# Molecular and Cellular Function of p63 in Skin Development and Genetic Diseases

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The transcription factor p63 is a master regulator of multiple ectodermal derivatives. During epidermal commitment, p63 interacts with several chromatin remodeling complexes to transactivate epidermal-specific genes and repress transcription of simple epithelial and nonepithelial genes. In the postnatal epidermis, p63 is required to control the proliferative potential of progenitor cells, maintain epidermal integrity, and contribute to epidermal differentiation. Autosomal dominant sequence variant in *p63* cause a spectrum of syndromic disorders that affect several tissues, including or derived from stratified epithelia. In this review, we describe the recent studies that have provided novel insights into disease pathogenesis and potential therapeutic targets.

**Keywords:** Ectodermal dysplasia, Embryonic development, Epidermis, Genetic disease

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## INTRODUCTION

Almost 20 years after the discovery of the p53 tumor suppressor (Lane and Crawford, 1979; Linzer and Levine, 1979), the scientific community was surprised to learn that 2 p53 paralog genes exist, namely *p63* (Yang et al, 1998) and *p73* (Jost et al, 1997; Kaghad et al, 1997), coding for proteins with highly similar domains (Figure 1a). Unlike its paralogs, the transcription factor p63 is highly expressed in the most proliferative layers of the epidermis and other stratified epithelia during embryonic development and in adulthood. Autosomal dominant sequence variants in the human *TP63* gene identified in syndromic disorders associated with ectodermal dysplasia (ED) and various mouse models that mimic ED to varying degrees have greatly contributed to establishing p63 as a master regulator of skin development and function. In this review, we discuss the recent evidence supporting the role of p63 as a regulator of several gene networks essential for epidermal development and maintenance. We also describe the molecular alterations that occur in p63-associated diseases and the therapeutic interventions that are being developed.

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*Abbreviations: ADULT, acro-dermato-ungual-lacrimal-tooth; AEC, ankyloblepharon-ectodermal defects cleft lip/palate; AER, apical ectodermal ridge; ChIP-seq, chromatin immunoprecipitation and sequencing; DBD, DNA-binding domain; EB, epidermolysis bullosa; ECM, extracellular matrix; ED, ectodermal dysplasia; EEC, ectrodactyly-ectodermal dysplasia-clefting; ESC, embryonic stem cell; FGF, fibroblast GF; HDF, human dermal fibroblast; iPSC, induced pluripotent stem cell; iKC, induced keratinocyte; K, keratin; LMS, limb mammary syndrome; OFC, orofacial cleft; OMIM, Online Mendelian Inheritance in Man; RHS, Rapp−Hodgkin syndrome; SAM, sterile alpha motif; SCC, squamous cell carcinoma; SHFM, split hand/foot malformation; siRNA, small interfering RNA; TID, transactivation inhibitory domain; WES, whole-exome sequencing; WGS, whole-genome sequencing*

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## THE COMPLEXITY OF THE *TP63* GENE

The human *TP63* gene consists of 17 exons spanning 265 kb on chromosome 3q28 in a genomic region that is frequently amplified in lung, esophageal, and head and neck squamous cell carcinomas (SCCs) (Campbell et al [2018] and references in it). Accordingly, *TP63* is often amplified and/or overexpressed in SCC (reviewed in Fisher et al [2023]). The structure of the *TP63* gene is relatively complex and has been described in detail elsewhere (Osterburg et al, 2021) (Figure 1b). In brief, all isoforms generated from the *TP63* gene share a zinc finger DNA-binding domain (DBD) and an oligomerization domain. Two alternative promoters and several alternative splicing at the 3′ end lead to the generation of several annotated transcripts. In the epidermis and in other stratified epithelia, an alternative transcription start site in the fourth exon (exon 3′) generates the ΔNp63 proteins that lack the strong transactivation domain at the N-terminus but has its own ectodermal-specific transactivation capacity (Figure 1b). At the 3′ end of the gene, several isoforms are formed by alternative splicing. p63a is the longest one including all exons, and it is the most highly expressed in ectodermal-derived structures (Figure 1b). Alternative splicing events generate other protein isoforms, of which the p63β, lacking exon 13, is the second most abundant one (ENCODE Project Consortium, 2012; Marshall et al, 2021) (Figure 1c).

The p63 protein forms a homotetramer (a p63 dimer of a dimer) or a heterotetramer with p73 (Gebel et al, 2016) and binds to the corresponding DNA consensus sequence. Interestingly, p73 is also expressed in the epidermal basal compartment, albeit at much lower level than p63 (Marshall et al, 2021), and positively regulates epidermal wound healing (Beeler et al, 2019). A recent comparison of several p63 chromatin immunoprecipitation and sequencing (ChIP-seq) with p53 ChIP-seq datasets revealed that p53 and p63 share many binding sites; however, p63 occupies more genomic regions than p53, likely owing, at least in part, to a less constrained binding sequence (Riege et al, 2020) and a higher abundance of the p63 protein in stratified epithelia under homeostatic conditions.

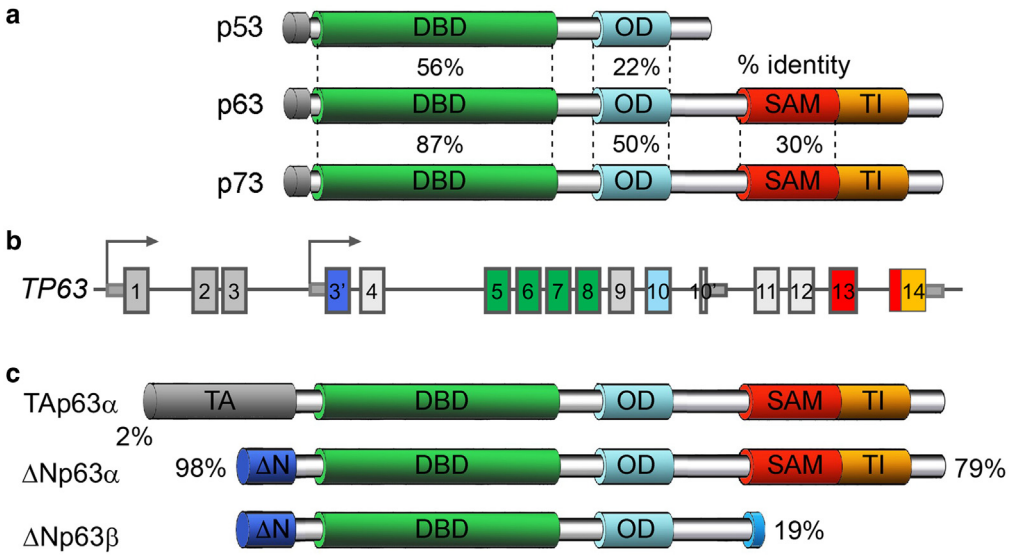


**Figure 1. Human *p63* gene and protein.** (**a**) Identity among the most conserved domains in the p53/p63/p73 family members. The domains were classified using Interpro (https://www.ebi.ac.uk/interpro/). IPR008967: p53-like transcription factor, DBD superfamily; IPR036674: p53 p53-like tetramerisation domain superfamily (OD); IPR013761: SAM domain. Alignment was performed using the pairwise Sequence Alignment EMBOSS Needle (https://www.ebi.ac.uk/jdispatcher/psa/emboss_needle; Matrix: EBLOSUM62) using p63 as a reference. The N-terminal domains are not indicated. (**b**) Structure of the human *TP63* gene. The color code corresponds to the protein domains indicated in **c**. Thinner blocks on the leading and trailing ends represent the 5′ and 3′ untranslated regions (in exon 3′ for the alternative transcript ΔNp63, in exon 10′ for the alternative p63γ isoform). (**c**) Summary of the major p63 protein isoforms expressed in the epidermis. Shown are TA domain (in gray), alternative transactivation domain (denoted with ΔN) (in blue), DBD (in green), OD (in light blue), SAM domain (in red), and TI domain (in orange). The ΔNp63β contains a short 3′-end terminal domain owing to the skipping of exon 13 (in azure blue). The percentage of TA and ΔN isoform mRNA is indicated on the left, whereas the percentage of the p63α and p63β isoforms is indicated on the right (Marshall et al, 2021). DBD, DNA-binding domain; OD, oligomerization domain; SAM, sterile alpha motif; TA, transactivation; TI, transactivation inhibitory.

The longest p63 isoform (p63α) contains a sterile alpha motif (SAM) and a transactivation inhibitory domain (TID) (Figure 1c). Despite considerable effort by several laboratories, the function of the p63 SAM domain has not been fully elucidated. This domain is also present in the p73α isoform but is absent in p53. Interestingly, the p63 SAM domain is highly conserved during evolution, even in invertebrates (Ou et al, 2007; Polinski et al, 2021), indicating that the function of the SAM domain is highly conserved in evolution. The role of the TID is well-characterized in the context of the TAp63α isoform in oocytes (reviewed in Osterburg et al [2021]), but its function in ΔNp63α is still unclear. Finally, in the last 25 C-terminal amino acids, sumoylation sequences are important for regulating protein stability (Huang et al, 2004; Straub et al, 2010).

## P63 AS A MASTER REGULATOR OF STRATIFIED EPITHELIUM DEVELOPMENT

Several studies have shown that p63 plays a critical role in the commitment of ectodermal cells to the keratinocyte cell fate. In early mouse embryonic development, ΔNp63 is the first stratified epithelial marker of the surface ectoderm (Fan et al, 2018; Tadeu and Horsley, 2013). p63 contributes to repress the simple epithelial keratins (eg, keratin K8 and K18) and other nonepidermal genes and activates the expression of basal layer epithelial keratins (K5 and K14) (Figure 2). Importantly, it activates developmental signaling pathways required for epidermal specification and stratification, including several components of signaling pathways, such as WNT, EDA, NOTCH, fibroblast GF (FGF), and BMP (Candi et al, 2007; De Rosa et al, 2009; Fan et al, 2018; Ferone et al, 2012; Laurikkala et al, 2006; Nguyen et al, 2006; Romano et al, 2012) (Figure 2).

A similar switch in expression from simple to stratified epithelial keratins occurs upon p63 expression during the maturation of human and mouse embryonic stem cell (ESC)–derived ectodermal cells toward the keratinocyte cell fate (Aberdam et al, 2008; Richardson et al, 2014; Shalom-Feuerstein et al, 2011). Human and mouse ESCs commit to the epithelial lineage through a retinoic acid– and BMP4-dependent initiation step. In human ESCs, these morphogens establish a chromatin landscape that in turn activates the expression of epithelial genes, including p63 itself and the simple epithelial keratins K8/K18 (Pattison et al, 2018). In addition, in ESC-derived ectodermal cells, p63 is required for repression of the neuronal cell fate and histone H3K27 methylation (Pattison et al, 2018). Subsequently, p63 activates the transcription of epidermal genes, including *K5* and *K14* as well as its own transcription through an autoregulatory loop (Antonini et al, 2015, 2006; Li et al, 2019). Notably, exogenous expression of ΔNp63 and KLF4 can convert human dermal fibroblasts (HDFs) into induced keratinocyte (iKC) (Chen et al, 2014). This assay has proven invaluable to test the effect of p63 missense mutations in p63-associated syndromes (Lin-Shiao et al, 2019; Osterburg et al, 2023; Russo et al, 2018) (discussed in subsequent section).

As a consequence of an impaired ectodermal development, the absence of p63 in mice causes severe malformations of all stratified epithelia leading to neonatal lethality (Mills et al, 1999; Romano et al, 2012; Yang et al, 1999). The epidermis is completely disorganized, likely owing to defective proliferative capacity of epidermal progenitor cells,

reduced cell–cell and cell–extracellular matrix (ECM) adhesion, and impaired differentiation (Ferone et al, 2015; Mills et al, 1999; Senoo et al, 2007; Yang et al, 1999) (Figure 2). In addition, p63-null mice display severe craniofacial defects due to aberrant morphogenesis of the nasal and maxillary processes and limb malformations, characterized by truncated forelimbs and the absence of hind limbs (Mills et al, 1999; Thomason et al, 2008; Yang et al, 1999). These phenotypes are recapitulated in homozygous ΔNp63-null mice (Romano et al, 2012), implying that ΔNp63 is required for the formation of stratified epithelia. Limb defects are caused by the absence of a properly formed apical ectodermal ridge (AER), a stratified epithelium at the tip of the growing limb that is essential for limb morphogenesis (Mills et al, 1999; Yang et al, 1999). AER fails to stratify and expresses low to undetectable levels of FGF8 and DLX5/DLX6, transcription factors involved in limb development (Lo Iacono et al, 2008). Notably, p63 is a substrate an E3 ubiquitin ligase complex, CRL4/cereblon (Asatsuma-Okumura et al, 2019), a primary target of thalidomide, which may explain the teratogenic effect of thalidomide causative of phocomelia and amelia, observed in the early 1960s, when thalidomide was used to relieve morning sickness in pregnant women.

## GENE NETWORKS REGULATED BY P63 IN EPIDERMAL CELLS

The epidermis is a fast-turnover tissue with a high regenerative capacity (reviewed in Banjac et al [2023]). Continuous regeneration of the epidermis is accomplished by basal epidermal progenitor keratinocytes, which undergo both symmetric and asymmetric divisions to maintain a dynamic balance between proliferation and differentiation.

Interestingly, ΔNp63 is essential to maintain the proliferative potential of basal epithelial cells and, at the same time, to initiate the early step of the differentiation program in transit amplifying epidermal cells by activating the transcription of key regulators of epidermal differentiation. Indeed, a number of transcription factors required for epidermal differentiation are p63 transcriptional targets such as NOTCH1, IRF6, ZNF750, and CASZ1 (Droll et al, 2024; Eyermann et al, 2024; Kouwenhoven et al, 2015; Nguyen et al, 2006; Oss-Ronen et al, 2024; Sen et al, 2012; Thomason et al, 2010) (Figure 2). In particular, cross-regulation between p63 and NOTCH signaling critically regulates the balance between keratinocyte proliferation and differentiation. Indeed, p63 is a positive modulator of the expression of the NOTCH ligands JAG1 and JAG2 and of NOTCH1 itself, whereas it negatively regulates HES1, a downstream effector of NOTCH signaling

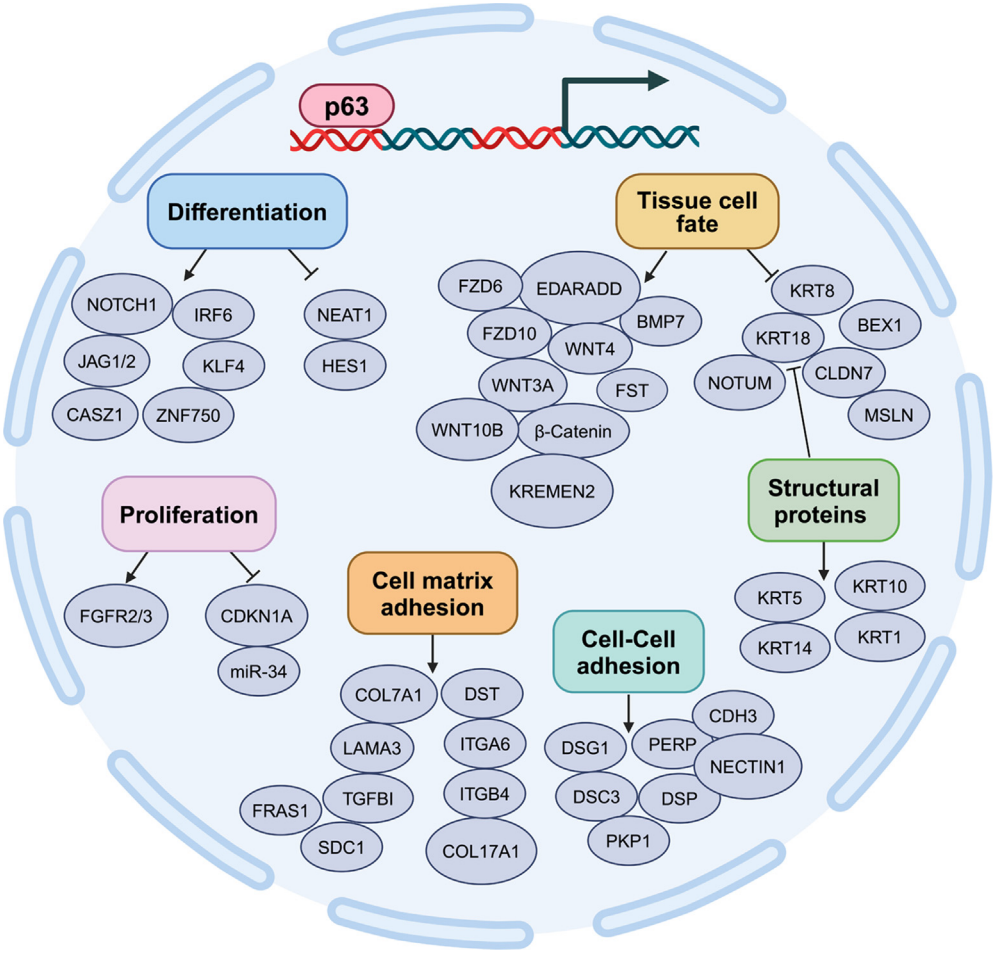


**Figure 2. Biological processes and genes regulated by p63.** Only the most representative p63 direct target genes are indicated (image was created with BioRender.com).

(Candi et al, 2007; Fan et al, 2018; Laurikkala et al, 2006; Moriyama et al, 2008; Nguyen et al, 2006) (Figure 2).

In addition, p63 plays a critical role in maintaining the self-renewal of adult stem cells in the epidermis and cornea in both humans and mice (Hirsch et al, 2017; Pellegrini et al, 2013, 2001; Senoo et al, 2007). Importantly, in skin replacement therapy, p63 is a prerequisite for successful cell transplantation and epidermal or corneal regeneration (Hirsch et al, 2017; Rama et al, 2010), underscoring the critical function of p63 in adult skin homeostasis. There are potentially multiple mechanisms through which p63 may control cell proliferation and long-term proliferative potential. One well-established mechanism is its ability to transactivate the *FGFR2* and *FGFR3* genes, which encode key GF receptors of the FGF family secreted by dermal fibroblasts (Candi et al, 2007; Ferone et al, 2012; Ramsey et al, 2013). In addition, p63 acts more directly on the cell cycle by repressing the cell cycle inhibitors CDKN1A (p21) and microRNA-34 family members, thereby promoting cell cycle progression (Antonini et al, 2010; Fan et al, 2018; Nguyen et al, 2006; Truong et al, 2006). Other microRNAs are also part of the *p63* gene network (reviewed in Candi et al [2015]).

Using a K14CreER-inducible mouse model, ΔNp63 was recently ablated in adult mice, revealing that its expression is required for cell proliferation and cell−cell and cell−ECM interactions even in the adult epidermis (Eyermann et al, 2024). ΔNp63-knockout keratinocytes are outcompeted by surrounding nontargeted cells that become progressively hyperproliferative possibly owing to ILs and alarmins secreted by ΔNp63-null cells. Interestingly, the simple keratins K8 and K18 are upregulated in ΔNp63-knockout cells, indicating that ΔNp63 is not only needed to repress them in embryonic development but also maintains their repression in adult cells.

In addition to controlling key regulators, p63 directly transactivates the expression of many crucial structural genes besides keratins, such as those coding for several hemidesmosome components (eg, *DST*, *COL17A1*, *ITGB4*) and basement membrane−associated genes (*COL7A1*, *SDC1*, *LAMA3*, *FRAS1*) (Carroll et al, 2006; Fan et al, 2018; Ferone et al, 2015; Koster et al, 2007; Osada et al, 2005; Romano et al, 2012; Truong et al, 2006). Moreover, p63 is a direct transcriptional activator of genes encoding desmosome components (eg, *DSG1*, *DSP*, *DSC3*) (Ferone et al, 2015, 2013; Ihrie et al, 2005) and adherens junctions (Mollo et al, 2015; Shimomura et al, 2008) (Figure 2). Thus, the transcription regulation of cell−ECM and cell−cell adhesion is a critical p63 function that contributes to the mechanical resistance of the epidermis by inducing and maintaining the proper expression of keratins, desmosomes, and hemidesmosomes. Alterations in these functions may underlie skin erosions in ankyloblepharon-ectodermal defects cleft lip/palate (AEC) syndrome (discussed in the following section).

## MECHANISMS OF TRANSCRIPTION REGULATION BY P63

Identification of direct p63-target genes has been greatly facilitated by a large number of high-throughput gene expression profiling in the presence or absence of p63, combined ChIP-seq (Fan et al, 2018; Kouwenhoven et al, 2015, 2010; McDade et al, 2012; Riege et al, 2020; Yu et al, 2021).

Collectively, several studies have indicated that p63 positively regulates epidermal development through a dual mechanism involving direct activation of differentiation-specific transcription factors and positive and negative modulation of the chromatin landscape. As discussed, p63 binds to thousands of enhancer regions, acting either as a transcriptional activator or repressor or bookmarking the enhancer for later activation during development or differentiation (Kouwenhoven et al, 2015). Therefore, p63 exerts a broad control on thousands of genes in stratified epithelia. Recent unpublished data suggest that p63 is more permissive than p53 in its ability to bind DNA sequences and may act as an activator more often in the presence of canonical p53/p63-binding sites rather than in noncanonical p63-only−binding sites (McCann et al, 2024[1]).

Strikingly, p63 acts as a pioneer factor in the expression of epidermal genes and is therefore required for chromatin opening together with the mammalian SWI/SNF (SWItch/Sucrose Non-Fermentable) chromatin remodeling complex (Bao et al, 2015; Lin-Shiao et al, 2019; Pattison et al, 2018). Indeed, p63-binding sites are mainly located in nucleosome-enriched genomic regions, and these sites are not accessible in cells that do not express p63 (Yu et al, 2021). In addition, p63 has been reported to directly regulate the expression of several chromatin regulators, such as SMARCA4 (BRG1), a catalytic subunit of the adenosine triphosphate−dependent BAF chromatin remodeling complex (Mardaryev et al, 2014), and HELLS (LSH), another member of the SNF2-like ATPase family (Keyes et al, 2011). In addition to the SWI/SNF chromatin remodeling complex, p63 has also been shown to interact with the H3K4 monomethyltransferase KMT2D; DMAP; DNA methyltransferase DNMT3A; the DNA-dependent ATPase; and DNA helicase activities RUVBL1, RUVBL2, and ACTL6A proteins, which are part of several chromatin remodeling complexes involved in the regulation of gene expression (Gallant-Behm et al, 2012; Lin-Shiao et al, 2018; Rinaldi et al, 2016; Saladi et al, 2017).

The repressive protein complexes associated with p63 have been less characterized, but it is known that p63 can recruit HDAC1 and HDAC2 (LeBoeuf et al, 2010; Ramsey et al, 2011). For instance, ΔNp63/HDAC1 complex represses the long noncoding RNA NEAT1, which binds to the promoters of key epidermal differentiation genes and contributes to their expression (Fierro et al, 2023).

As discussed earlier, p63-mediated transcriptional repression has also been linked to p63's ability to inhibit early ectodermal, neural, and mesenchymal genes in differentiating human ESCs. Such repressive function is achieved by p63 through the accumulation of trimethylated histone H3 lysine 27 at several genomic regulatory regions, including those surrounding TFAP2C (Li et al, 2019; Pattison et al, 2018). Thus, in specific genomic regions, p63 cooperates with PRC2 to repress transcription. A similar repressive function has been described during zebrafish ectodermal development, where p63 directly binds enhancer regions

[1] McCann AA, Baniulyte G, Woodstock DL, Sammons MA. Context dependent activity of p63-bound gene regulatory elements. bio Rxiv 2024.

associated with neural genes, thus impairing binding of the neural transcription activator SOX3 (Santos-Pereira et al, 2019).

## *P63* SEQUENCE VARIANTS IN SYNDROMIC DISORDERS

Proper specification of epidermal cell fate is defective in ED-associated syndromes. Autosomal dominant allelic variants in the *TP63* gene cause syndromic disorders associated with defective development of stratified epithelia and characterized by various combinations of ED, limb abnormalities (syndactyly and/or ectrodactyly [discussed in the following section]), and facial clefting, that is, cleft lip and palate. ED includes dry skin, hypohidrosis, sparse and coarse hair, dystrophic nails, tooth agenesis, and hypoplastic peg-shaped teeth (Figure 3). Facial cleft is associated with conductive ear loss and other minor features, including hypoplasia of the maxillary region, broad nasal bridge, choanal atresia, short philtrum, small mouth, and cup-shaped ears. A severe clinical condition often found in p63-related patients is underdeveloped lacrimal and Meibomian glands, associated with defective limbal stem compartment, leading to corneal scarring and opacification (reviewed in Di Iorio et al [2023]). Additional abnormalities, including genitourinary abnormalities, supernumerary nipples, short stature, heart defects, have been reported. Intrafamilial phenotype variability has been observed, suggesting the putative presence of modifier genes.

Other characteristics differ significantly among the syndromes (discussed in the following section), and their specific sequence variants cluster in specific domains of the *TP63* gene, thus indicating a genotype–phenotype correlation and suggesting specific pathogenic mechanisms for each syndrome (van Bokhoven and Brunner, 2002) (Table 1). However, in a few case reports, an overlap of different features has been observed in patients, and the correlation between genotype and phenotype has not held true (Celik et al, 2011; Chiu et al, 2011; Helenius et al, 2023).

As it can be anticipated, many p63-target genes are themselves mutated in skin genetic diseases with overlapping features (eg, *WNT10A*, *CDH3*, *RIPK4*, *NECTIN1*, *EDAR*, *PKP1*, *RHBDF2*) (Wright et al, 2019).

With some notable exceptions, the vast majority of *TP63* variants found in ED-associated syndromes give rise to amino acid substitutions or small insertion or deletions exerting dominant-negative and/or gain-of-function effects. Chromosomal deletion involving 3q28, where the *TP63* gene is located, have been found rarely (Khandelwal et al, 2019; Ponzi et al, 2015), implying that haploinsufficiency and loss of function are usually not the cause of disease.

In this section, we provide a brief description of the p63-associated syndromes and their corresponding mutations (Table 1).

AEC syndrome (Online Mendelian Inheritance in Man [OMIM] 106260) or Hay–Wells syndrome is the second most common p63-associated disorder, characterized by skin erosions and/or ankyloblepharon (complete or partial eyelid fusion) (Bree, 2009; Hay and Wells, 1976; Maillard et al, 2019; McGrath et al, 2001). Cleft lip/palate is also found in the vast majority of patients, whereas limb defects are typically mild and include syndactyly but not ectrodactyly (Bree, 2009; McGrath et al, 2001). Skin abnormalities associated with AEC syndrome include congenital erythroderma, skin fragility, blisters or bullae, extensive erosions in areas of friction, or trauma (Julapalli et al, 2009; McGrath et al, 2001). They frequently affect scalp, head and neck, skin folds, palms, and/or soles and are often associated with crusting, granulation tissue, and secondary infection (Figure 3a and b). Healing results in scarring alopecia on the scalp or reticular

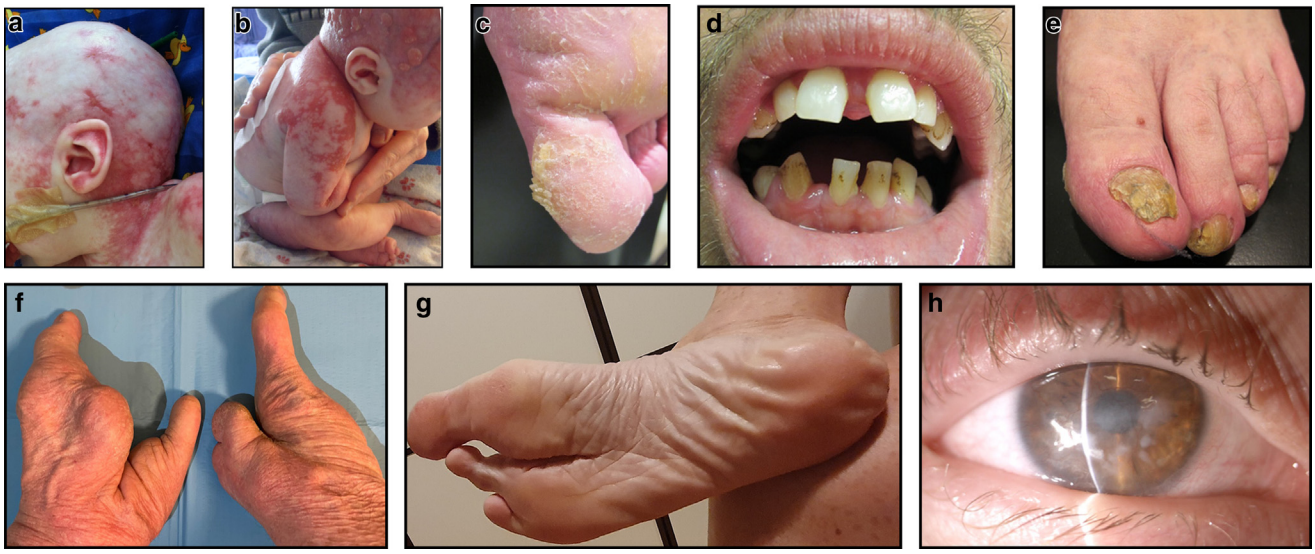


**Figure 3. Phenotypic features of p63-associated syndromes.** (**a**) Residual scarring and scalp alopecia typically seen in infants with AEC syndrome as a result of skin erosion. (**b**) Erythema and reticular scarring on the back and shoulder of infant with AEC syndrome. Bullae can be observed on the scalp. (**c**) Plantar keratoderma in adult with AEC syndrome. (**d**) Tooth abnormalities in an individual with AEC syndrome. (**e**) Nail dystrophy in individual with AEC syndrome. (**f**) Ectrodactyly in both hands of individual with EEC syndrome. (**g**) Ectrodactyly in feet of individual with EEC syndrome. (**h**) Meibomian gland aplasia, inflammation, and neovascularization of individual with EEC syndrome. There is strong photophobia. Human subjects or parents have provided written consent. AEC, ankyloblepharon-ectodermal defects cleft lip/palate.

**Table 1. Protein Domain Distribution of *TP63* Allelic Variants Identified in Genetic Disorders and Relative Clinical Features**

| | p63 Protein Domains | | | | | | | Clinical Features | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Syndrome** | **TAD** | **ΔN** | **—** | **DBD** | **OD/QP** | **SAM** | **TID** | **Skin and Adnexa** | **Eyes** | **Facial Features** | **Hands and Feet** | **Other Features** |
| AEC/RHS | | Q9fsX23, Q11X, Q16X | | | Δex11, 1456InsA | S509I, I510T, F513S/V, **L514**F/S/V, G518V, C519R, **C522**R (EEC/AEC), C522G/W/D, L523P, **F526**C/L, G530V/W, L531P, T533P, 534InsF, Q536L/R, I537T/N, **S541**F/P/Y, D544V/Y, L545P, **P551**H/K/L/T, R555P, I558T/F, **G561**D/V/R, I562N/T, 1697delG, 1709delA, 1716dup11bp, 1721delC | 1742DelC, R598, D601V, I611T, 1783delC, 1787delG, 1846delC, 1859delA | Very common: ED, skin fragility, blisters, erosions, erythema. Common: congenital erythroderma, hypohidrosis, palmoplantar keratoderma | Common: lacrimal and meibomian gland aplasia or hypoplasia; Rare: corneal scarring | Very common: cleft palate or cleft lip and palate. Common: hearing impairment, hypoplasia of the maxillary region, broad nasal bridge, choanal atresia, short philtrum, small mouth, and cup-shaped ears | Common: Syndactyly; rare ectrodactyly | Common: ankyloblepharon. Rare: breast and nipples hypoplasia, hypospadias and urinary malformation |
| ELA (EEC/LMS/ADULT) | G76W | N6H | S90W, P127L, S128F, G134D/V | 154InsP, L162P, Y163C/D, Y192C/D, V202M, **R204Q/W**/L, H208D/L/R/Y, **R227**Q/P, E229K,C269Y, N270D, S271T, S272N/T, C273Y, G275E, **R279H**/C/Q/S, **R280C**/H/S, I285delI, R298Q/G/L, **R304Q**/G/P/W, C306R/Y, A307D/G, C308Y (EEC/AEC), C308S, P309S, R311G, D312E/G/H/N, R313G, A315E, C347Y | 1217delACTT | 1572insA,1576delTT, L563P | 1743delAA,R604X, K632X | Common: ED, eczema-like dermatitis, atopic dermatitis | Very common: lacrimal and meibomian gland malformation, corneal scarring scarring, ulceration, and neovascularization | Very common: cleft palate or cleft lip and palate. Common: hearing impairment, hypoplasia of the maxillary region, broad nasal bridge, choanal atresia, short philtrum, small mouth, and cup-shaped ears | Very common: split hand/foot, oligodactyly, syndactyly, digital duplication | Rare: hypospadias, urethral stenosis; breast and nipple aplasia (ADULT) or hypoplasia. Very rare: polycystic kidney |
| SHFM4 | R58C | | | K161E, K193E, K194E, R204Q, M268I, M277I, G310E, R280C, R298L | | | Q634X, E639X | Rare: ED | — | — | Very common: split hand/foot, oligodactyly, syndactyly, ectrodactyly | |

(*continued*)

**Table 1. Continued**

| Syndrome | p63 Protein Domains | | | | | | Clinical Features | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | TAD | ΔN | — | DBD | OD/QP | SAM | TID | Skin and Adnexa | Eyes | Facial Features | Hands and Feet | Other Features |
| OFC8 | | | S90L | 452del8bp, V185I, Q294X, R313G | V366M, R369H, Q403X, R448C | D564H | | Hypodontia and occasionally other ED features, plantar hyperkeratosis | — | Very common: unilateral or bilateral cleft lip or cleft lip/palate | Very common: cleft lip/palate; choanal atresia | |

Abbreviations: ΔN, alternative transactivation domain; ADULT, acro-dermato-ungual-lacrimal-tooth; AEC, ankyloblepharon-ectodermal defects cleft lip/palate; DBD, DNA-binding domain; ED, ectodermal dysplasia; EEC, ectrodactyly-ectodermal dysplasia-clefting; LMS, limb mammary syndrome; OD, oligomerization domain; OFC8, orofacial cleft 8; QP, glutamine- and proline-rich domain; RHS, Rapp–Hodgkin syndrome; SAM, sterile alpha motif; SHFM4, split hand/foot malformation 4; TAD, transactivation domain; TID, transactivation inhibitory domain.
The mutations are indicated on the basis of GenBank AAF43487.1. In recent years, the NM_003722.4 has been considered the canonical isoform. As a result, to convert the indicated mutations to the canonical nomenclature, 39 amino acid have to be added. Hotspot mutations are in bold. Underlined are mutations that share clinical features of AEC and EEC.

scarring on the rest of the body. Erosions occur at birth or in infancy and may recur and/or persist for a very long time, leading to secondary infections and affecting the patient's QOL. A colloidal skin manifestation and frequent extensive erosions at birth may lead to an initial misdiagnosis of epidermolysis bullosa (EB). In adults, skin erosions are generally found in areas subjected to friction or pressure, such as the palms and soles, and are associated with chronic dermatitis (Dishop et al, 2009; Hay and Wells, 1976; Julapalli et al, 2009; McGrath et al, 2001). Management of these wounds can be challenging owing to bleeding, increased risk of infection, and slow healing. In addition, adult patients with AEC are often characterized by palmar and plantar keratoderma (Figure 3c). Other common features of patients with AEC are alopecia or hypotrichosis (not shown), nail dystrophy, and oligodontia associated with dysplastic teeth (Figure 3d and e).

Rapp–Hodgkin syndrome (RHS) (OMIM 129400) is very similar to AEC syndrome and is currently considered a variable manifestation of the same clinical entity. Patients with RHS rarely exhibit ankyloblepharon and have a milder skin phenotype. The connection between RHS and AEC syndrome is based on both overlapping clinical features and the identification of the same mutation in individuals exhibiting symptoms of either RHS or AEC (Bertola et al, 2004; Clements et al, 2012, 2010; Rinne et al, 2007) (Table 1).

AEC mutations are mainly clustered in the C-terminal domain of the p63a isoform and are either missense mutations found mainly in the SAM domain or single nucleotide frame shift mutations found mainly in the TID domain causing an elongation of the amino acid sequence (Rinne et al, 2009) (Table 1). RHS/AEC syndrome can also be caused by mutations that introduce a premature termination in the N-terminal part of the p63 protein, leading to a reinitiation of translation with a second start codon in exon 4, resulting in the expression of a ΔΔNp63 isoform. This isoform, which lacks the N-terminal transactivation domain is also expressed in wild-type keratinocytes but at much lower levels and may exert a dominant negative effect (Rinne et al, 2008) (Table 1).

ELA (EEC/LMS/ADULT) syndrome is a relatively recent classification that includes ectrodactyly-ectodermal dysplasia-clefting (EEC) syndrome (OMIM 604292), limb mammary syndrome (LMS) (OMIM 603543), and acro-dermato-ungual-lacrimal-tooth (ADULT) syndrome (OMIM 103285). These syndromes share several characteristics and are often associated with the same sequence variant (Osterburg et al, 2023, 2021; Prontera et al, 2011) (Table 1). The major features of each individual syndrome are listed below.

EEC syndrome is the most prevalent of all TP63-associated diseases and is characterized by ED; cleft lip/palate; and limb malformations, including split hand/foot, oligodactyly, digital duplication, and syndactyly (Figure 3f and g). Ocular defects are more common in EEC syndrome than in the other p63-associated disorders and can be very severe (Di Iorio et al, 2023, 2012) (Figure 3h). EEC syndrome is mainly caused by single nucleotide sequence variants in the DBD, which can either directly affect the protein–DNA interaction or alter the domain structure. The most common sequence variants are in

arginine residues R204, R227, R279, R280, and R304, which account for the majority of EEC syndrome cases (Celli et al, 1999; Osterburg et al, 2021). Interestingly, sequence variants of these residues can affect the phenotypic manifestation in different ways. For example, a sequence alteration in arginine R304 is associated with a severe cleft lip/palate phenotype, whereas patients with a sequence alteration in arginine R227 rarely present this malformation (Rinne et al, 2006), suggesting that different mechanisms of impairment are at work (Figure 4).

LMS is relatively rare and is characterized by mild ED manifestations, including nail dysplasia, hypohidrosis, hypodontia, lacrimal duct atresia without hair, and skin abnormalities (van Bokhoven et al, 1999). Mammary gland and/or nipple hypoplasia or aplasia and limb malformations are characteristic. Clefting usually affects only the palate and not the lip. LMS is caused by missense variants in the DBD, such as R204Q and R227Q, which have been identified in LMS and EEC (Table 1). In addition, SAM domain truncating variants in exon 13 or 14 have been observed, leading to premature termination of the protein (Figure 4).

ADULT syndrome is relatively rare and differs from the other syndromes mainly by the presence of skin and hair defects and the absence of facial cleft. A characteristic feature of ADULT syndrome is excessive freckling and neurodermitis signs. The pathogenic variants typically associated with this disorder are mainly located in the DBD, with the hotspot mutation in residue R298Q but also R204Q (Figure 4 and Table 1).

### Nonsyndromic genetic disorders

Nonsyndromic split hand/foot malformation (SHFM)4 (OMIM 605289) is a pure limb malformation (ectrodactyly and syndactyly) without orofacial cleft (OFC) or ED. SHFM4 is not very common and is caused by mutations in either the DBD or the C-terminal domain involving sumoylation sites that regulate protein stability (Huang et al, 2004; Ianakiev et al, 2001, 2000; Jin et al, 2019; Osterburg et al, 2023; Otsuki et al, 2016; Rinne et al, 2007; van Bokhoven and Brunner, 2002) (Table 1).

OFC8 (OMIM 618149) is characterized by unilateral or bilateral cleft lip or cleft lip/palate and is associated with heterozygous allelic variants in the *TP63* gene found throughout the p63 sequence (Basha et al, 2018; Leoyklang et al, 2006). A recent screening of *TP63* in a large cohort of families affected by OFC with or without mild ED symptoms has identified the presence of several *TP63* loss-of-function variants, including frameshifts that introduce a premature stop codon in the DBD or missense variants that impair tetramerization (Khandelwal et al, 2019). Interestingly, the loss-of-function variants were all inherited from unaffected parents, suggesting a reduced penetrance of such loss-of-function alleles (Khandelwal et al, 2019).

## MOLECULAR DYSFUNCTION AND MODELING OF P63-ASSOCIATED ED

### AEC syndrome

AEC-associated skin erosions are caused by mild mechanical stress, suggesting a potential defect in the mechanical resistance of the epidermis. The limited changes in gene expression observed in patients with AEC syndrome are consistent with the histologic appearance of unaffected skin showing only mild hyperkeratosis and mild epidermal atrophy in half of the samples (Clements et al, 2012; Dishop et al, 2009).

Interestingly, reduced gene expression has been observed for key ECM-associated genes, such as the basement membrane proteins FRAS1 and COL7A1, providing a possible explanation for the severe skin erosions observed in AEC syndrome (Clements et al, 2012). Consistent with this hypothesis, loss-of-function mutations of the *FRAS1* gene result in Fraser syndrome (OMIM 219000), an autosomal recessive disorder characterized by embryonic epidermal blistering, fused eyelids, syndactyly, and genital and urinary tract abnormalities (McGregor et al, 2003). In addition, loss of *Fras1* in mice leads to similar malformations (McGregor et al, 2003; Vrontou et al, 2003). Equally relevant is the downregulation of the *COL7A1* gene, which encodes a critical component of anchoring fibrils located beneath the basal lamina at the dermal–epidermal basement membrane. Specific variations in the *COL7A1* gene cause autosomal-dominant or recessive forms of dystrophic EB (Christiano et al, 1995; Gurevich et al, 2022), a devastating skin disorder characterized by recurrent blistering and scarring of the skin and mucous membranes in response to mechanical

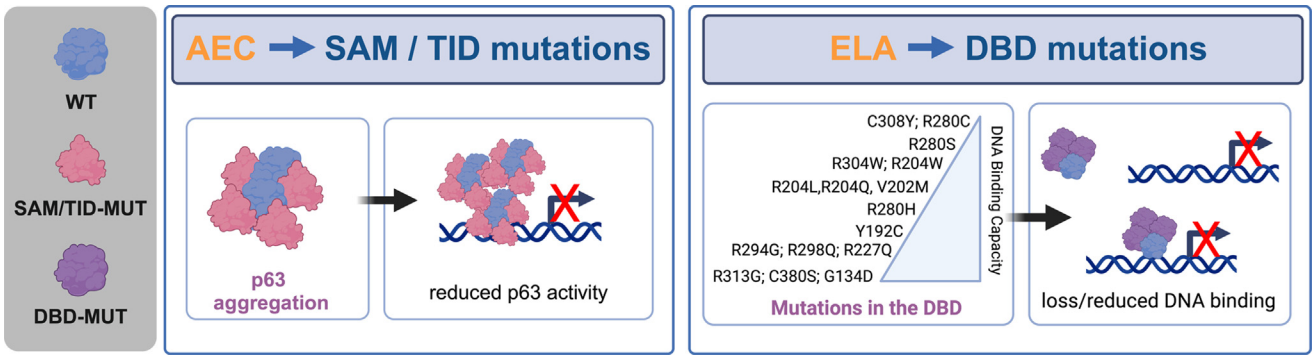


**Figure 4. Scheme of p63 functional alterations in syndromic and nonsyndromic disorders.** AEC mutations are mainly clustered in p63 SAM domain or TID, causing domain destabilization and p63 protein aggregation. ELA syndromes are mainly caused by mutations in the DBD, which can either directly affect the protein–DNA interaction or alter the domain structure, resulting in reduced DNA-binding capacity according to the type of mutation (image was created with BioRender.com). AEC, ankyloblepharon-ectodermal defects cleft lip/palate; DBD, DNA-binding domain; SAM, sterile alpha motif; TID, transactivation inhibitory domain; WT, wild type.

force. It is important to note that due to the pleiotropic effects of p63, it is likely that other alterations in gene expression also contribute to the overall phenotype.

Given the paucity of human skin samples, mouse models have been generated to investigate the cause of skin erosions in AEC syndrome in vivo. In inducible p63-knockdown mouse, altered terminal differentiation and reduced basement membrane integrity, accompanied with FRAS1 reduced expression and overt inflammation, were observed (Koster et al, 2009, 2007). To mimic AEC syndrome from a genetic point of view, we generated a constitutive knock-in mouse model carrying the L514F mutation to closely mimic the corresponding alleilc variant found in individuals with AEC (Ferone et al, 2012). As anticipated, AEC heterozygous mutant mice were born with cleft palate, dental defects, and hypoplastic epidermis with underdeveloped hair follicles. Reduced cell proliferation of the embryonic epidermis and oral epithelium were associated with reduced expression of the *Fgfr2* and *Fgfr3* genes. A similar hypoplastic phenotype was previously observed in *Fgfr2*-null epidermis (De Moerlooze et al, 2000). Consistent with a defective stem cell compartment, AEC mouse keratinocytes and *Fgfr2*-null keratinocytes in culture were impaired in their clonogenic potential (De Moerlooze et al, 2000; Ferone et al, 2012), and cell proliferation in AEC keratinocytes was restored by reactivating the FGF signaling pathway. Thus, dysregulation of the FGF signaling pathway is likely to contribute to the skin atrophy and delayed recovery from skin erosions observed in individuals with AEC syndrome. Skin fragility was associated with microblistering between the basal and suprabasal layers with reduced desmosome and adherens junction contacts (Ferone et al, 2015, 2013; Mollo et al, 2015).

To overcome the inability to study the postnatal consequences of the AEC mutations, a novel conditional knock-in mouse model carrying the same mutation (L514F) was generated (Russo et al, 2018). Early embryonic activation of the mutant allele resulted in a phenotype identical to that observed in the constitutive model, with perinatal lethality due to cleft palate. When inducible mutant mice were crossed with mice carrying the Cre recombinase under the control of a late K14 promoter, a mild phenotype was observed in heterozygous mutant mice after birth; however, mice with homozygous AEC recapitulated skin erosion typical of AEC syndrome (Russo et al, 2018).

The generation of the AEC mouse model coupled with rigorous biochemical studies provided important mechanistic insights into the molecular causes of AEC syndrome: missense and frameshift mutations in the carboxy-terminal domain lead to destabilization and misfolding of the SAM domain and aggregation of the p63 protein (Russo et al, 2018). Transcriptional activity was restored by insertion of amino acid variants known to reduce the intrinsic aggregation propensity of the C-terminal domain, opening a window for therapeutic intervention. The presence of p63 protein aggregates has been confirmed in primary keratinocytes isolated from patients with AEC syndrome (Aberdam et al, 2020). Aggregation of p63 is a unique feature of AEC syndrome that is not observed for EEC/LMS/ADULT-causing alterations.

### EEC syndrome

Most sequence variations associated with EEC syndrome are clustered in the DBD and affect directly or indirectly DNA binding. These variants encode for proteins with an increased half-life which accumulates, likely causing an imbalance between the variant and the wild-type protein in the p63 tetramers and leading to a more effective dominant-negative function of the variant protein (Browne et al, 2011).

To model EEC syndrome, human primary keratinocytes from affected individuals were compared with wild-type keratinocytes under proliferating and differentiating conditions. Similar to AEC models in vitro, EEC mutant keratinocytes showed a disruption of the entire differentiation program (Qu et al, 2018), which is not observed in vivo, likely owing to compensatory mechanisms. The entire enhancer landscape is modified in isolated keratinocytes from patients with EEC, with a decrease in active enhancers associated with p63-binding sites and a redistribution of active enhancers. Single-cell transcriptomics and epigenetic analysis showed that induced pluripotent stem cells (iPSCs))derived from patients with EEC were impaired to commit to keratinocyte differentiation and confirmed p63 ability to transactivate epidermal epidermal genes while repressing nonepidermal genes (Soares et al, 2019). In a knock-in mouse model with a heterozygous missense mutation corresponding to human R279H, mild malformations of the digits, dystrophic nails, sparse hair, dental abnormalities, and infrequent cleft palate were observed (Vernersson Lindahl et al, 2013). In homozygous mice, AER stratification was disrupted, and expression of the distal-less class homeobox genes *Dlx5* and *Dlx6*, which normally colocalize with p63 in the AER, was reduced (Lo Iacono et al, 2008). In humans, the genomic region comprising *DLX5* and *DLX6* has been implicated in SHFM (SHFM1) (OMIM 183600) (Duijf et al, 2003), and in mice, simultaneous targeted deletion of both *Dlx5* and *Dlx6* genes causes SHFM (Merlo et al, 2002a, 2002b; Robledo et al, 2002). Notably, heterozygous mice with EEC with a concomitant homozygous loss of *Dlx5* or heterozygous loss of *Dlx6* developed SHFM syndrome (Lo Iacono et al, 2008). Microdeletion of the p63-bound enhancer in the SHFM1 locus was identified in a patient with SHFM (Kouwenhoven et al, 2010).

Recently, a novel classification of the p63 DBD mutations has been proposed on the basis of 4 different kinds of mutations (Osterburg et al, 2023): (i) in the DNA contact interface, which directly interfere with DNA binding; (ii) in the zinc-binding region, which favors a zinc-free form; (iii) in the H2 region perturbing the local fold; and (iv) in the DBD−DBD dimer interface, essential to form dimers and dimers of dimers for high affinity binding to DNA.

HDF-to-iKC conversion assay coupled with ChIP-seq experiments was used to characterize several different EEC/LMS/ADULT mutations. Most EEC mutants (R204W, R304W, R313G, R279H, R280S, R280H) were unable to induce conversion (Osterburg et al, 2023). Interestingly, in partial agreement with their residual DNA-binding affinity, the p63R227Q (mild EEC phenotype) and the ADULT syndrome variant R298Q (no OFC) retained a relatively high DNA-binding affinity and were able to partially convert HDF into iKC. Interestingly, ChIP-seq experiments showed that the R227Q and R298Q mutants predominantly bind to p63 hemisites and incomplete p63 consensus sequences, respectively, explaining their partial impairment (Osterburg et al, 2023). Mechanistically, the amino acid variants are

located in the DBD−DBD interface and affect intermolecular interaction and tetramer formation. Thus, loss of the tetrameric structure impairs binding to the full DNA consensus, which consists of 2 hemisites, each bound by a p63 dimer (Osterburg et al, 2023).

## DIAGNOSIS AND TREATMENT OF P63-ASSOCIATED DISORDERS

The diagnosis of p63-associated syndromes involves integration of clinical assessment and genetic testing. Genetic testing is usually performed by targeted gene sequencing of the *TP63* gene to identify single nucleotide variations, small deletions, or insertions. For large patient cohorts of individuals, multiplex sequencing using molecular inversion probes has been recently used (Khandelwal et al, 2019). However, whole-exome sequencing (WES) or whole-genome sequencing (WGS) can be employed to detect sequence variations in coding regions or in coding and noncoding regions of the entire genome, respectively. The cost of WES and WGS is becoming progressively lower, and these comprehensive strategies may be advantageous when clinical signs are not clearly distinct and only 1−2 tissues are affected, raising the possibility that a different gene may be responsible for the observed phenotype. Finally, loss of heterozygosity is not a typical mechanism of p63-associated disorders, but copy number variation arrays have revealed loss-of-function variants of *TP63* in OFC (Khandelwal et al, 2019).

Owing to the rarity of p63-associated syndromes, relatively few variants have been identified as causative *p63* mutations in patients. Once a new variant in *TP63* has been identified, a simple functional test for its activity is a luciferase assay using a p63-responsive promoter (Osterburg et al, 2023). Novel high-throughput approaches are being developed to simultaneously evaluate the functional effect of all possible missense variants using unbiased multiplexed assays of variant effect (Davide Cacchiarelli, personal communication; Fowler et al, 2023; Melnikov et al, 2014).

After genetic identification, the p63-associated disorders are very difficult to treat given the variety and the complexity of the symptoms. The first line of treatment in p63-associated disorders is based on surgery to correct anatomical abnormalities such as cleft lip and palate or other surgically treatable malformations. Dermatological care is focused on managing skin fragility and preventing dehydration and infections. Ophthalmologic care is also required and should be applied as soon as possible to avoid corneal opacification.

Pharmacological experimental approaches involve the development of small molecules designed to enhance the function of the remaining wild-type p63 protein. An example is APR246/PRIMA-1$^{MET}$, which was originally used to target and reactivate p53 function in tumor cells (Wiman, 2010). Specifically, APR246/PRIMA-1$^{MET}$ can revert epithelial lineage commitment in EEC-iPSCs derived from reprogramming of fibroblasts from patients with EEC (Shalom-Feuerstein et al, 2013; Shen et al, 2013). In primary keratinocytes isolated from a patient with AEC, treatment with APR246 caused induction of some differentiation markers and ameliorated severe skin erosions in 2 individuals, suggesting that it may have some beneficial effect even though the mechanism remains to be elucidated (Aberdam et al, 2020).

As discussed earlier, the balance between keratinocyte proliferation and differentiation is mediated by a cross-regulation between p63 and NOTCH signaling. Therefore, the g-secretase and NOTCH signaling inhibitor DAPT (N-[N-(3, 5-difluorophenacetyl)-L-Alanyl]-S-Phenylglycine T-butyl ester) has been shown to extend the relatively short lifespan of epithelial stem cells of the oral mucosa of a patient with EEC (Barbaro et al, 2022). Interestingly, DAPT treatment accelerates corneal epithelial wound closure (Movahedan et al, 2012), suggesting that it could be a potential treatment for the EEC corneal epithelium.

On the other end, targeted RNA therapies, such as small interfering RNA (siRNA) and antisense oligonucleotides, have been investigated to specifically downregulate the expression of altered *TP63* mRNA, therefore reducing the production of dysfunctional p63 protein (Barbaro et al, 2016; Novelli et al, 2016). Allele-specific siRNA against p63 mutants (R279H and R304W) have been used to restore the limbus function by grafting genetically corrected autologous corneal limbal stem cells. However, such a strategy should be developed ad hoc for each specific mutation, and the efficiency of their internalization in limbal stem cells should be tested in vivo (Barbaro et al, 2016; Novelli et al, 2016).

## CONCLUSIONS

Studies in both humans and mice have provided important insights into the regulatory networks of p63. Sequence alterations in the *TP63* gene result in a spectrum of syndromic disorders that affect multiple stratified epithelia and are primarily characterized by ED, cleft lip/palate, and limb malformations in line with the known p63 gene network. Overall, understanding the molecular dysfunction of p63-associated ED syndromes has revealed disease pathogenesis and potential therapeutic targets. However, further studies are needed to elucidate the precise mechanisms underlying the different disorders and to develop targeted interventions to alleviate their most severe manifestations. Novel emerging genome-editing technologies coupled with regenerative medicine or topical treatment are likely to be a future therapeutic option for AEC skin erosions and/or corneal defects.

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### CONFLICT OF INTEREST

The authors state no conflict of interest.

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### AUTHOR CONTRIBUTIONS

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