermal dysplasia with severe involvement of the skin, and cleft palate with or without cleft lip. Clinical manifestations have different penetrance: about

75% of patients have severe skin erosions at birth, with some AEC patients reported to have up to 70% denuded skin. By 4 – 5 years age erosions could disappear, except for the head and auricular region. Clefting occurs approximately in 80% of AEC patients. The ankyloblepharon occurs only in 44% of AEC cases (Fig. 6). Hearing loss has been reported in about 40% of the patients. AEC patients have nails and teeth defects in about 75–80% of cases (Fig. 6). About half of the patients have lacrimal duct atresia. Dermatological features include thin and dry skin, often associated with congenital erythroderma, widespread skin erosions at or soon after birth, and erosive keratoderma of palms, soles and thin hair and/or alopecia. The most severe cutaneous manifestation of this disorder is the skin fragility associated with severe skin erosions after birth. Erosions typically involve the scalp, head and neck, skin folds, palms, and/or soles and are often accompanied by crusting and secondary infection (Fig. 6). Skin lesions are a distinctive signs of AEC syndrome. Adult patients can be affected by palmoplantar hyperkeratosis and erosive palmoplantar keratoderma with bleeding after extensive walking. The biological mechanisms underlying the skin erosions remain unveiled, and treatment is limited to gentle wound care and antibiotic treatment to prevent or cure infections. Healing is slow and recurrent breakdown is typical.

In AEC syndrome pathogenic mutations mainly fall in the C-terminus of p63 protein and include twenty-five missense and only two frameshift mutations in the SAM domain, whereas in the PS domain predominate the frameshift mutations that extend p63 protein (Rinne et al., 2009). These mutations give rise to mutant p63α proteins with dominant effects towards their wild-type counterparts. Recently, novel AEC causative mutations have been identified that result in translation re-initiation downstream of the non-canonical transactivation domain in the ΔN-specific isoforms, leading to expression of truncated ΔNp63 protein with dominant negative effects (Rinne et al., 2008).

AEC syndrome differs from the other conditions by the severity of the skin phenotype, the occurrence of ankyloblepharon, and the absence or mild limb malformations.

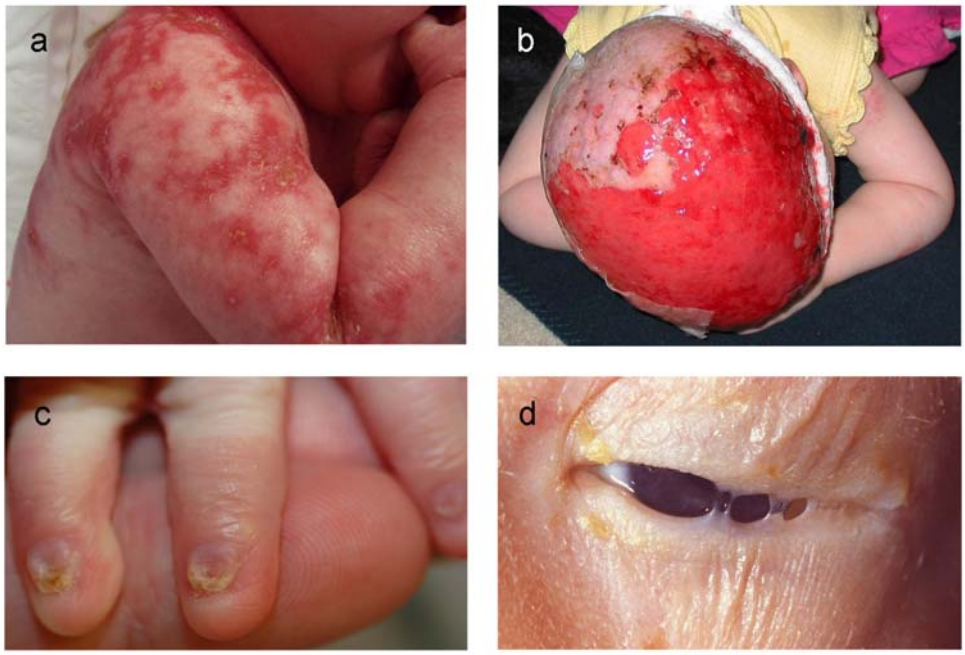


Figure 6. Clinical presentation of AEC infants. a) A close up of reticulated scarring that is typically seen in AEC patients as the results of skin erosions healing processes. b) Scalp erosions with hemorrhagic crusting, granulation tissue and secondary infection. c) Dystrophic fingernails of AEC patients, characterized by small nails with partial loss/absence and distal fraying of the nail plate. d) Focal fusion of the eyelids (ankyloblepharon) (Adapted from Ferone et al., 2015).

1.6 AEC mouse models

To study the functional activity of mutant p63 in AEC syndrome a first knock-in mouse model ($p63^{+/L514F}$) was generated in our laboratory (Ferone et al., 2012).
This model carries a phenylalanine substitution in position 514 (L514F) and closely resembles the human disease (Ferone et al., 2012). This mutation falls in the first helix of the SAM domain and disrupts the folding of the protein. Among the AEC causative mutations we decided to focus our attention on L514 amino acids for three reasons: first of all, this amino acid is mutated in three different amino acids (phenylalanine, valine or serine); this mutation affects an amino acid that is predicted to be buried inside the protein and has a small solvent accessible surface, so any mutation in this region is likely to affect the overall structure and stability of the protein by altering the packing of the helices, and moreover the substitution of a leucine with a phenylalanine probably cause a severe steric clash between two phenylalanine rings that are located close to each other (McGrath et al., 2001; Rinne et al., 2007).

$p63^{+/L514F}$ mouse model is characterized by hypoplastic and fragile skin, ectodermal dysplasia and cleft palate (Fig. 7). Ferone et al., 2010, found that epidermal hypoplasia and cleft palate are associated with a transient reduction in epithelial cell proliferation during development. These defects closely resemble those observed in the Fgfr2b-/- mice (De Moerlooze et al., 2000; van Bokhoven et al., 2001; Petiot et al., 2003; Rice et al., 2004; Candi et al., 2006). Since p63 transcriptionally controls the Fibroblast growth factor receptors Fgfr2 and Fgfr3 and their expression, they found that impaired FGF signaling downstream of p63 is likely an important determinant of reduced ectodermal cell proliferation and defective self-renewing compartment in AEC syndrome. Unfortunately, a neonatal lethality due to cleft palate prevented the generation of a mouse line and the studying of the adult phenotype.

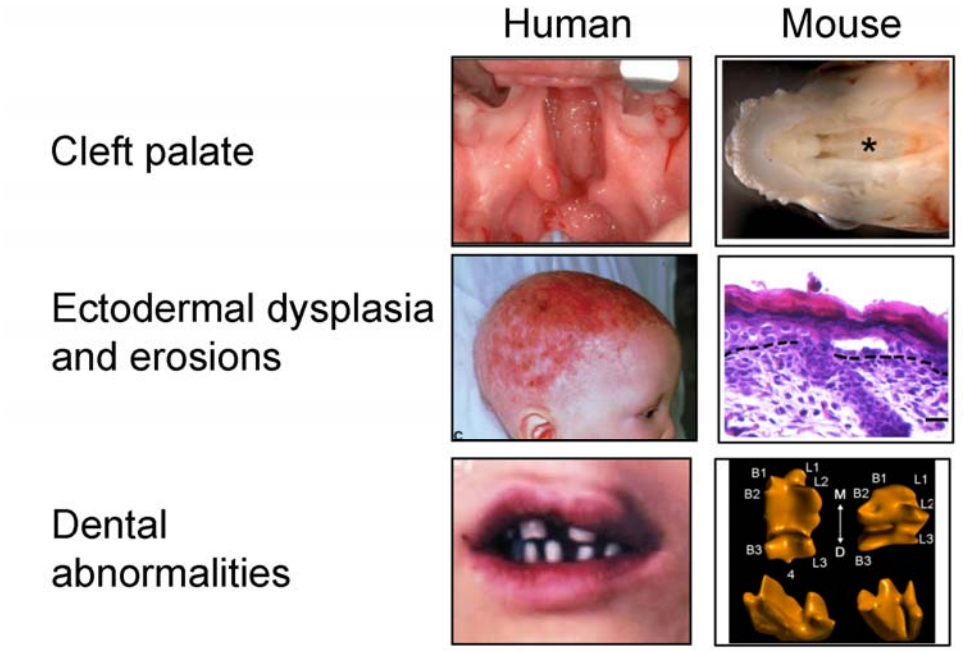


Figure 7. Constitutive AEC mouse model. Comparison between AEC syndrome phenotype and constitutive AEC mouse model indicated that the mouse model faithfully recapitulated some human features, as cleft palate, skin erosions and dental abnormalities.

1.6 p63 and adhesion molecules

In the epidermis cell adhesion has a fundamental role in supporting epithelia formation, structure and maintenance thus enabling skin to support mechanical stress and to be impermeable. Three different types of junction complexes mediate cell-cell adhesion in epithelia: tight junction, adherens junction and desmosomes junction. While the tight junction perform a role in establishment

of barrier formation and in para-cellular transport, the adherens junction and desmosome are crucial in promoting cell adhesion.

Several studies indicate that p63 is a crucial transcriptional regulator of genes involved in cell-matrix adhesion and cell-cell adhesion genes, directly regulating a subset of extracellular matrix, hemidesmosomal, desmosomal, adherens junction and tight junction components (reviewed in (Ferone et al., 2015)). The importance of these junctions for epidermal integrity is highlighted by autoimmune or genetic blistering diseases in humans caused by dysfunctional desmosome components (McMillan and Shimizu, 2001).

Cell-matrix adhesion molecules allow the anchorage of keratinocytes to the basement membrane. In skin as well as in other complex epithelia, multiprotein complexes called hemidesmosomes hold this role. Hemidesmosomes consist mostly of three single-span transmembrane proteins: (1) heterodimers of integrin α6β4 (ITGA6 and ITGB4) that contact laminin filaments; (2) the bullous pemphigoid antigen BP180 (COL17A1, or BPAG2) with a long C-terminal collagenous domain that projects into the extracellular matrix below the complex; (3) the tetraspanin CD151 that associates laterally with integrin α6β4 (Simpson et al., 2011; Yang et al., 2012).

p63 was found to regulate the expression of several integrin subunits, including integrin alpha 3, 4, 5 and 6 (Kurata et al., 2004; Carroll et al., 2006; Truong et al., 2006) and of the hemidesmosome protein dystonin (Osada et al., 2005).

In addition, p63 is required for expression of some extracellular matrix components such as laminin2 (Carroll et al., 2006) and FRAS1 (Koster et al., 2007), an epidermal ECM protein that, when defective, results in severe blistering (McGregor et al., 2003). Direct regulation of *Fras1* gene was demonstrated *in vivo* and *in vitro* (Koster et al., 2007).

Importantly, global gene expression profiling performed in different cell types or tissues indicate that p63 is crucial for expression of integrins and other hemidesmosome genes, as well as basement membrane components (Carroll et al., 2006; Vigano et al., 2006; Yang et al., 2006; Della Gatta et al., 2008; Shalom-Feuerstein et al., 2011).

Desmosomes are specialized intercellular complexes, which confer stability to tissues by anchoring the plasma membrane of two adjacent cells to their IF cytoskeleton (reviewed in (Michels et al., 2009; Simpson et al., 2011). The main components of desmosomes belong to three protein families: cadherins, armadillo proteins and plakins. Desmogleins (DSG1-4) and desmocollins (DSC1-3), members of the cadherin superfamily, are transmembrane proteins, which form heterophilic and homophilic interactions in the extracellular space between adjacent cells. Their cytoplasmic domain is anchored to the IF cytoskeleton via a series of protein– protein interactions involving members of the armadillo family such as plakoglobin (JUP) and plakophilins (PKP1-PKP3), which in turn bind desmoplakin (DSP), a plakin family member, which associates directly to IF (Getsios et al., 2004). Additional desmosomal components are required for specialized functions in the epidermis in a cell-

type-specific manner,for example envoplakin, periplakin and corneodesmosin together with DSG1 are essential for corneodesmosomes formation in the stratum corneum of the epidermis (Ruhrberg et al., 1996; Ruhrberg et al., 1997; Jonca et al., 2002). In addition to these canonical desmosome proteins, a small transmembrane polypeptide PERP is an integral component of desmosomes and is required for their function (Ihrie et al., 2005).

p63 was found to directly regulate the expression of the desmosomal component Perp (Ihrie et al., 2005). In the absence of Perp expression, mice display severe intra-epidermal blistering underscoring the importance of Perp in cell adhesion.

In the knock-in mouse model for AEC syndrome (Ferone et al., 2012), epidermal blisters are observed primarily between the basal and suprabasal layers and are associated to a significant reduction in desmosome contacts compared to other type of adhesion molecules. Accordingly, a strong reduction in Dsp, Dsc3 and Dsg1 is observed in AEC newborn skin, in isolated AEC primary keratinocytes and in human keratinocytes derived from AEC (Ferone et al., 2013; Koster et al., 2014). In addition, Dsp, Dsc3, and Dsg1 are also downregulated in p63-depleted keratinocytes, and are shown to be direct p63 target genes (Ferone et al., 2013; Johnson et al., 2014).

Adherens junctions (AJ) are intercellular structures that connect actin cytoskeleton between neighboring cells (reviewed in (Brandner J.M., 2010). They consist of two basic adhesive units: the classical calcium-dependent cadherin–catenin complex and the calcium-independent nectin–afadin complex. Classical cadherins are single-pass, transmembrane glycoproteins that share a common structure composed by five characteristic extracellular cadherin (EC) repeats and an intracellular region containing a catenin-binding domain. EC domains bind calcium (Ca^{2+}) undergoing a Ca^{2+}-dependent conformational change that allows homodimerization. Through extracellular interactions with E-cadherins on a neighboring cell, opposing cadherin dimers can integrate the actin cytoskeleton. Stabilization of intercellular adhesion requires the cytoplasmic domain of E-cadherin, which binds to β-catenin that, in turn, binds α-catenin, recruiting a number of cytoskeletal proteins essential in polymerizing and organizing actin cables to seal membranes (Halbleib and Nelson, 2006; Brasch et al., 2012). The other basic adhesive unit composing the AJ is the nectin–afadin complex. Nectins are a family of calcium independent immunoglobulin-like cell–cell adhesion molecules, with the ability of homophilic and heterophilic interaction (reviewed in (Rikitake et al., 2012)). Four family members (nectin 1-4) share a common structure characterized by an extracellular region with three immunoglobulin-like loops, a single membrane-spanning region and a cytoplasmic tail that take contact with afadin. Through their immunoglobulin-like loops nectins contact each other on the surface of the adjacent cells and in particular, nectin-1 can interact with nectin-3 and nectin-4, whereas nectin-2 is able to interact with nectin-3. Afadin, a F-actin binding protein that connect nectins to the actin cytoskeleton,

is also able to directly bind α-catenin to mediate the assembly of multiple complexes (Tachibana et al., 2000; Yokoyama et al., 2001).

Different groups demonstrated that p63 regulates different components of AJ. Indeed, Christiano and coworkers found that p63 directly interacts with two distinct regions of the human *CDH3* promoter but not *CDH1* (Shimomura et al., 2008) whereas McDade and colleagues demonstrated a direct p63 binding on the human *PVRL1* promoter in human keratinocytes, and its responsiveness to p63 in heterologous cells (McDade et al., 2012). More recently we demonstrated that Pvrl1 expression is impaired in p63 depleted keratinocytes and in *p63* null embryonic skin, and that *Pvrl1* is a p63 direct target gene (Mollo et al., 2014). Interestingly, Pvrl1 and Pvrl4 expression were reduced in keratinocytes derived from a conditional mouse model for AEC syndrome and from human patients, indicating that Pvrl4 expression is affected by mutant p63, possibly through a mechanism dependent on the transcription factor Irf6, as shown in Irf6 depleted keratinocytes (Mollo et al., 2014).

Tight junctons are responsible for intercellular sealing and the compartmentalization of extracellular environments, exhibiting ion and size selectivity (reviewed in (Van Itallie and Anderson, 2014)). They form an almost continuous plasma membrane contact zone around each cell and are composed of two kinds of tetraspan transmembrane proteins, occludin and members of the claudin family and single span transmebrane proteins JAMs that act as regulators of tight junction assembly in epithelia.

It is reported that *Cldn1* is a p63 direct target gene (Rinne et al., 2007; Lopardo et al., 2008), indeed p63 deficiency leads to strong inhibition of Cldn1 expression both in *p63* null mice and in mouse keratinocytes. Among the claudins, Cldn3 and Cldn7 are expressed in simple epithelia and are ectopically expressed in p63-depleted keratinocytes and in *p63* null embryonic epidermis (De Rosa et al., 2009). Consistent with these data in mouse, CLDN7 is overexpressed in AEC patient keratinocytes in conjunction with reduced CLDN1 expression (Zarnegar et al., 2012; Shen et al., 2013), suggesting that an unbalance among claudins in AEC patients may be at the origin of some of the observed defects. This possibility is supported by previous studies showing that ectopic overexpression of periderm-specific claudin 6, in the upper layers of the epidermis induces a phenotype that is identical to *Cldn1* null mice (Morita et al., 1998; Turksen and Troy, 2002), indicating that unbalanced claudin expression might alter TJ function. Thus a functional p63 is required for appropriate claudin expression in the epidermis, although the functional significance of this finding in AEC syndrome is unclear at the moment.

1.7 The Notch pathway

Stratification of the epidermis is a tightly controlled process that requires the orchestrated action of different key signal transduction pathways, such as e.g. Wnt/□-catenin, Notch, TGF-□ and NF-□B (Koster and Roop, 2007; Blanpain and Fuchs, 2009). In particular, Notch signaling has a crucial role in the proper

stratification and development of the epidermis and its appendages (Vauclair et al., 2005; Moriyama et al., 2008). Keratinocytes require Notch signaling to downregulate basal genes and induce spinous layer markers, thereby switching from proliferation to differentiation (Rangarajan et al., 2001; Blanpain et al., 2006; Blanpain and Fuchs, 2009).

p63 expression counteracts the ability of Notch1 to restrict growth and promote differentiation in keratinocytes.

In mammals, there are four Notch receptors (Notch1-4) and five canonical ligands of the Delta-Serrate-Lag (DSL) type (Jag1 and Jag2 and delta-like 1 (Dll1), Dll3 and Dll4) (reviewed in (D'Souza et al., 2010)).

Notch receptors are a single pass trans-membrane protein expressed on the cell surface as heterodimers composed of a large extracellular domain non-covalently linked to the intracellular domain. The extracellular domain of all Notch receptors contains epidermal growth-factor-like repeats (EGFLR) and three LIN Notch (LNR) repeats. The intracellular domain contains the RAM23 domain and seven Ankyrin/CDC10 repeats (ANK), necessary for protein-protein interactions. In addition, Notch receptors 1-3 contain two nuclear localization signals (NLS), in contrast Notch4 contain only one NLS. The NSL target the intracellular domain to the nucleus where the transcriptional activation domain (TAD) activates downstream events (Kopan, 2012).

Notch receptors are cleaved by protein convertases during exocytosis at site 1 (S1), which regulates their trafficking and signaling activity (Jarriault et al., 1998; Gordon et al., 2009). The response of the Notch receptors to different subfamilies of ligands and the subsequent activated downstream pathway are regulates by a series of post-translational modifications. Indeed, they can be glycosylated by glycosyltransferases such as Fringe during passage through the Golgi whereas, E3 ubiquitin ligases (e.g., Deltex and Nedd4), Numb, and □-adaptin, regulate the steady-state levels of the Notch receptor at the cell surface (Fig. 7).

The signaling pathways initiated in response to Notch ligands are known as the canonical and non-canonical Notch signaling pathway. In canonical Notch signaling, a Notch transmembrane receptor interacts extracellularly with a canonical Notch transmembrane ligand on a contacting cell, initiating proteolytic cleavage of the receptor by metalloprotease of ADAM/TACE family which removes the Notch extracellular domain by cleaving at site 2 (S2); □-secretase then cleaves Notch within its transmembrane domain at site 3 (S3) to release various forms of the NICD, the Notch intracellular domain of the receptor. Notch ICD then translocates to the nucleus where it interacts with a CBF1/Suppressor of Hairless/LAG-1 (CSL) family DNA-binding protein (or recombination signal binding protein for immunoglobulin kappa J region (RBPJ-□) in mammals) and initiates the transcription of Notch target genes (Fig. 7). Non-canonical Notch signaling differs from canonical signaling in that it can be initiated by a non-canonical ligand, or may not require cleavage of the Notch receptor.

The role of Notch in epidermal development and maintenance has been the

subject of number studies. Ablation of Notch signaling during skin embryogenesis invoked early postnatal death mainly due to a disturbed epidermal barrier (Demehri et al., 2008). Postnatal knockout of Notch-1 led to a hyperproliferative epidermis, hair loss and epidermal cyst formation (Nicolas et al., 2003; Blanpain et al., 2006). Loss of epidermal Notch-1 or Notch-1/Notch2 disturbed skin homeostasis and resulted in inflammatory skin disease (Demehri et al., 2009; Dumortier et al., 2010). Mice with impaired Notch-1 signaling developed skin tumors, indicating a tumor suppressor function for Notch-1 in the skin (Nicolas et al., 2003; Proweller et al., 2006). All these studies supported the idea that Notch is the main regulator of skin differentiation since the perturbation of this pathway leads to dramatic changes in epidermis structure and function.

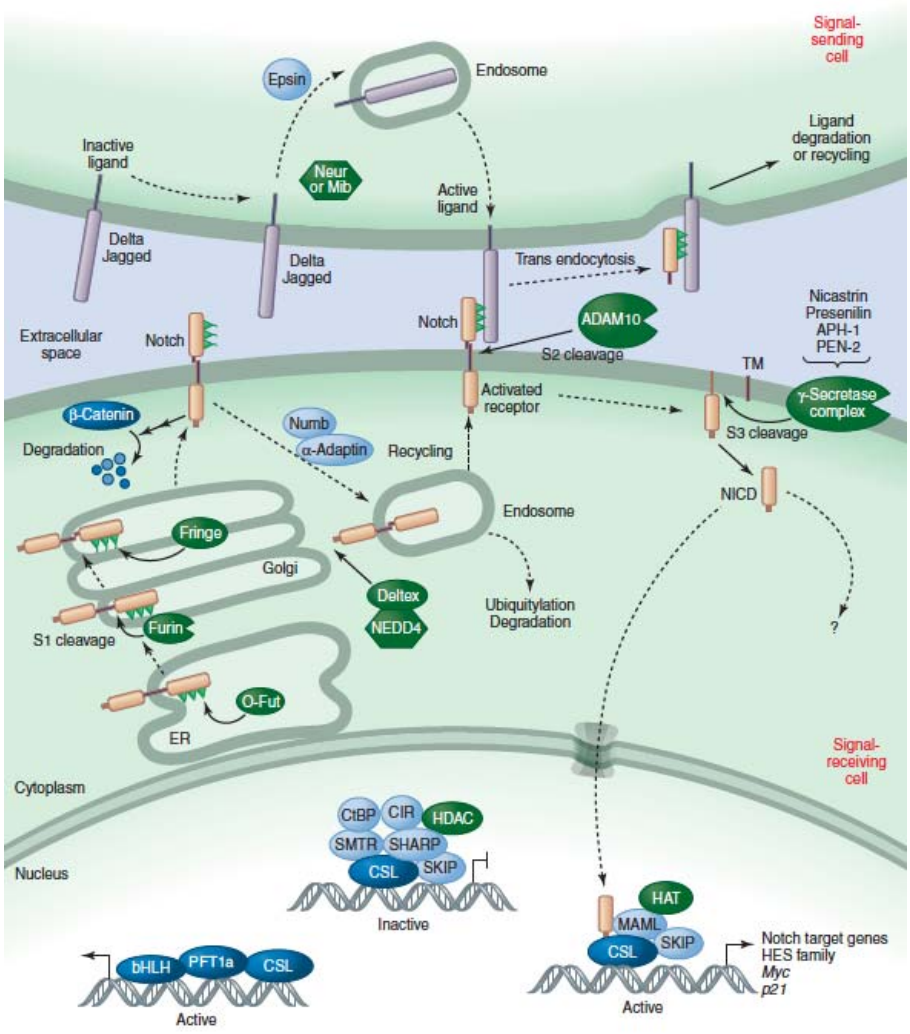


Figure 8. Notch signaling. Notch proteins are synthesized as single precursor proteins, which are cleaved in the Golgi by a Furin-like convertase at site S1. Cleavage at S1 generates two subunits held together noncovalently. EGF-like repeats present within the extracellular domain of the receptors are glycosylated by Fringe proteins in the Golgi before receptors are transported to the cell surface. Notch signaling is initiated by ligand–receptor interaction, which induces a second cleavage at site S2 (close to the transmembrane domain) mediated by ADAM-type metalloproteases, followed by a third cleavage at S3 within the transmembrane domain mediated by the g-secretase activity of a multiprotein complex containing presenilins. This last proteolytic cleavage liberates the cytoplasmic domain of Notch receptors (NICD), which translocate to the nucleus and bind to the transcription factor RBP-J, converting it from a transcriptional repressor into a transcriptional activator by recruiting co-activators to induce target gene expression (Adapted from Kopan R., 2012).

2. AIM OF THE STUDY

My research activity, during the last three years, focused on the elucidation of the molecular mechanisms at the basis of epidermal defects and skin fragility in AEC syndrome. To these aim I characterized a newly generated conditional mouse model for the AEC syndrome.

3. MATERIALS AND METHODS

3.1 Generation of conditional $p63^{+/FloxL514F}$ mouse model

We recently generated a conditional knock-in model ($p63^{+/FloxL514F}$), in which the L514F mutation is expressed only in the presence of the CRE recombinase. The knock-in/replacement strategy was designed to replace the wild-type amino acid leucine encoded by codon 514 with a sequence coding for phenylalanine in the p63 protein in murine embryonic stem (ES) cells by homologous recombination. The targeting contruct contained two LoxP flanking fused wild-type exons 13 and 14 placed upstream a neomycin resistence cassette flanked by FRT loci and a mutant exons 13 followed by the exon 14 and an immunoepitope tag (3xFlag) at the end of it.

3.2 Mouse genotyping

Conditional knock-in $p63^{+/FloxL514F}$, $p63^{FloxL514F/FloxL514F}$, K14Cre;$p63^{+/FloxL514F}$ (+/L) and K14Cre;$p63^{FloxL514F/FloxL514F}$ (L/L) were genotyped by PCR using genomic DNA isolated from mouse tails.
Oligonucletide primers used for PCR:
Conditional knock-in $p63^{+/FloxL514F}$, $p63^{FloxL514F/FloxL514F}$

Forward primer (5'-3')	**Reverse primer (5'-3')**
CAGCGTATCAAAGAGG AAGGAGA	AGCCAGAATCAGAATCAGGTG AC

The expected bands were of 250bp for the wild-type mice, 337bp for the mutant homozygous mice and both for the heterozygous ones.

To distinguish the presence of Cre-recombinase in p63 heterozygous and homozygous mice we used the following primers for PCR:

Forward primer (5'-3')	**Reverse primer (5'-3')**
AGGGATCTGATCGGGA GTTG	CTTGCGAACCTCATCACTCG

Western blot

For immunoblotting cells were lysed in sample buffer (10% glycerol, 0.01 % Bromophenol Blue, 0.0625 M Tris-HCl pH 6.8, 3 % SDS, 5 % ßmercaptoethanol) supplemented with protease inhibitors. For immunoblotting of epidermal extracts, epidermis was isolated from dermis by producing a

termic shock at 55°C or by floating skin biopsies, epidermis side up, in a Dispase solution. After isolation from dermis, epidermis was snap frozen in liquid nitrogen and then homogenized with a tissue lyser in lysis buffer supplemented with phosphatase and protease inhibitors. Proteins were run on a denaturing SDS-PAGE gel and subsequently transferred to Immobilon-P transfer membranes (Millipore) probed with primary antibodies and detected by chemiluminescence (ECL, GE Healthcare Life Sciences). Antibodies used for immunoblotting were: p63 (4A4 Santa Cruz Biotechnology), Cdh1 (BD Bioscience), krt5, krt14, krt1, krt10, ivl, lor (all Covance), krt15 (a gift from doct. Langbein), Nectin1 (Santa Cruz Biotechnology), b-actin (Santa Cruz Biotechnology), Notch1 (Santa Cruz Biotechnology), ERK-1 (Santa Cruz Biotechnology).

Real-time RT-PCR

Mouse epidermis was isolated from dermis by digestion with Dispase solution. Total RNA was extracted from primary keratinocytes or epidermis using TRIzol reagent (Invitrogen). cDNA was synthesized using SuperScript Vilo (Invitrogen). Two-step real-time reverse transcription RT-PCR was performed using the SYBR Green PCR master mix in an ABI PRISM 7500 (Applied Biosystems). Levels of the target genes were quantified using specific oligonucleotide primers and normalized for Actin (ß-actin) or RPLP0 expression.

Oligonucletide primers used for Real-Time RT-PCR on mouse samples:

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
ß-actin	CTAAGGCCAACCGTGAAAAGAT	GCCTGGATGGCTACGTACATG
p63 wt	ACTCTCCATGCCCTCCAC	GAGCAGCCCAACCTTGCT
p63 L514F	ACTCTCCATGCCCTCCAC	GAGCAGCCCAACCTTGCA
Krt5	CAACGTCAAGAAGCAGTGTGC	TTGCTCAGCTTCAGCAATGG
Krt14	ACCACGAGGAGGAAATGGC	TGACGTCTCCACCCACCTG
Dsc3	CCACCGTCTCTCACTACATGGA	TGTCCTGAACTTTCATTATCAGTTTGT

Dsp	CACCGTCAACGACCAG AACTC	GATGGTGTTCTGATTCTGATGT CTAGA
Pvrl1	TTCTTCCTCCCAGGCAC TCA	AAGCCATACATGGAGTCGTTCA
Pvrl2	TGCTGCCAGTGACCCTC TCT	CATCATAGCCGGAGATGGATAC T
Pvrl3	AAACCCTCCACCCTTCA AGTCC	CAATAAACCATCAGGCCATTGT C
Pvrl4	GGCATCGTTTACAGGCC AAT	AGCACCACTGTCACTACGTCAG A
Mllt4	TCGGGAAGCGAGAGAG AAAC	GCTCCAGAACTGCCAGGTAAGT
Cdh1	GGGCTGGACCGAGAGA GTTAC	AAGCCTTCACCTTGAAGGTCAG
Notch1	ATGGAGGGAGGTGCGA AGT	ATTGGAGTCCTGGCATCGTT
Jag2	CCTCCTCCTGCTGCTTT GTG	TCTGGATCAGGCTGCTGTCA
Tslp	GCCAGGGATAGGATTG AGAGTATAGT	GACTGTGAGAGCAAGCCAGCTT
Alox12	CCTTGGCCGAGAAGGTT CA	GCGCCATTGAGGAACTGGTA
Hes1	CTGTGAGGTCAGGCCAG CTT	AGGCCCATGGAAAATGAGC

Oligonucleotide primers for Real Time RT-PCR on human samples

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
RPLP0	GACGGATTACACCTTC CCACTT	GGCAGATGGATCAGCCAAGA
PVRL1	CCCTACGGGCAATCGA GAA	GGTGGGTTTGGCCATCAC
PVRL4	ATGCTCAAGTGCCTGA GTGAAG	TCCAGCCGTGTCCAGTTGTA

KRT5	CCTCAACAATAAGTTT GCCTCCTT	GCAGCAGGGTCCACTTGGT
KRT14	GGATGACTTCCGCACC AAGT	TCCACACTCATGCGCAGGT

Primary keratinocytes and cell cultures.

Newborn mice were placed in petri dishes with ice and inserted in an ice bucket. After 30-45' newborn mice were washed twice with 70% ethanol and twice with water to remove ethanol completely. Using sterile techniques, mice tails and limbs were amputated with sterile surgical scissors. Single skin was carefully separated from the rest of the viscera and flattened in a empty 6 well dish with the dermis facing down; 2 ml of Dispase solution (0.5mg Dispase-GIBCO, Na-bicarbonate 0.75%, Hepes 10mM, Antibiotic-Antimycotic in PBS) were added to each 6 well dish and incubated o/n at 4 °C. Next day epidermis was separated from the dermis and placed in a 100mm Petri dish in 2ml (for each epidermis) of 0.125% trypsin- 0.1mM EDTA. Epidermis was minced with tweezers and scissors until was reduced in very small fragments and placed at 37°C for 5-8 minutes. Then trypsin was inactivated with DMEM+10% FBS and filtered by applying it to cell strainer in order to remove the floating particles. Cells were placed into the centrifuge for 5 minutes at 1000 rpm; then were plated on collagen coated plates (1.2x106 cells/ml) and incubated at 34°C, 8% CO2. Primary mouse keratinocytes were isolated from newborn mice and cultured under low calcium conditions (0.05 mM) or treated with 2mM calcium chloride as previously described (Antonini et al., 2010).

Human keratinocytes obtained from AEC patients Q11X and T533P (kindly provided by J. Zhou, (Ferone et al., 2013)), as well as control keratinocytes obtained by unaffected individuals were plated at a density of 10^4cells/cm^2 and cultured in KBM Gold medium (Lonza) till they reach confluency. Confluent cells were treated with 0.3mM calcium for subsequent RNA analysis.

Knockdown was achieved by transient transfection of 100nM small interfering RNA (siRNA) for pan-p63, ΔNp63 (De Rosa et al., 2009), Irf6 (Mm_Irf6_2; Qiagen) or negative control (Invitrogen).

Normal human epidermal keratinocytes (NHEK-Neo, Lonza; Cat. No. 00192907) were plated at a density of 10^4cells/cm^2 and cultured in KBM Gold medium (Lonza) until they reach confluence. p63 and ΔNp63 knockdown were obtained by transient transfection of 100nM small interfering RNA (siRNA) for pan-p63 (5'- CAGAACACACAUGGUAUCCAGAUGA-3', Stealth RNAi Invitrogen), ΔNp63 (De Rosa et al., 2009) or negative control (Invitrogen).

Skin Barrier Assays

To perform X-gal staining, unfixed, untreated newborn mice or embryos were washed in PBS and then incubated overnight at 37°C in 5-bromo-4-chloro-3-indlyl-b-D-galactopyranoside (X-gal) reaction mix (100 mM NaPO4,

1.3 mM MgCl2, 3 mM K3Fe[CN]6, 3 mM K4Fe[CN]6, and 1 mg/ml X-gal [pH 4.5]). At pH 4.5, the skin exhibits endogenous b-galactosidase activity, so increased X-gal staining indicates epidermal permeability to X-gal, a sign of compromised barrier function (Hardman et al., 1998).

Gene expression microarrays

We measured the differential expression of 22000 RNA on freshly isolated epidermis from three mutant versus three wild-type newborn mice. We hybridized the RNA samples to the Affymetrix Mouse Genome 430A 2.0 chips. We processed the microarrays using the RMA algorithm. False Discovery Rate (FDR) correction was perfomed on the estimated p-value to correct for multiple hypothesis test.

Histology and Immunostaining

Dorsal skin was dissected, fixed in 4% paraformaldehyde (PFA) and embedded in paraffin or in OCT, from which 7 μm sections were cut and stained with Haematoxylin and Eosin (H&E) and immunofluorescence according to standard methods. For paraffin sections, permeabilization for antigen retrieval was performed by microwaving samples in 0.01 M citrate buffer at pH 6.0. The following antibodies were used: keratin 6, keratin 5, keratin 14 (Convance), p63 (4A4, Santa Cruz Biotechnology), Ecadherin (Zymed laboratories-Invitrogen), guinea pig antibody to keratin 15 (a gift from doct. Langbein), FLAG M2 (Sigma), Col7a1 (Sigma), Dsp and Dsc3 (a gift from doct. H. Thomason)
The following secondary antibodies were used for immunofluorescence staining: Alexa Fluor ® 488 goat anti-mouse (Invitrogen), Alexa Fluor ® 594 goat anti-rabbit (Invitrogen), Alexa Fluor ® 594 goat anti-rat (Invitrogen). Fluorescent signals were monitored under a Zeiss Axioskop2 plus image microscope or under a Zeiss confocal microscope LSM510meta.

Chromatin Immunoprecipitation (ChIP)

1 x 10^6 keratinocytes were fixed with 1% formaldehyde in growth medium at 37°C for 10 min. Extracts were sonicated using BIORUPTOR (Diagenode) to obtain DNA fragments ranging from 400 to 800 bp in length. Chromatin was immunoprecipitated as in the Upstate protocol (http://www.upstate.com). Immunoprecipitation was performed using anti-p63 (H-137; Santa Cruz Biotechnology) and anti-ERK-1 (K23; Santa Cruz Biotechnology) antibodies as negative control. Real-time PCR was performed using the SYBR Green PCR master mix in an ABI PRISM 7500 (Applied Biosystems).

Oligonucleotide primers for ChIP analysis on human genomic DNA.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
PVRL1 reg 1	TGAGCCTGTAGGACCAGAATCA	TTTCCCACTCAAGCTGTGTC TCT
PVRL1 reg 2	TCTGCCCAACACGAATCTATCA	CTGAAACCCCGACAAGTCT GA

Oligonucleotide primers for ChIP analysis on mouse genomic DNA.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Pvrl1 reg 1	CTGTGTCTCCAGGACGTGTCA	GCACAGCCAAGCCTGTAG GA
Pvrl1 reg 2	GAAAGACCCCACACTTGAAACTG	TCCTCCCGGGCTTCCTT

Dispase-based dissociation assay

Mechanical integrity of cell monolayers was assessed as described previously (Calautti et al., 1998; Huen et al., 2002). Keratinocytes were grown to confluence in low-calcium medium containing 0.05 mM CaCl2 and switched to medium containing 0.6 mM CaCl2 for 1–3 days. At that time, keratinocyte sheets were lifted from the culture dishes by treating with 2.4 U/ml Dispase II (Roche) for 30 min at 378C. Intact sheets were transferred to 15 ml conical tubes containing 5 ml of PBS. Tubes were placed in a rack and inverted were inverted on a rotor at 0.28 g for 30–50 inversion cycles. Cellular fragments were transferred to 35 mm tissue culture plates and counted using a dissecting microscope.

Adenoviral infection

Mouse primary keratinocytes were infected after 4-5 days of plating, when they reached confluence. Adenovirus carring the GFP or the NIC (provided by OKAIROS) at MOI 100 were diluted in 250□L for 12-well of LCM without

serum and EGF. After 2 hours of infection, supplemented medium was added and left o/n at 34°C.

Retroviral infection

Mouse primary keratinocytes were infected the day after plated with retroviruses carrying the GFP (PINCO), Notch1 or MAML1 for 2 hours. After the infection cells were washed with PBS and were cultured in LCM4% for a week at 34°C.

4. RESULTS

4.1 Epidermal-specific L514F mutation results in severe skin phenotype.

To dissect the molecular defects underlying AEC syndrome and to overcome the lethality of the constitutive AEC mouse model (Ferone et al., 2012), we generated a conditional knock-in $p63^{floxL514F}$ mouse model, in which the L514F mutation was expressed only in the presence of the CRE recombinase. The knock-in strategy was designed to replace the wild-type amino acid leucine encoded by codon 514 with phenylalanine in the p63 protein in murine embryonic stem (ES) cells by homologous recombination (Fig. 9A). To study the adult phenotype we crossed $p63^{+/floxL514F}$ mice with a knock-in mouse expressing Cre by E17.5 in epidermis and hair follicle under the control of the endogenous keratin 14 (K14) promoter (K14-Cre Δneo) (Huelsken et al., 2001) to obtain both the heterozygous and the homozygous model (K14-Cre;$p63^{+/floxL514F}$ and K14-Cre;$p63^{floxL514F/floxL514F}$). The expression of the mutant protein was confirmed in K14-Cre;$p63^{+/floxL514F}$ (+/L) mutant newborn mice by immunofluorescence analysis with FLAG antibody, which specifically recognizes the mutant protein (Fig. 9B). At birth a nearly uniform expression of the mutant protein in the interfollicular epidermal cells was observed by a co-staining with FLAG antibody and Krt14 antibody that marked the basal layer of the epidermis and the hair follicle (Fig. 9B). The wild-type and mutant alleles were also revealed by quantitative real time RT-PCR (qRT-PCR) and by immunoblot in both heterozygous +/L and homozygous K14-Cre; $p63^{floxL524F/floxL514F}$ (L/L) mutant mice. qRT-PCR and immunoblot analysis confirmed that by post-natal day 3 (P3) wild-type p63 was virtually absent in L/L epidermis and about 50% expressed in +/L epidermis (Fig.9 C, D). The expression of mutant p63 mRNA and protein was more abundant in mutant than in wild-type epidermis, consistent with the previously reported p63 accumulation in the skin of AEC patients and in agreement with our previous data (Moretti et al., 2010; Browne et al., 2011; Ferone et al., 2012).

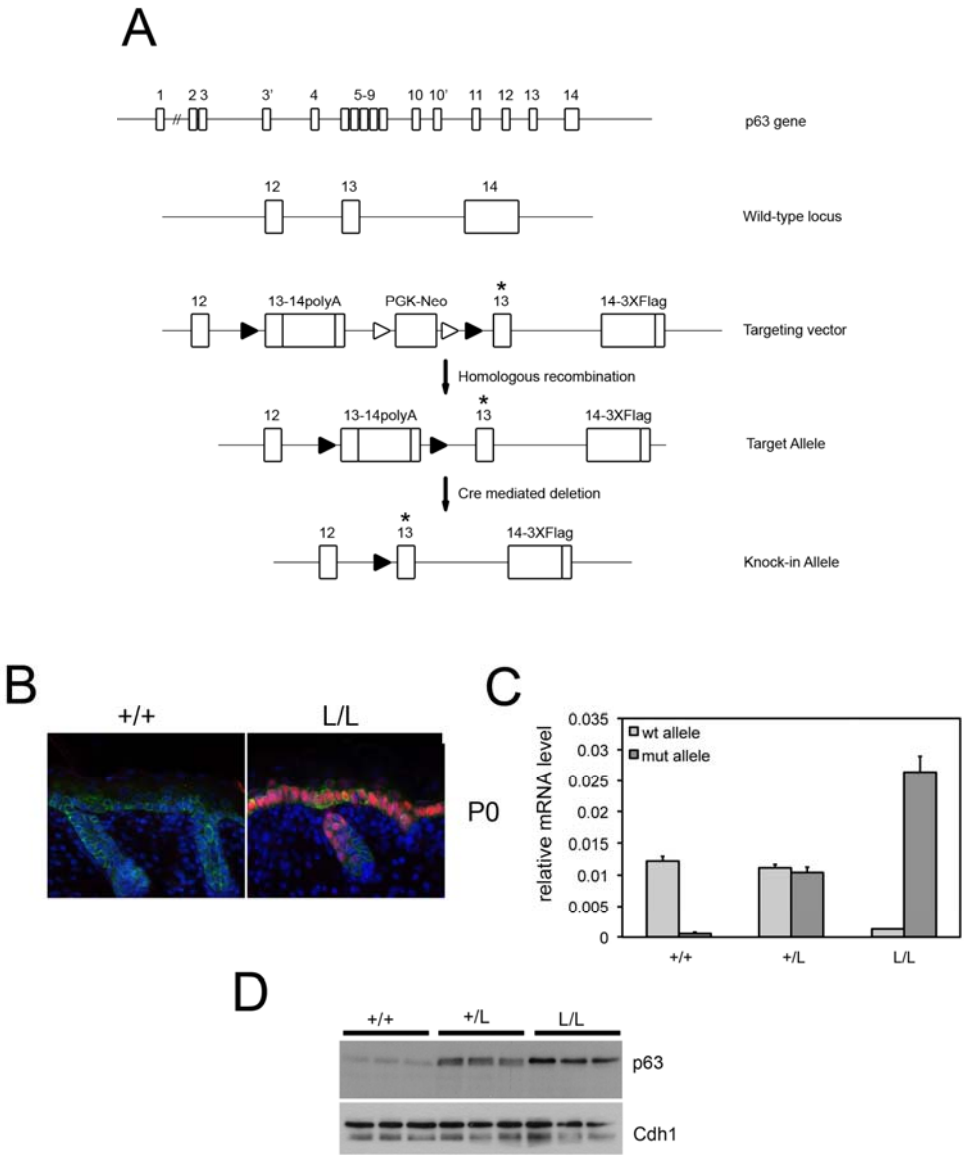


Figure 9. Generation of AEC mutant mice: A) Gene targeting strategy used to generate the $p63^{+/L514F}$ knock-in mice. The L514F mutation is indicated with * The targeting contruct contained two LoxP flanking wild-type exons 13 and 14 fused together and placed upstream a neomycin resistence cassette flanked by FRT loci and a mutant exon 13 followed by the exon 14 and an 3xFLAG epitope tag at the end of it. B) Double immunofluorescence for Krt14 and Flag revealed that all the cells of the basal layer of the epidermis in AEC mutant newborn mice are positive for the mutant protein. C) Real-Time RT-PCR performed on RNA from controls (+/+), heterozygous (+/L) and homozygous (L/L) epidermis at P3, revealed the proper expression of mutant allele. D) Immunoblotting of total cell extracts from controls (+/+), heterozygous (+/L)

and homozygous (L/L) neonatal epidermis (P3) using antibodies against p63 and cdh1 as loading control.

+/L mice showed no gross abnormalities and were characterized by striped hair and partial hair loss (Fig. 10A). At the histological level +/L skin appeared hypoplastic at birth with a reduction of keratin 15 (Krt15) expression at later stage, consistent with the previous generated data in the constitutive mouse model (Fig. 10B and C) (Ferone et al., 2012).

At later stages hypoplasia of the epidermis and hypercellularized dermis was visible in +/L skin respect to controls (Fig. 10B).

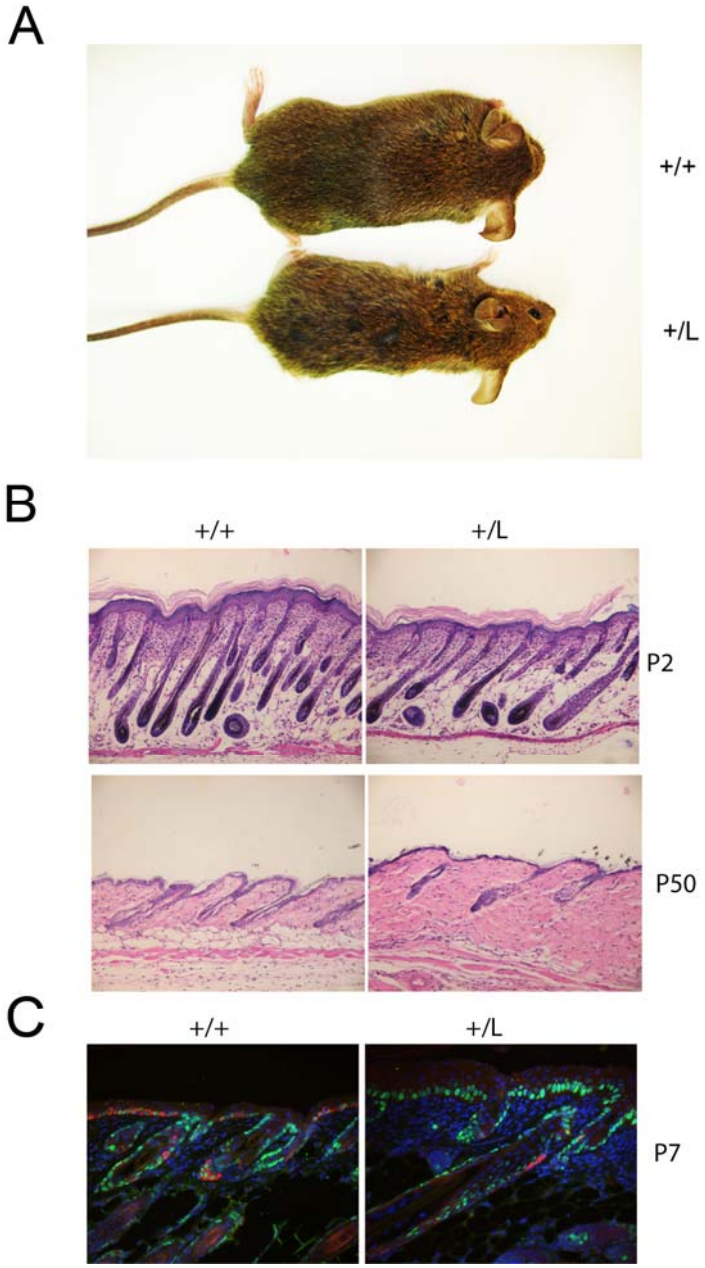


Figure 10. Characterization of +/L mouse model. A) Photographs were taken of +/L and L/L at 30 days. B) H&E of back skin of +/+ and +/L mice at P2 revealed a slight hypoplasia that is maintained also at P50, when dermis became hyper-cellularised and fat layer diminished. C) Double immunofluorescence for p63 and Krt15 of back skin of +/+ and +/L mice at P7 revealed a decreased staining of Krt15 in the interfollicular epidermis respect to the control.

Interestingly, L/L mice were indistinguishable from their wild-type littermates at birth, but in few cases, 3-5 days old pups (P3-P5) had areas of severe skin erosion with an otherwise apparently normal phenotype. Starting from P5-P7, mutant mice developed a progressively severe phenotype, characterized by skin erosions, skin crusting and hair loss. About 75% of mutant mice died between P10 and P20, although some lived up to 12 months. A significant reduction of body weight was observed at different time points (Fig.11 A, B).

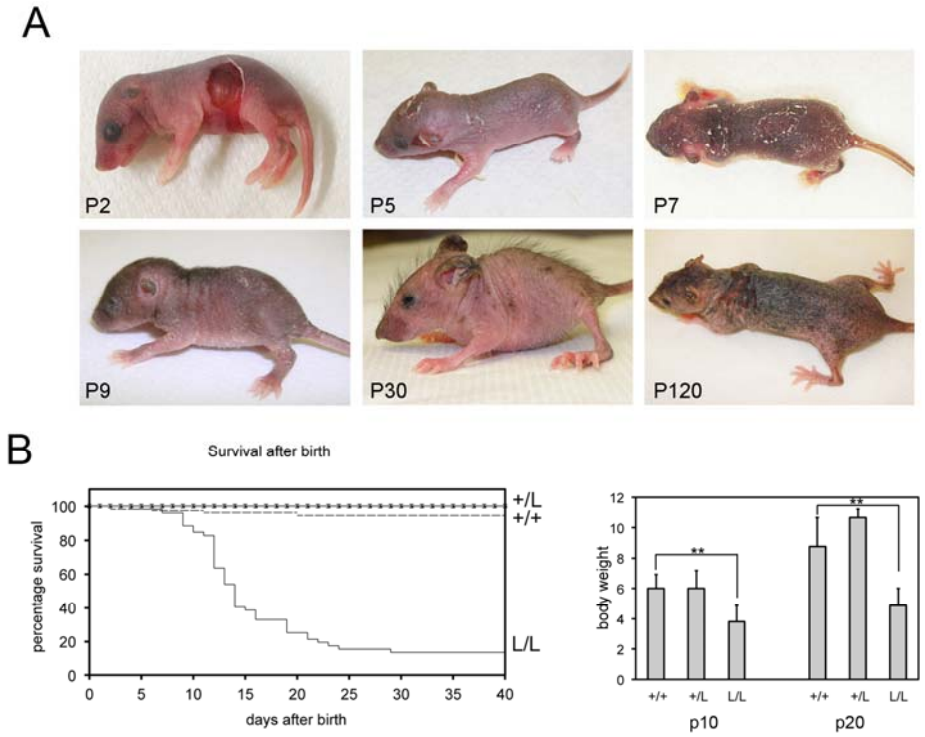


Figure 11. Phenotype of AEC mutant mice: A) Photographs were taken from L/L mice at different stages, as indicated in the figure. Five days after birth L/L mice showed a strong phenotype characterized by skin erosion, skin crusting, hair loss and ulcerations that worsen throughout their life. B) Survival curve of L/L, +/L and +/+ mice (n= 184); on the left side body weight at p10 and p20 of

L/L, +/L and +/+ mice, (p10: n= 72 p-value=2.6847E-07; p20: n= 47 p-value= 6.38948E-06).

4.2 Epidermal blistering and defective adhesion molecules in AEC mice.

Although the visible phenotype appeared after many days after birth, histological analyses of the back skin from L/L mice and littermates controls revealed epidermal hypoplasia at P0 with consistently thin epidermis and dermis. Blistering skin lesions, localized only in focal points of mutant epidermis, were observed both between the basal and suprabasal layers and between basal layer and basal lamina, as previously observed for the constitutive mouse model and consistent with a fundamental role of p63 in cell adhesion (Fig. 12A)(Ferone et al., 2013; Ferone et al., 2015). Epidermal blisters were also accompanied by both acantholysis and cytolysis, as display by the immunostaining for basal keratin Krt14 that revealed both loss of cohesion between keratinocytes and cell disruption (Fig. 12B).

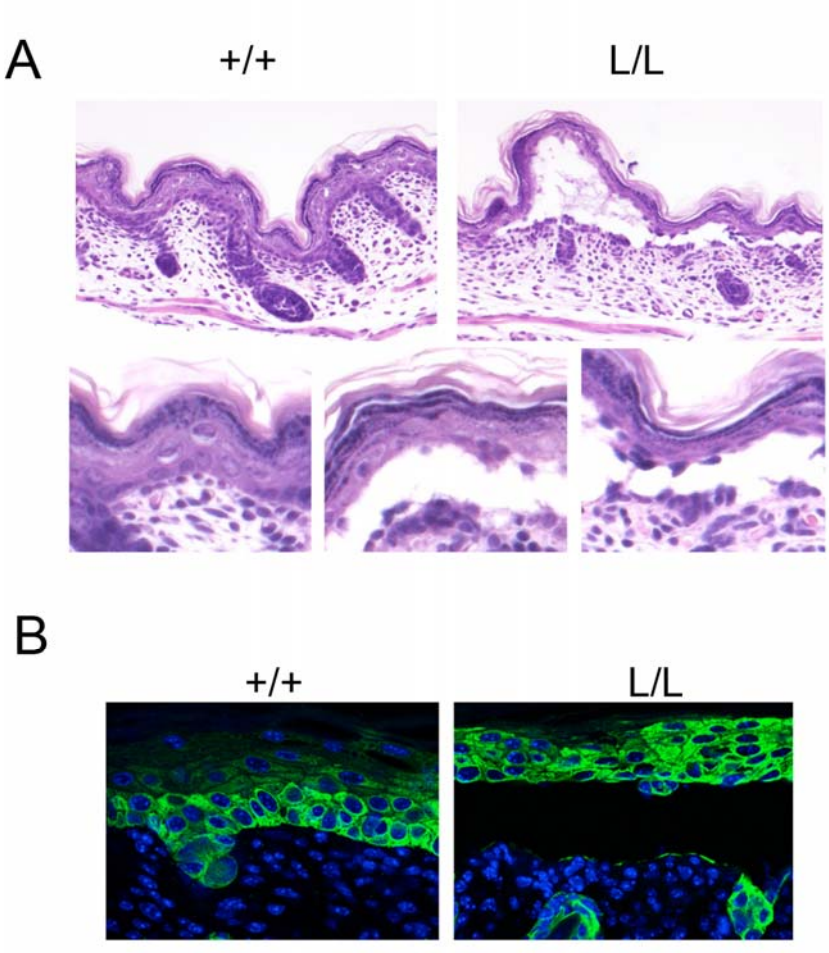


Figure 12. Epidermal blistering and skin fragility in AEC mutant mice. A) H&E staining of dorsal skin of L/L mice at P0. Mutant epidermis was thinner, with less developed hair follicles and focal gaps were visible in L/L epidermis. B) Immunofluorescence analysis of dorsal skin for Krt14 revealed that in L/L skin focal blistering were accompained by acantholysis and cytolysis.

Given the presence of skin blistering, we evaluated the epidermal barrier integrity using a permeability assay based on X-gal substrate. We observed no abnormalities at E17.5 but newborn mutant mice showed focal gaps in the epidermis, consistent with the focal skin blistering observed at histological level (Fig. 13A and B).

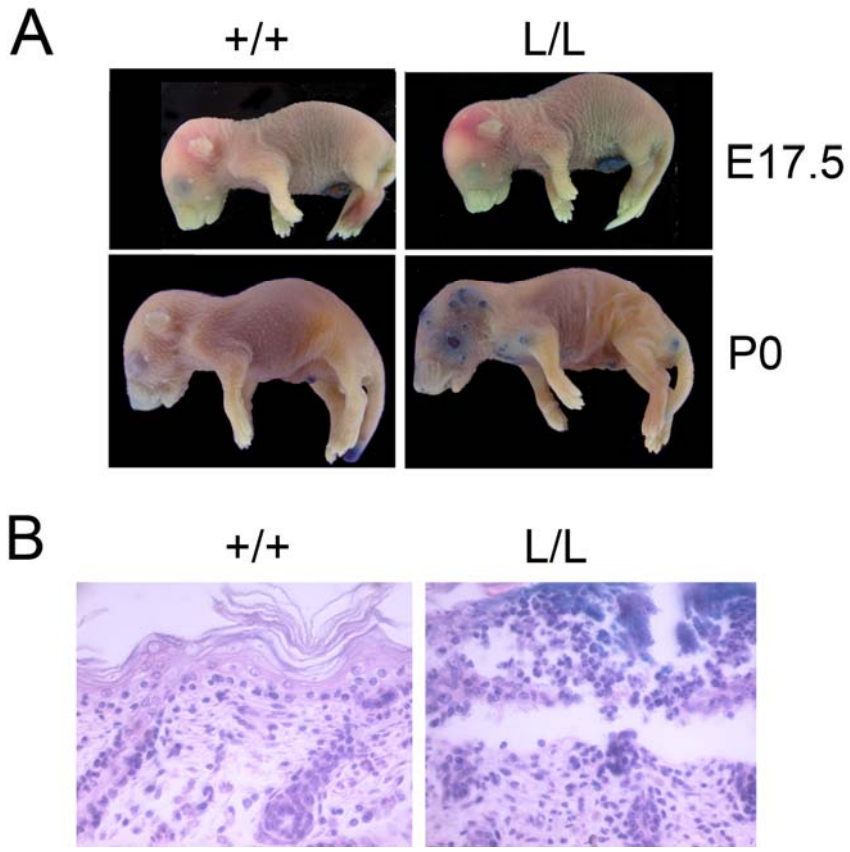


Figure 13: Focal epidermal barrier disruption in AEC mutant mice. A) Barrier assay at E17.5 and P0 on AEC mutant and control mice revealed the presence of focal gaps in mutant mice at P0 (p-value=1.58596E-09;n= 34). B) H&E of focal gaps revealed by barrier assay.

Because epidermal lipids are crucial to form the protective barrier of the skin in the stratum corneum we stained P3 skin sections with the lipophilic dye Nile Red. No significant differences in epidermal lipid content were observed. Impaired integrity of the cornified envelope (CE) has been linked to skin

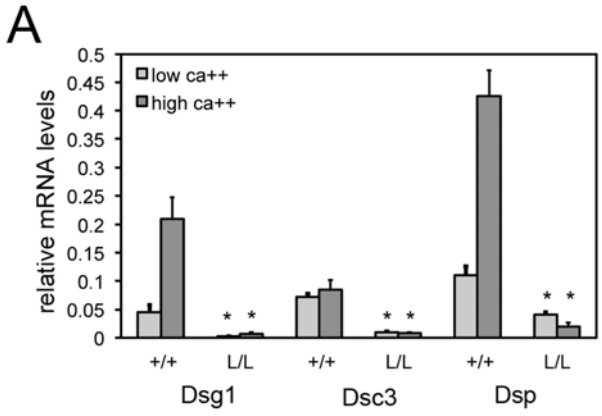
barrier defects (Sevilla et al., 2007) therefore CE preparations derived from back skin of P3 mice was analyzed. Also this analysis revealed no evident differences in mutant versus control preparations (data not shown).

4.3 Skin blistering in AEC mouse model is due to alteration in different adhesion molecules.

Since epidermal blisters may be caused by defects in adhesion molecules and we previously observed that in both the AEC patients and the constitutive AEC mouse model desmosomes were impaired, we analyzed the expression of desmosomal genes in our mouse model (Ferone et al., 2013).
Accordingly with previous data, we observed a strong reduction of several desmosome components in L/L keratinocytes, with the most significantly affected desmosome protein being desmoglein 1 (Dsg1), desmocollin 3 (Dsc3) and desmoplakin (Dsp) (Fig.14A).

At the functional level, desmosome defects are associated with weaken cell–cell adhesion and reduced mechanical stress resistance of keratinocyte monolayers. To determine whether defects in desmosome gene expression resulted in weakened cell–cell adhesion, the mechanical stress resistance of mutant keratinocyte monolayers in culture was compared with controls. Consistent with reduced expression of several desmosomal proteins, cell sheets of mutant keratinocytes generated under high-calcium conditions were significantly less resistant to mechanical stress than control wild-type keratinocytes in a shearing assay (Fig. 14B).

Interestingly, immunofluorescence analysis revealed that also the cellular localization of desmosomal protein Dsp and Dsc3 was lower and abnormal in cultured mutant keratinocytes respect to controls (Fig.14C). These data were obtained in mouse keratinocytes, whereas mutant epidermis of the conditional mouse model, in spite of focal skin fragility, expressed properly desmosomes (data not shown) thus indicating that other mechanism may be account for the observed epidermal blistering in mutant mice.



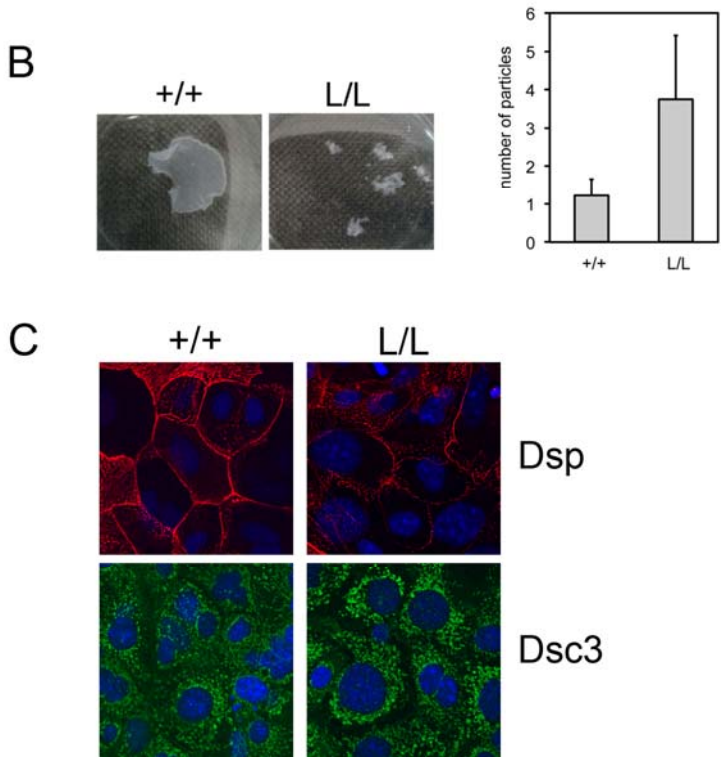


Figure 14. Desmosomes expression and mechanical resistance in vitro in AEC keratinocytes. A) Real-time RT-PCR on mouse keratynocytes derived from L/L and control mice harvested under basal (low Ca^{++}) and differentiating conditions (high Ca^{++}, 2mM). B) Detached monolayers of keratinocytes were subjected to mechanical stress, and resulting fragments of the cell sheet were imaged and counted. Quantitative evaluation of particles generated in the experiment is shown on the right. C) Immunofluorescence analysis on mouse primary keratinocytes isolated from control or mutant mice cultured for one week on glass slides. Cells were treated (Dsp and Dsc3) with 2mM calcium chloride to induce differentiation for 24 hours.

Beside desmosomes, adherens junction components *Pvrl1* and *Pvrl4* were expressed in epidermis and are fundamental for cell-cell cohesion. We analyzed the expression of *PVRL1* and *PVRL4* in human keratinocytes derived from individuals affected by AEC syndrome. Interestingly, both *PVRL1* and *PVRL4* were strongly reduced (Fig. 15A). Similar results were obtained in keratinocytes derived from AEC mouse model respect to control. Both *Pvrl1* and *Pvrl4* were significantly lower in mutant keratinocytes versus controls under basal and differentiating conditions (Fig. 15B).

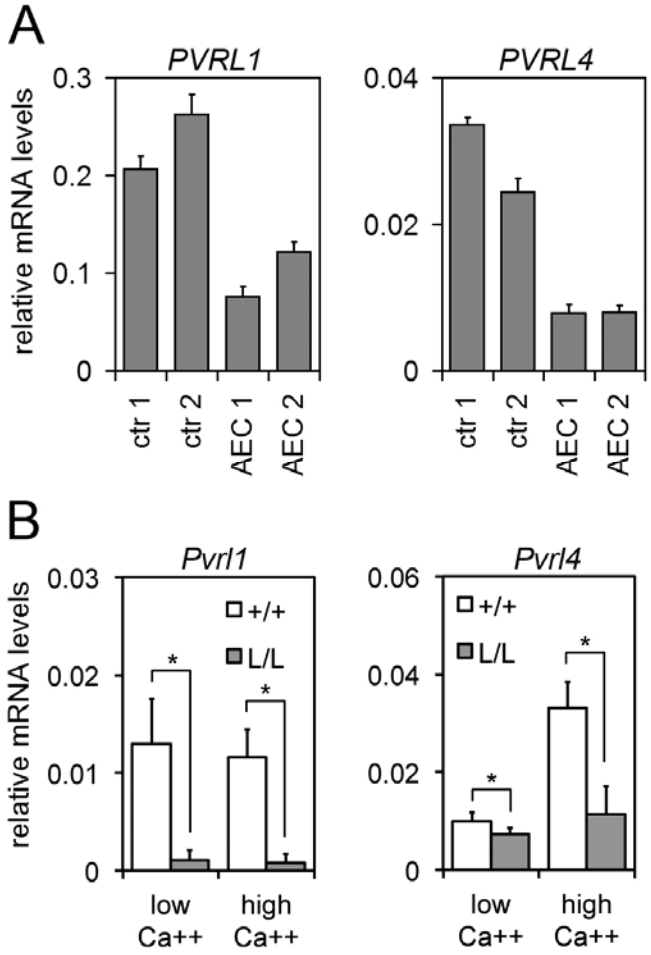


Figure 15. *PVRL1* and *PVRL4* expression in p63 mutant keratinocytes: A) *PVRL1* and *PVRL4* mRNA expression levels in human keratinocytes derived from two affected individuals (AEC1 and AEC2) and controls (ctr1 and ctr2) harvested under differentiating (0.3 mM Ca^{++}) conditions. Data are normalized for RPLP0 mRNA levels and are represented as mean ± SD normalized mRNA levels. B) *Pvrl1* and *Pvrl4* mRNA expression in L/L keratinocytes harvested under basal (low Ca^{++}) and differentiating conditions (high Ca^{++}, 2mM). *Pvrl1* mRNA levels were significantly decreased both under basal (*p-value=8.13E-05; n=14) and differentiating conditions (*p-value=9.7E-05; n=10). Similarly *Pvrl4* mRNA levels were significantly decreased under basal (*p-value=0.012; n=14) and differentiating conditions (*p-value= 0.008; n=6). Data are normalized for β-actin mRNA levels and are represented as mean ± SD.

Skin blistering could be due to alterations in different components of cell-cell and cell-matrix adhesion. Indeed further analysis revealed a strong reduction also in cell-matrix adhesion molecule collagen VII (Col7a1) (Fig. 16), thus

reinforcing the idea that skin fragility in AEC mouse model was due to alteration of different adhesion molecules.

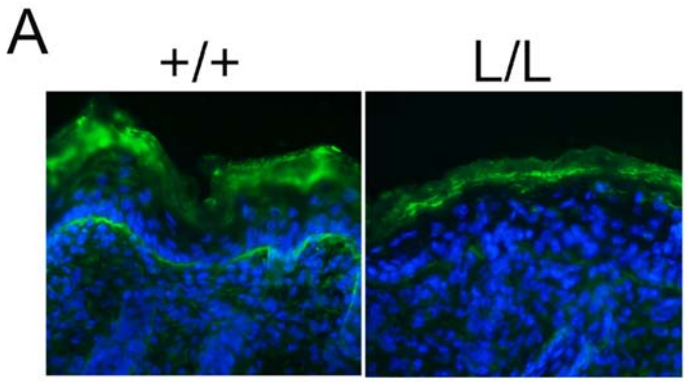


Figure 16: Skin blistering in the AEC mouse model. A) Immunofluorescence on frozen section of dorsal skin at P3 for Collagen VII revealed a strong reduction of the protein in AEC mutant epidermis respect to the controls.

Since it has been suggested from the literature that the overall function of epidermal intermediate filaments (IF), also known as keratins, is to impart mechanical integrity to the cells, without which, the cells become fragile and prone to rupturing, we analyzed the expression levels of keratins in epidermis of AEC mutant and control mice. Keratins represent an apparatus through which the cell becomes more resistant to mechanical stress, on the other hand they are an index of differentiation of the epidermis, being expressed in a specific manner by the different layers of the epidermis.

At P3 epidermal differentiation was critically altered in L/L mice since expression of Krt5, Krt10 and Krt15 decreased in mutant mice respect to the controls. In contrast, late differentiation markes such as loricrin and involucrin are properly expressed in mutant mice (Fig.17A). In accordance with the alteration of keratins of the basal layer, ultrastructural analysis demonstrated reduces keratin bundles in the basal layer of the epidermis of mutant mice compared to controls (Fig. 17B). Importantly reduced KRT5 was observed also in human samples derived from AEC patients both at the RNA and protein levels (Fig. 17C).

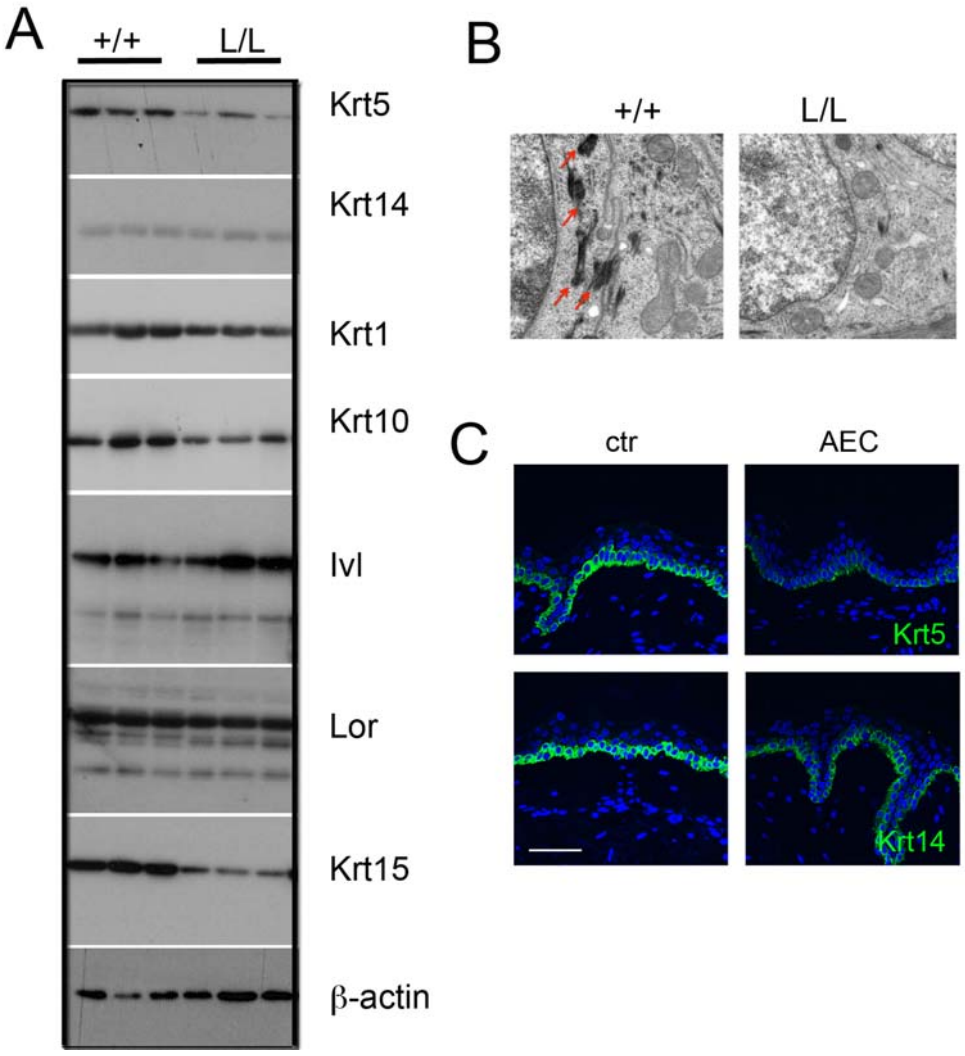


Figure 17. Expression of keratins and other differentiation markers in the AEC mouse model. A) Immunoblotting of total cell extracts from neonatal epidermis of L/L and +/+ mice using antibodies against the indicated differentiation markers. B-actin was used to normalize samples. B) Tissue-electron microscopy (TEM) of newborn skin. C) Immunofluorescence analysis of human AEC patients and controls with antibodies against KRT5 and KRT14.

Interestingly, we observed that both *Krt5* and *Krt14* expression, that were known p63 target genes (Romano et al., 2009), was strongly downregulated in p63 knockdown and in AEC mutant keratinocytes (Fig. 18 A and B), whereas in AEC mutant epidermis only Krt5 was significantly affected indicating that in vivo p63 plays a crucial function in Krt5 regulation (Fig. 18 C).

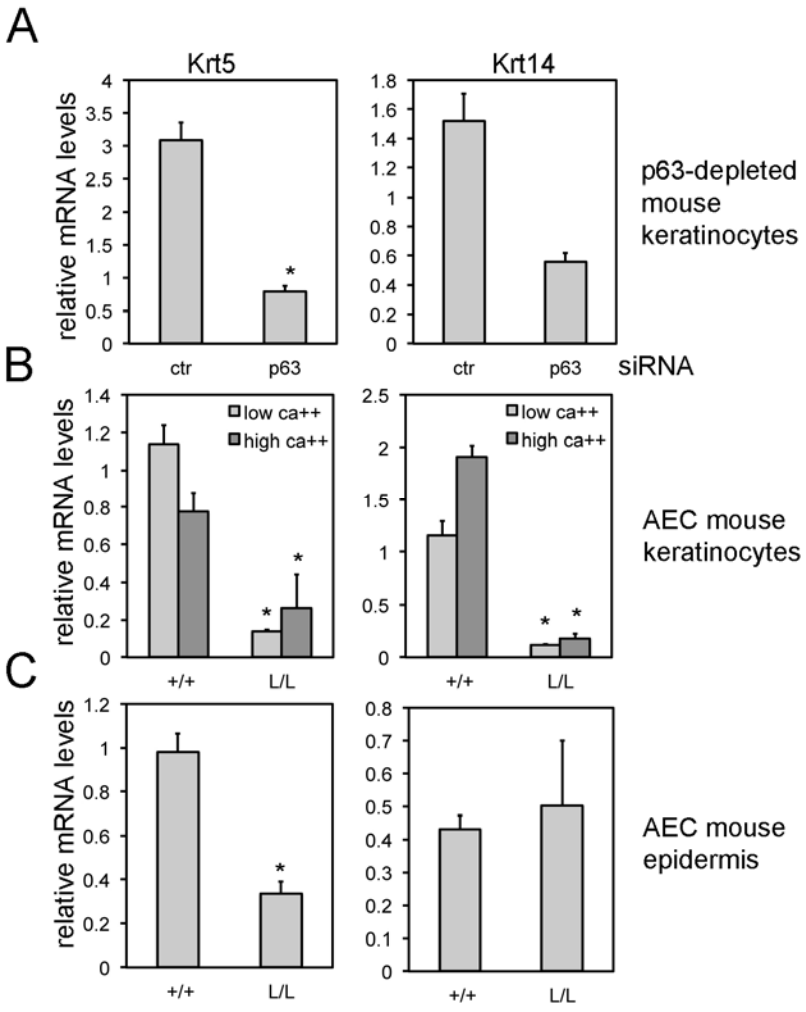


Figure 18 Differential regulation of basal intermediate filaments. A) Real time RT-PCR for *Krt5* and *Krt14* in keratinocytes depleted of p63. *Krt5* and *Krt14* mRNA expression was strongly downregulated in p63 depleted keratinocytes as compared to controls (*p-value= 0.0002; n=4). Data are normalized for β-actin mRNA levels and are represented as mean ± SD normalized mRNA levels. B) Real-time RT-PCR for *Krt5* and *Krt14* in mouse keratynocytes derived from L/L and control mice harvested under basal (low Ca^{++}) and differentiating conditions (high Ca^{++}, 2mM). Data are normalized for β-actin mRNA levels and are represented as mean ± SD normalized mRNA levels. C) Real-time RT-PCR for *Krt5* and *Krt14* in mouse epidermis derived from L/L and control mice (*p-value= 0.0004; n=6). Data are normalized for β-actin mRNA levels and are represented as mean ± SD normalized mRNA levels.

4.3.1 Adherens junction component *Pvrl1* is a p63 target gene in skin.

A possible regulation of nectins by p63 has never been explored, although a putative genetic interaction between *p63* and *Pvrl1* has been postulated, since mutations in both genes lead to cleft palate (McGrath et al., 2001). Given that *PVRL1* and *PVRL4* associated syndromes have many features in common with those caused by *p63* mutations, we investigated the possibility that p63 may regulate Pvrls expression in epidermis.

In *p63* null embryonic epidermis *Pvrl1* mRNA was strongly diminished in *p63* null mice, whereas an equal amount of the other Pvrl genes, afadin (*Mllt4*) and E-cadherin (*Cdh1*) was observed (Fig. 19A). We next analyzed Pvrl1 expression in primary mouse keratinocytes depleted for p63 or ΔNp63 using specific siRNA (Fig. 19B e C). In both p63 and ΔNp63 depleted keratinocytes *Pvrl1* was significantly reduced both at the mRNA and protein levels as compared to controls, whereas *Pvrl4* mRNA levels were not affected by p63 knockdown, similar results were obtained in human (Fig. 19B and 18C).

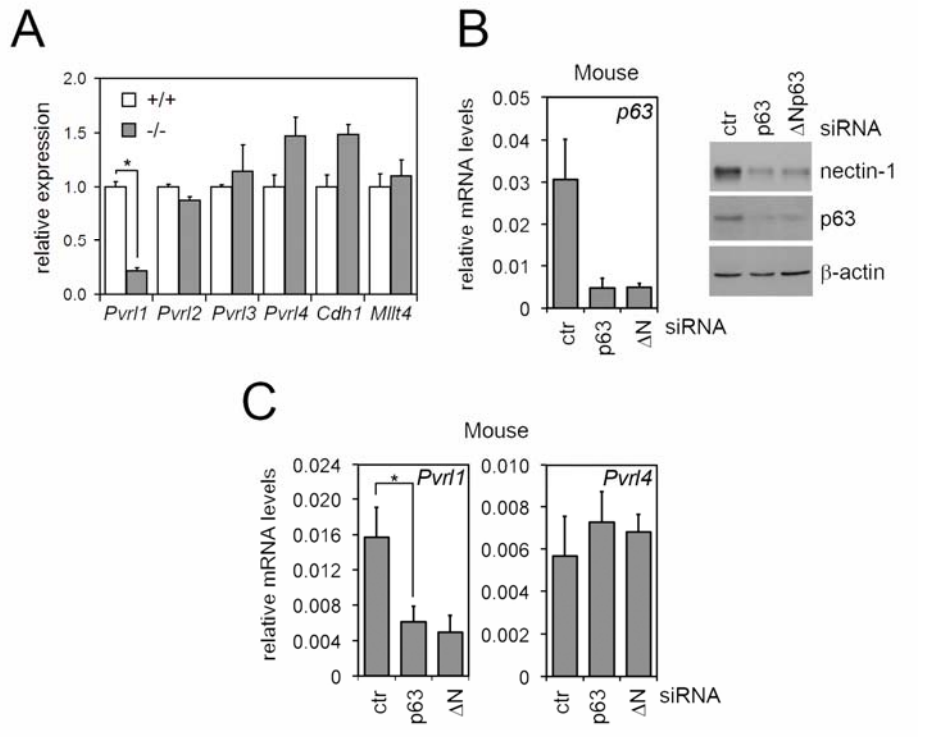


Figure 19: Pvrl1 is regulated by p63. A) Expression of the indicated genes was performed by real time RT-PCR in E16.5 p63 null (-/-) and controls (+/+) skin. *Pvrl1* mRNA levels were strongly decreased in the skin of *p63*-/- mice compared to wild-type (*p-value=0.007; n=6). Data are normalized for β-actin mRNA levels and are expressed relative to controls. Error bars denote SD. B)

p63 mRNA expression in control mouse keratinocytes or in keratinocytes depleted of p63 or □Np63. Data are normalized for β-actin mRNA levels and are represented as mean of ± SD (left panel); immunoblotting analysis for nectin-1 and p63 in cell extracts depleted of p63 or ΔNp63, or controls (ctr). Data are normalized for β-actin protein expression (right panel). C) Real time RT-PCR for *Pvrl1* in keratinocytes depleted of p63 or □Np63. *Pvrl1* mRNA expression was strongly downregulated in p63 depleted keratinocytes as compared to controls (*p-value=0.002; n=8) whereas *Pvrl4* expression is similar to control. Data are normalized for β-actin mRNA levels and are represented as mean ± SD normalized mRNA levels.

To obtain further insights into the regulation of *Pvrl1* by p63 we analyzed a genome-wide ChIP-seq analysis previously performed in human primary keratinocytes (Kouwenhoven et al., 2010) which revealed at least two strong p63 binding regions (p63BS1 and p63BS2) in the first intron of *PVRL1*. p63BS1 and p63BS2 were highly conserved in mammals and contained DNAse hypersensitive sites, consistent with their open chromatin state(Crawford et al., 2006; Nord et al., 2013). In addition p63BS2, and to a lesser extent p63BS1, were enriched for histone H3 acetylation on lysine 27 (H3K27ac), a chromatin marks known to associate with active regulatory regions(Creyghton et al., 2010; Ernst et al., 2011; Rada-Iglesias et al., 2011). ChIP-qPCR analysis performed in human keratinocytes and in primary mouse epidermis confirmed that p63 efficiently bound both the identified regions and more strongly to p63BS2 in mouse epidermis (Fig. 20A and B). To gain further insights, we cloned p63BS2 upstream of the luciferase gene. Luciferase assays revealed that the p63BS2 activity was efficiently reduced upon p63 depletion in mouse keratinocytes (Fig. 20C). Taken together these data indicate that *Pvrl1* is a *bona fide* p63 transcriptional target gene.

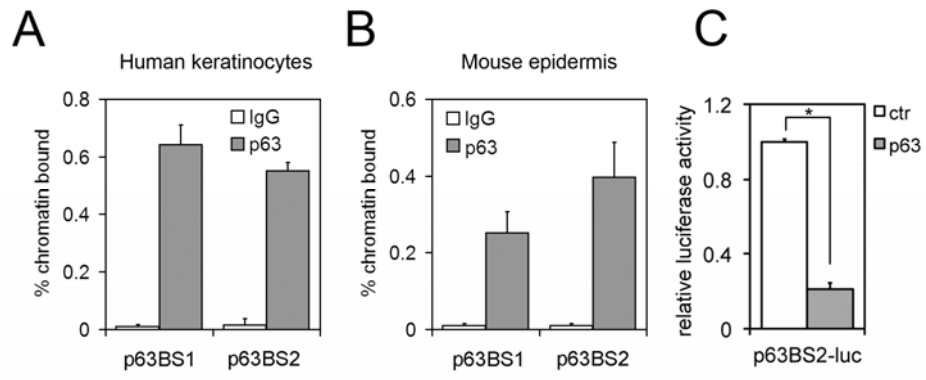


Figure 20: Pvrl1 is a direct p63 target gene both in human and in mouse. A) ChIP-qPCR of the two identified p63 binding regions (p63BS) in the *PVRL1* genomic locus was performed in human primary keratinocytes and in mouse epidermis. B) p63BS2 enhancer activity was analyzed by luciferase

assay in p63 depleted or control keratinocytes (*p-value= 3.12E-06; n=6). Data are represented as mean of ± SD of three independent experiments.

4.4 Gene expression analysis of AEC mouse epidermis.

To obtain a global view of changes in gene expression of AEC mutant mice, we performed a comparison of gene expression profiling of mutant versus wild-type epidermis at P3 using Affymetrix gene chip.

Analysis of the data revealed that a subset of genes, known to be p63 target genes, is down-regulated by L514F mutation. Among the p63 targets affected in mutant epidermis we found the two fibroblast growth factor receptors *Fgfr2* and *Fgfr3* and *Krt15* (Ferone et al., 2012), *Col17a1* and *Dst*, two genes encoding for hemidesmosome components (Carroll et al., 2006), *Ddit4* gene, also called *Redd1*, a developmentally regulated transcriptional target of p63 and p53, involved in DNA damage (Ellisen et al., 2002), and *Notch1* and *Jag2*, two key components of the Notch signaling pathway (Nguyen et al., 2006)(Fig. 21A).

Interestingly, a number of biological processes were de-regulated. In particular we observed that epidermal barrier genes involved in arachidonic acid and lipid metabolism were strongly downregulated in AEC mutant skin (42 genes; p-value= 5.3x10-7), including *Alox12*, *Alox12e* and *Adh1* (Fig. 21B).

In contrast, AEC mutant epidermis displayed increased focal expression of some keratins, including *Krt6* and *Keratin 16* (*Krt16*) that were essential to maintain keratinocyte integrity in wounded epidermis, and *Keratin* 8 (*Krt8*) and *Keratin 18* (*Krt18*), simple epithelial keratins, that for their nature could participate to intermediate filaments but did not confer strong mechanical resistance to the epithelium. Also kallikreins, enzymes with serine protease activity that regulate the desquamation of the epidermis (Candi et al., 2005) were strongly up-regulated thus indicating an altered equilibrium in epidermis structure (Fig. 21C and D).

Surprisingly, we found a strong up-regulation of inflammatory genes. In particular, we found an up-regulation of one hundred fold of *Tslp*, an IL-7-like cytokine produced by epithelial cells, including keratinocytes that was highly expressed in the epidermis of atopic dermatitis and asthmatic patients (Ziegler and Artis, 2010) (Fig. 21D). TSLP was produced by epithelial cells and had different roles. Indeed, Tslp caused a polarization of dendritic cells to drive T helper (Th) 2 cytokine production, promoted T-cell proliferation in response to T-cell receptor activation and Th2 cytokine production, and supported B-cell expansion and differentiation. TSLP further amplifies Th2 cytokine production by mast cells and natural killer T cells.

In addition to Tslp induction, our microarray data revealed us also the induction of damage-associated molecular pattern molecules (DAMPs) that initiate the inflammatory response in the event of a disruption of the epidermal barrier, such as the inflammatory complex of calprotectin (*S100a8*/ *S100a9*

complex), and -to a lesser extent- antimicrobial peptides, *Defb4* and *Defb6* (Fig. 21D).

A

p63 target genes

Gene Symbol	Fold +/ctr
Fgfr2	0.43
Fgfr3	0.36
Krt15	0.30
Col17a1	0.44
Dst	0.57
Ddit4	0.49
Notch1	0.44
Jag2	0.63

B

Lipid metabolism

Gene Symbol	Fold +/ctr
Alox12	0.17
Alox12e	0.34
Adh1	0.36

C

Terminal differentiation genes

Gene Symbol	Fold +/ctr
Klk10	3.36
Klk5	1.20
Klk6	5.12
Klk7	1.42
Klk9	1.56

D

Biological processes

Gene Symbol	Fold +/ctr
Defb4	2.58
Defb6	2.31
S100a8	9.03
S100a9	7.81
Tslp	112.93
Krt6a	11.09
Krt6b	14.18
Krt16	9.24

Figure 21. Gene expression analysis. A) Microarray data confirmed that a subsets of known p63 target genes are affected in the epidermis of L/L mice at postnatal day 3 compared to wild-type controls B-D) Microarray data indicated that a subset of genes involved in different biological processes were down-regulated and up-regulated consistently with what we observed in AEC mutant mice.

4.5 Inflammation in AEC mouse model

Skin of mutant mice progressively became hyperplastic with clear signs of inflammation at P12. Few days after the first biochemical sign of inflammation, epidermis was hyperplastic and hyperkeratotic with neutrophils accumulation in the epidermis, a rich cellularization of the dermis accompained by a strong induction of Krt6 (Fig. 22 A and B). The presence of infiltrating inflammatory cells in the dermis and the upregulation of Krt6 in the epidermis of mutant mice suggested that an inflammatory response may be implicated in the development of the severe skin phenotype observed at later stages that could be related to Tslp induction.

To confirm the microarray data, we verified the expression of Tslp in AEC mutant mice respect to controls. QRT-PCR analysis in epidermis of mutant mice at P3 confirmed the strong up-regulation of Tslp, which usually is not expressed in epidermis. Since Tslp was able to reach the bloodstream to arrive in different body districts, we evaluated its expression also in blood serum derived from AEC mutant and control mice and we found a high expression of Tslp also in blood (Fig. 22C). Interestingly, also in human AEC patients we observed an induction of Tslp, thus indicating that Tslp may be involved in AEC syndrome and could be the cause of the progressive severe phenotype that characterized the AEC mutant mice.

Luisa Cirillo, a PhD student in the laboratory is carrying out this part of the project.

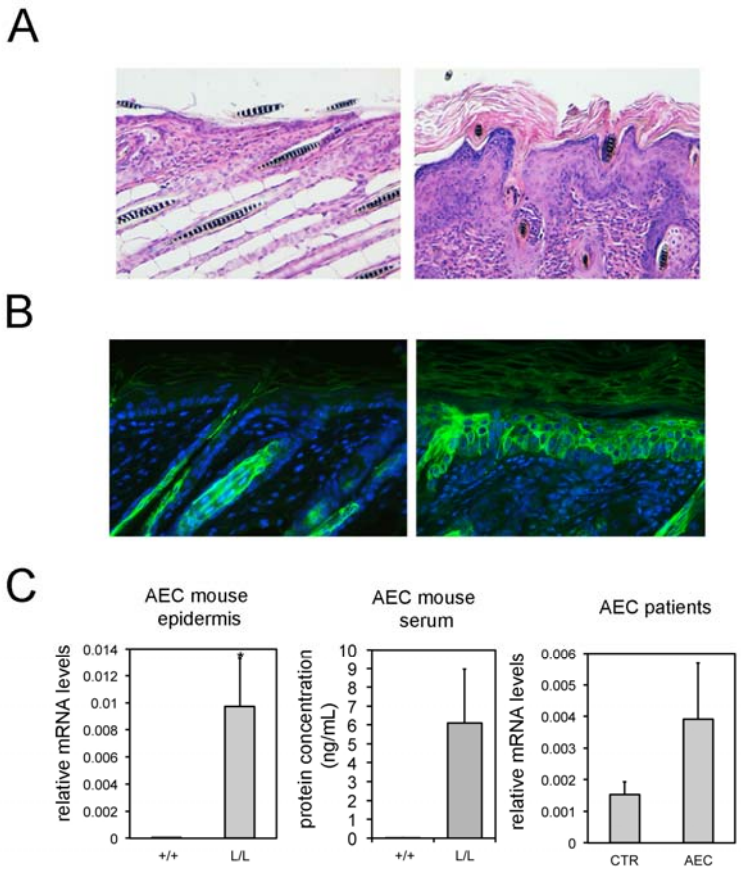


Figure 22: Inflammation in AEC mouse model. A) H&E staining of mutant skin sections at P12 revealed a hyperplastic epidermis with clear signs of massive inflammation. B) Immunofluorescence analysis of mutant skin sections at P7 revealed a strong and abnormal expression of Krt6 in mutant epidermis respect to controls. C) (from the left panel) Real-time RT-PCR for Tslp in epidermis at P3 confirmed the strong up-regulation of the mRNA levels of Tslp (n=7; p-value= 0.01). Data are normalized for β-actin mRNA levels

and are represented as mean ± SD normalized mRNA levels; systemic accumulation of Tslp protein (ng/ml) in blood serum of mutant mice at P15 measured by ELISA; Real-time RT-PCR for Tslp in skin derived from AEC patients revealed Tslp mRNA up-regulation also in human patients. Data are normalized for RPLP0 mRNA levels and are represented as mean ± SD normalized mRNA levels.

4.6 Impaired Notch signaling in AEC mutant mice.

Global gene expression profiling revealed that several biological processes are altered in AEC mutant epidermis. Indeed, we confirmed a strong reduction of *Notch1* and *Jag2* expression levels accompanied with reduced expression of the Notch1 direct target gene *Hes1* in AEC mutant epidermis (Fig. 23A), in agreement with previous work indicating that p63 and Notch signaling were interconnected and that *Notch* and *Jag2* were direct p63 target genes (Sasaki et al., 2002; Laurikkala et al., 2006; Nguyen et al., 2006; Candi et al., 2007). In mice loss of Notch signaling led to strong TSLP induction, which was suggested to be a consequence of the epidermal barrier failure (Demehri et al., 2008). Similarly to AEC conditional mouse model, conditional Notch1/Notch2 mouse model showed severe inflammation, an altered gene expression of genes involved in barrier formation and a strong induction of Tslp. Indeed, in Notch-deficient epidermis Tslp induction was accompanied by defective in skin lipid biosynthetic enzymes, with the most strongly inhibited gene being the Arachidonate 12-Lipoxigenase (Alox12), and to a lesser extent Alox12e, Adh1, and Liph (Demehri et al., 2008)(Fig. 23B and data not shown).

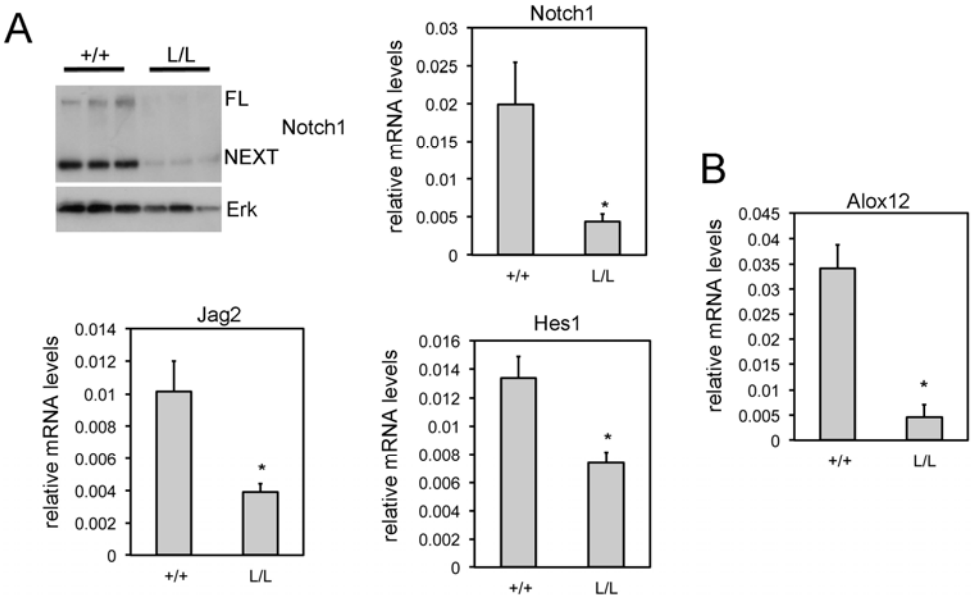


Figure 23: Notch1 pathway in AEC mouse model. A) Immunoblotting of total cell extracts from P3 epidermis of L/L and +/+ mice using antibodies

against full-length and NEXT fragment of Notch1. FL: Notch1 Full Length protein; NEXT: Notch Extracellular Truncation, cleaved by gamma-secretase. Erk was used to normalize samples (on the left). Real-time RT-PCR for *Notch1* (*p-value=0.03; n=7), *Jag2* (*p-value=0.02; n=7) and *Hes1* (*p-value=0.01; n=7) in mouse epidermis derived from L/L and control mice. Data are normalized for β-actin mRNA levels and are represented as mean ± SD normalized mRNA levels. B) Real-time RT-PCR for *Alox12* in mouse epidermis derived from L/L and control mice (*p-value=0.002; n=7). Data are normalized for β-actin mRNA levels and are represented as mean ± SD normalized mRNA levels.

4.7 Notch1 is responsible for Alox12 downregulation and Tslp induction in AEC mouse model.

Given the strong similarities between AEC mouse model and conditional Notch1/Notch2 mouse model, we asked whether restoration of Notch1 pathway in our system could rescue the expression of genes involved in arachidonic acid metabolism, such as *Alox12* and could explain the strong induction of Tslp. To this aim, I infected keratinocytes derived from AEC mutant and control mice both with either retroviruses or adenoviruses carrying an active form of Notch1. Following the Notch1 overexpression, I evaluated the expression of some known target genes and genes disrupted in AEC mutant mice that could be linked to Notch1 signaling. I found that *Alox12* was rescued by re-activation of Notch pathway, as well as *Hes1* (Fig. 24A and B).

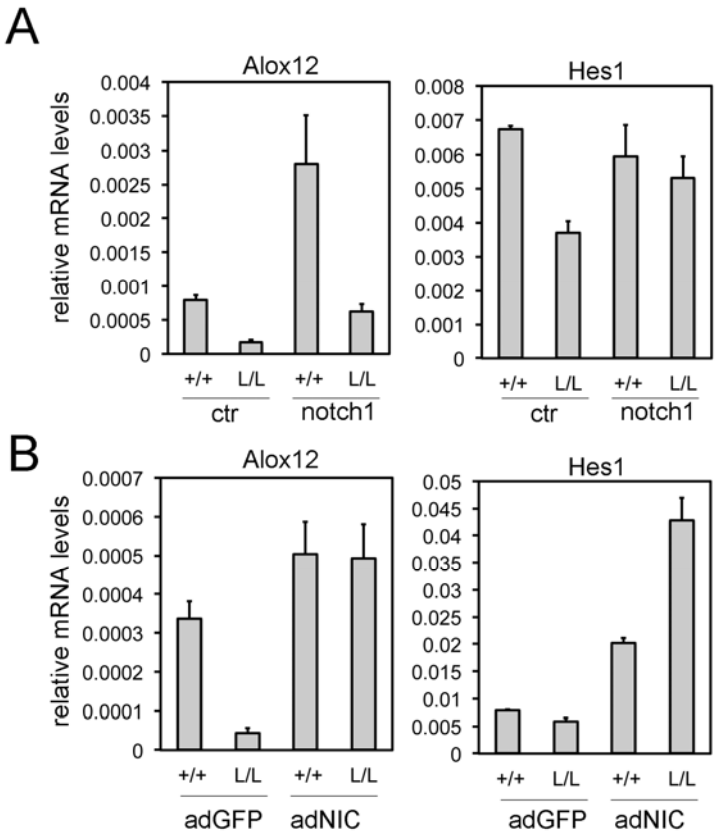


Figure 24. Alox12 and Hes1 were rescued by active Notch in AEC mutant keratinocytes. A) Real-time RT-PCR for *Alox12* and *Hes1*. mRNA of both genes were rescued by retroviral transduction of an active form of Notch1 in mutant mouse keratinocytes. Data are normalized for β-actin mRNA levels and are represented as mean ± SD normalized mRNA levels. B) Similar results were obtained by Adenovirus expressing the transcriptional active form of Notch (Ad-NIC).

Interestingly also *Tslp* expression was influenced by re-activation of Notch1 pathway since its expression was strongly downregulated by upregulation on Notch1 (Fig. 25 A).

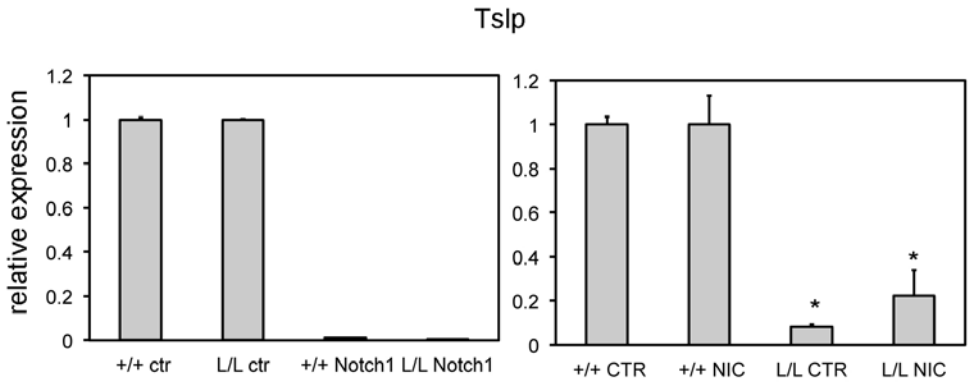


Figure 25. Tslp expression was regulated by Notch1. Real-time RT-PCR for *Tslp* revealed that both retroviral and adenoviral transduction of an active form of Notch1 downregulated the expression of Tslp in vitro (*p<0.001; n=34). Data are normalized for β-actin mRNA levels and are expressed relative to controls. Error bars denote SD.

I next inhibited Notch1 activity by using a retrovirus carrying a peptide that competes for MAML1 binding to the Notch–CBF-1 complex and prevents downstream transcription (Weng et al., 2003). In agreement with previous data, suppression of Notch pathway led to an inhibition of *Alox12* expression as well as of *Hes1* whereas, in this condition we were not able to detect a significant upregulation of *Tslp* expression (Fig. 26).

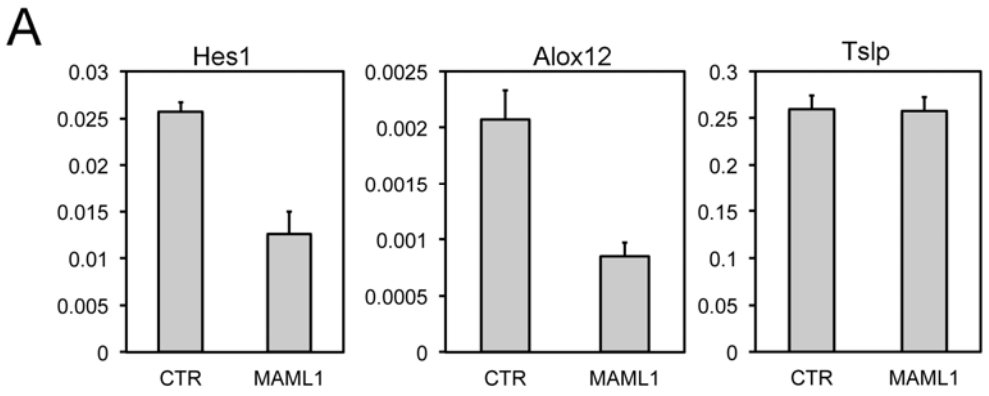


Figure 26. Inhibition of Notch1 in cultured keratinocytes led to inhibition of Alox12 and Hes1 expression. A) Real-time RT-PCR for *Hes1, Alox12* and *Tslp* in cultured keratinocytes derived from mutant and control mice harvested under differentiating conditions (2mM Ca++) and infected with a retrovirus containing a dominant-negative form of MAML1 reveled that inhibition of Notch1 pathway led to a downregulation of *Alox12* and *Hes1*, whereas *Tslp* expression was not affected in these conditions. Data are normalized for β-actin mRNA levels and are represented as mean ± SD normalized mRNA levels.

5. DISCUSSION and CONCLUSIONS

Ankyloblepharon, ectodermal defects, cleft lip/palate (AEC) syndrome is a rare autosomal dominant disorder caused by mutations in the p63 gene, essential for embryonic development of stratified epithelia. The most severe cutaneous manifestation of this disorder is the long-lasting skin fragility associated with severe skin erosions after birth. Indeed, in AEC syndrome skin is severely affected with approximately 80% of the patients having congenital erythroderma and extensive erosions that can lead to the initial misdiagnosis of epidermolysis bullosa. Erosions typically involve the scalp, head and neck, skin folds, palm and soles, and are often accompanied by crusting, granulation tissue and secondary infections and treatments are supportive but not curative (Julapalli et al., 2009).

Since little is known about the causes of AEC skin erosions, the histological and molecular defects, and feasible treatments, we previously generated a constitutive mouse model that carries a clinically relevant point mutation in the SAM domain of the p63 alpha isoform (L514F) reported in AEC patients.

p63$^{+/\ L514F}$ mice died at birth with severe cleft palate, skin defects and ectodermal dysplasia (Ferone et al., 2012), thus faithfully recapitulating the defects observed in AEC syndrome. The cause of death in *p63*$^{+/L514F}$ mice was cleft palate, which is lethal in mice but not in humans. *p63*$^{+/\ L514F}$ skin displayed an overall reduction in skin thickness, accompanied by a significant epidermal atrophy and hair follicle hypoplasia. At the molecular level we found that the AEC mutation affected proliferation of the epithelial cells of the palate and skin, and led to a reduced number of epidermal stem cells. These phenotypes were associated with aberrantly low FGF signaling due to reduced expression of the FGF receptor *Fgfr2*, a direct p63 target gene. Importantly, we obtained evidences that humans affected by AEC syndrome have similar defects of the epidermal stem cell compartment (Ferone et al., 2012). AEC mouse skin displayed focal blistering skin lesions, localized mainly between the basal and suprabasal layers of the epidermis accompanied with acantholysis (loss of intercellular adhesions) and only rarely by cytolysis (rupture of the cell membrane). These phenotypes were associated with reduced desmosomal contacts in AEC mutant skin. We demonstrated that skin fragility observed in the constitutive mouse model was caused by a strong reduction of desmosomes and that Dsg1, Dsc3 and Dsp were strongly reduced also in human keratinocytes derived from AEC patients and focal downregulation of DSP and DSC3 expression was observed in non-lesional skin of AEC patients (Ferone et al., 2013; Koster et al., 2014), confirming their possible causative role in the pathogenesis of AEC syndrome.

To overcome the lethality of the constitutive mouse model and to better characterize the molecular mechanisms underlined skin erosions and hypothetical in vivo treatments, we generated a conditional AEC mouse model. The conditional knock-in model (p63$^{+/floxL514F}$), express the L514F mutation

only in the presence of the CRE recombinase. Thus the newly generated $p63^{+/floxL514F}$ mouse model expresses only the wild-type p63 and has no phenotype until a portion of the inserted DNA is removed by CRE-mediated recombination. We cross $p63^{+/floxL514F}$ with a knock-in mouse expressing Cre by E17.5 in epidermis and hair follicle under the control of the endogenous keratin 14 (K14) promoter (K14-Cre Δneo) to obtain both the heterozygous and the homozygous model (+/L and L/L) (Huelsken et al., 2001).

We begin to characterize the heterozygous model, +/L that show no gross abnormalities. We hypothesize that, since the expression of the mutation is late in the development a number of biological processes that required p63 presence are not affected because they happen when the mutation is still off and only one copy of mutant p63 is not able to negatively influence these processes later. Our observations are quite in contrast with what we observe in the constitutive model, in which gene expression analysis lead us to hypothesize that L514F mutation acts in a dominant negative fashion. To shed light on this discrepancy further analysis on the molecular mechanisms by which mutant p63 influences its transcriptional activity will be needed. On the other hand it has been reported that sometimes mouse model and human disorders are quite different in the genetic contest, therefore our model need to be in homozygousity to recapitulate the syndrome. Importantly, alteration of the p63 target genes in the conditional homozygous model is comparable to the constitutive heterozygous mutant in spite of nearly complete absence of the wild-type copy by P0, thus indicating that the L/L model is comparable to the constitutive one.

L/L mice display a strong phenotype characterized by skin erosions and skin crusting. Due to the complex and progressive phenotype a high percentage of mutant mice die between 10 and 20 days after birth, whereas those that become adults are affected by skin ulcerations, complete hair loss, and severely reduced body weight, thus indicating that not only skin but all body districts are influenced by p63 mutation.

The histological analysis of epidermis reveal altered skin architecture in focal points with cells undergoing cytolysis and acantholysis since cells detach both from the basal lamina and each other.

The focal gaps in the epidermal barrier that we observe in mutant mice are consistent with focal alteration of the epidermal barrier revealed by X-gal penetration assay. The structural integrity of epidermis is ensured by an organized cytoarchitecture, thus we hypothesize that the defects that we observed in this model are due to alteration in adhesion molecules at different levels.

It has been postulated that intra-epidermal blistering with areas of both acantholysis and cytolysis in perilesional skin of a newborn AEC patient after mechanical rubbing are consistent with a disruption in desmosomal components (Payne et al., 2005; Ferone et al., 2013). In addition, mutations in genes encoding desmosomal components lead to a range of disorders characterize by epidermal fragility, blistering, and keratoderma depending on

the affected gene (Lai-Cheong et al., 2007). In line with these observations, we find a decreased expression of desmosome genes Dsg1, Dsc3 and Dsp and reduced mechanical stress resistance, similarly to the constitutive AEC model (Ferone et al., 2013).

Beside desmosomes, it has been previously suggested that p63 and adherens junction component Pvrl1 may be genetically linked because *PVRL1* mutations in humans cause ectodermal dysplasia and cleft lip/palate (Zlotogora et al., 1987; Bustos et al., 1991; Suzuki et al., 2000) resembling p63-associated syndromes (McGrath et al., 2001). In addition, since *PVRL1* and *PVRL4* have a crucial and partially overlapping role and human mutations in these two genes are causative of partially overlapping defects, we hypothesize that also Pvrl4 may be regulate by p63. Consistent with these hypotheses, we find that *Pvrl1* expression is reduced in keratinocytes derived from AEC patients and from conditional AEC mouse models. Surprisingly, *Pvrl4* expression is also reduced in differentiated AEC keratinocytes, even though its expression is not altered in p63 depleted keratinocytes or epidermis. McDade and colleagues demonstrate a direct p63 binding on the human PVRL1 promoter in human keratinocytes and its responsiveness to p63 in heterologous cells (McDade et al., 2012). We demonstrate that *Pvrl1* expression is impaired in p63-depleted keratinocytes and in p63 null embryonic skin and that two conserved intronic regions are strongly bound by p63 in mouse keratinocytes, again indicating that *Pvrl1* is a p63 direct target gene (Mollo et al., 2014).

A transcriptomic profile of AEC patients skin has been published and postulated that skin erosions in AEC patients could be due to defects in *FRAS1* and *COL7A1* expression (Clements et al., 2012). The extracellular matrix protein Col7a1 is a major component of the anchoring fibrils at the dermal–epidermal junctions (Burgeson, 1993). Mutations in *COL7A1* result in dystrophic epidermolysis bullosa (DEB); a major type of EB caused by disrupted dermal/epidermal adhesion (Dang and Murrell, 2008). We find that in our mouse model *Col7A1* but not *Fras1* expression is disrupted, thus supporting the idea that epidermal blistering in AEC mouse model may be due also to alteration in different structures that regulate adhesion processes. Accordingly, several studies indicate that p63 is a crucial transcriptional regulator of genes involved in cell-matrix adhesion and cell-cell adhesion genes, directly regulating a subset of extracellular matrix, hemidesmosomal, desmosomal, adherens junction and tight junction components (reviewed in (Ferone et al., 2015)).

Given that alteration in desmosomes and adherens junctions are evident only in mutant keratinocytes but not in epidermis, an hypothesis is that compensatory mechanisms may act in vivo thus protect the weakness of these structures, but given the strong phenotype, other molecules have to be involved in the skin fragility and erosions in mutant mice.

The structural integrity of epidermal keratinocytes is maintained also by a filamentous network made up of keratins (Magin et al., 2000). Since it has been suggested that the overall function of epidermal keratin filaments is to

impart mechanical integrity to the cells, without which, the cells become fragile and prone to rupturing, we hypothesize that alteration in intermediate filaments in the basal layer of the epidermis may explain epidermal blisters in AEC syndrome.

Keratins belong to the superfamily of intermediate filament (IF) proteins, which have the capacity to assemble to form obligatory heteropolymers, indeed they are often coexpressed as specific pairs in vivo (Hutton et al., 1998). In particular, the basal layer of the epidermis is characterized by heteropolymers of Krt5 and Krt14. It is now well established that mechanically induced blistering disorders of the epidermis involve mutations in keratins and often the severity of these diseases seems to correlate with the degree to which the keratin mutants perturb IF assembly in vitro (Bonifas et al., 1991; Coulombe et al., 1991). In addition, since p63 induces expression of keratins typical of stratified epithelia and suppresses simple epithelium keratins (De Rosa et al., 2009), we hypothesize that in mutant keratinocytes the balance between these keratins could be affected thereby reducing epidermal resilience.

Interestingly, we find that in AEC mutant epidermis *Krt5* is strongly downregulated, in spite of a normal amount of *Krt14*. Ultrastructural analysis demonstrate reduce keratin bundles in the basal layer of the epidermis, thus indicating that the reduction of Krt5 is able to perturb the assembly of keratin bundles in mutant epidermis. In contrast, we observe that both *Krt5* and *Krt14* expression is strongly downregulated in p63 knockdown and in AEC mutant keratinocytes indicating that in vivo p63 plays a crucial function in *Krt5* regulation whereas *Krt14* expression maybe sustain by other transcription factors, such as AP1 and AP2 (Sinha et al., 2000; Romano et al., 2007).

The severity of skin erosion in AEC patients often leads to misdiagnosis, confusing AEC syndrome with epidermolysis bullosa simplex, a genetic disorder caused by mutations in intermediate filaments as *Krt5* (Muller et al., 2006). Importantly reduced *KRT5* is observed also in human samples derived from AEC patients both at the RNA and protein levels, thus reinforcing the idea that in AEC syndrome impaired intermediate filaments network may be responsible for skin fragility and epidermal blistering.

Global gene expression profile of AEC mutant epidermis reveals that a number of epidermal genes are affected. Indeed, p63 is a master regulator of gene expression in the epidermis and in other stratified epithelia and positively controls a large number of tissue-specific genes. As the main regulator of skin, p63 mutation affects different processes related to skin development and manteinance. Indeed , as indicators of altered epidermal structure, we find that by P3, *Krt6* and *Krt16* are strongly upregulated. Krt6 and Krt16 are being regarded as the most sensitive indicators of disturbances in epidermal homeostasis (McGowan and Coulombe, 1998). In control animals they are restricted to hair follicles and foot sole epidermis and are absent in interfollicular epidermis but after mechanical stress or tissue injury or as a result of hyperproliferation they are strongly induced in interfollicular

epidermis (Peters et al., 2001). In addition, we find an upregulation of *Klk5*, *Klk6*, *Klk7* and *Klk10*, serin-protease named kallikreins that coordinate the desquamation mechanism, a process that lead to the shedding of the outer epidermis. The correct balance between these protease and their inhibitors preserve epidermal homeostasis and provide an environmental barrier (Candi et al., 2005). Concomitant with *Krt6* and *Krt16* induction, we observe significant induction of damage-associated molecular pattern molecules (DAMPs) that initiate the inflammatory response in the event of a disruption of the epidermal barrier, such as the inflammatory complex calprotectin (*S100a8*/ *S100a9* complex), and -to a lesser extent- antimicrobial peptides, *Defb4* and *Defb6*. Taken together these data lead us to hypothesize that the epidermal barrier formation and the differentiation program of mutant epidermis does not occur properly and may be at the basis of the inflammatory phenotype that we observe at later stages.

Skin of mutant mice is characterized by a strong induction of Tslp, an IL-7-like cytokine produced by epidermal cells. Interestingly, Tslp is impicated in different disorders beyond allergic inflammation including atopic dermatitis (AD), a chronic inflammatory skin disease in which Tslp protein is highly expressed in acute and chronic lesions (Ziegler and Artis, 2010). In mice, overexpression of Tslp specifically in the skin was sufficient to induce a disease phenotype characterized by all the hallmark features of AD (Yoo et al., 2005). In addition, several models of induced Tslp expression in skin result in subsequent allergic airway inflammation, suggesting that Tslp may reach and act in different body districts leading to different systemic disorders (Demehri et al., 2009; Fontenot et al., 2009; Leyva-Castillo et al., 2013). Tslp is also overexpressed in the skin of individuals with Netherton syndrome (NS), a severe skin disease characterized by AD-like lesions as well as other allergic manifestations that result from mutations in the serine peptidase inhibitor Kazal-type 5 (SPINK5) gene. These observations lead us to hypothesize a fundamental role of Tslp in our mouse model in the development and progression of inflammatory phenotype throughout the body. Indeed, few days after the expression of Tslp, surviving L/L mice are characterized by a chronically thickened epidermis, dry skin, severe inflammatory dermal infiltrates, and a systemic myeloproliferative disease that may be the cause of the high mortality rate of mutant mice. Interestingly, an autoimmune disorder is observed in at least one AEC patient with severe skin erosions, indicating that the release of TSLP may be at basis of a systemic autoimmune condition also in individuals affected by AEC syndrome. This patient had severe skin erosions occurring soon after birth in 70% of the body. Unfortunately, AEC syndrome was associated later in life (age 6) with the systemic disorder that was later diagnosed as an autoimmune proliferative disorder (age 8) (http://nfed.org/index.php/meet_our_families/single/the-miracle-child),
suggesting that skin inflammation may have systemic consequences, possibly due to excess TSLP release in the blood stream. The disease was not

investigated in depth at the time, and the child was placed under prednisone, and at the age of 16 is still on a very low dose of mycophenolate mofetil (Mark Atlas, MD Pediatric Hematology-Oncology doctor; personal communication). We tested his levels of TSLP in the blood, but found no difference with matching controls, consistent with the remission of the disease. In spite of this data, we evaluate *TSLP* mRNA levels in epidermis of human AEC patients and we observe an induction of *Tslp*, thus indicating that Tslp may be involved in AEC syndrome and could be the cause of the progressive severe phenotype that characterized the AEC mutant mice. In addition, gene expression profile in human AEC samples reveals that in eroded scalp skin there is a significant upregulation of several S100 genes as *S100A8* and *S100A9* that are proteins associated with acute and chronic skin inflammation (Clements et al., 2012). Interestingly, in AEC mouse model we find the same upregulation that, together with the induction of Tslp indicate that acute and chronic inflammation may play a key role in the progression of the disease both in human and mouse.

In a search for other mouse models in which Tslp may be similarly massively upregulated, we find that comparable Tslp production is observed in mice with a Notch-deficient skin (Demehri et al., 2008; Dumortier et al., 2010; Murthy et al., 2012). Indeed, ablation of cutaneous Notch signaling in mice results in the overexpression of Tslp causing a chronic inflammatory disorder resembling atopic dermatitis (Demehri et al., 2008; Dumortier et al., 2010). In spite of the fact that inflammation appears to be extremely sensitive to aberrations in epidermal Notch signaling, the mechanism by which Notch signaling is able to regulate inflammation and in particular Tslp remains unclear. Of note, TSLP has been proposed to be produced as a consequence of skin barrier defects in general and does not depend on loss of Notch signaling directly (Demehri et al., 2008) whereas others postulated a direct regulation of Notch1 on Tslp promoter (Murthy et al., 2012).
These data lead us to hypothesize that the induction of Tslp in AEC mouse model may be due to impair Notch signaling.

Notch signaling is necessary for proper epidermal differentiation and lipid deposition. Altered epidermal differentiation and lipid deposition lead to release of high levels of TSLP into the systemic circulation. Therefore TSLP is a keratinocyte ‘‘quality control’’ response to defective differentiation/epidermal barrier (Demehri et al., 2008).

Importantly, in AEC mutant epidermis we observe a strong reduction of Notch1 and Jag2 expression levels accompanied with reduced expression of the Notch1 direct target gene Hes1. In addition a strong reduction of lipid biosynthetic enzymes was observed in AEC mutant skin at P3, with reduced expression of genes involved in arachidonic acid metabolism and lipid metabolism (42 genes; p-value= 5.3x10-7), including *Alox12*, *Alox12e*, *Adh1*.

Interestingly, it has been reported that the dry skin phenotype of intact AEC syndrome skin and the eroded skin sample are also associated with changes in

several structural proteins implicated in lipid metabolism that may have a role in barrier formation. Consistently, the expression of genes involved in arachidonic acid metabolism, such as *ALOX15B* and *ALOX5AP*, is affected also in skin of human individuals affected by AEC syndrome (Clements et al., 2012).

To better understand the role of Notch1 deficiency in AEC mouse model in regulating the expression of both *Tslp* and *Alox12* we exogenously expressed Notch1 in mutant keratinocytes.

Surprisingly, although *Alox12* is also directly controlled by p63 (Kim et al., 2009), expression in mouse primary keratinocytes isolated from AEC mutant mice is rescued by overexpression of exogenous active Notch1, whereas infection with a retrovirus carrying a peptide that competes for MAML1 binding to the Notch–CBF-1 complex and prevents downstream transcription (Weng et al., 2003), inhibits *Hes1* and *Alox12* in wild-type keratinocytes.

These data indicate that the severity of skin phenotypes of AEC patients is linked to different altered pathway, among which arachidonic acid metabolism may be play an unexplored role.

Interestingly also *Tslp* expression is influenced by re-activation of Notch1 pathway since its expression is strongly downregulated by upregulation of Notch1. These results support the idea that Notch1 may directly regulate Tslp expression; therefore we need to further investigate this point.

In conclusion, we demonstrate that the basal cell fragility and blistering observed in AEC mutant mice are due to alteration in adhesion molecules belonged to different categories. Interestingly, we establish a key role of intermediate filaments in the basal layer of the epidermis of AEC mouse model, which integrity is fundamental to give mechanical resilience to the epidermis. For the first time we evaluate the contribution of Krt5 in the progression of skin erosions in AEC syndrome therefore paving the way for new feasible treatments aimed at restoration of keratins functionality.

Importantly, we explore the possibility that altered skin barrier due to unbalanced keratin bundles in the basal layer and alteration of lipid and arachidonic acid metabolism, leads to inflammation, driven by upregulation of Tslp. An active role in this process may be play by Notch signaling which deficiency is responsible for the aberrant expression of Tslp.

Of note, these observations are true also in human AEC patients, thus reinforcing the idea that L/L mouse model may be a useful model to study the AEC syndrome.

Taken together, these data shed light on the clarification of the molecular mechanisms underling skin defects observed in AEC patients.

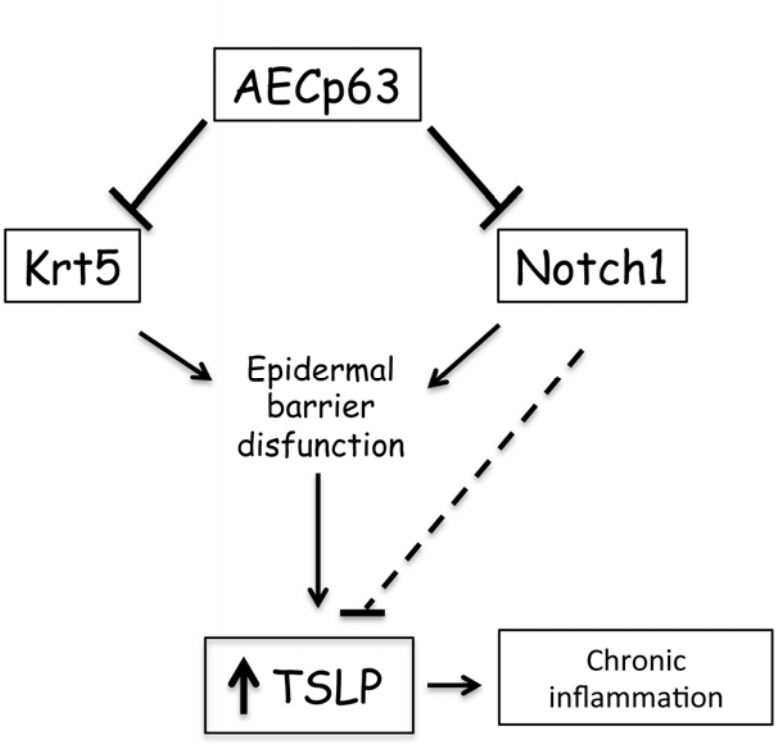


Figure 27. Proposed model. Mutant p63 affects both intermediate filaments network, through a strong reduction of Krt5 expression and inhibit Notch1 signaling. Krt5 reduction may be account for the skin fragility observed in AEC mouse model and, together with impaired Notch1 signaling lead to an epidermal barrier dysfunction. The absence of a negative control of Notch1 and the epidermal barrier failure lead to an upregulation of Tslp that may be explain the chronic inflammation that is observed in AEC mouse model.

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