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Hay–Wells syndrome is caused by heterozygous missense mutations in the SAM domain of p63

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Hay–Wells syndrome, also known as ankyloblepharon–ectodermal dysplasia-clefting (AEC) syndrome (OMIM 106260), is a rare autosomal dominant disorder characterized by congenital ectodermal dysplasia, including alopecia, scalp infections, dystrophic nails, hypodontia, ankyloblepharon and cleft lip and/or cleft palate. This constellation of clinical signs is unique, but some overlap can be recognized with other ectodermal dysplasia syndromes, for example ectrodactyly–ectodermal dysplasia–cleft lip/palate (EEC; OMIM 604292), limb–mammary syndrome (LMS; OMIM 603543), acro-dermato-ungual-lacrimal-tooth syndrome (ADULT; OMIM 103285) and recessive cleft lip/palate–ectodermal dysplasia (CLPED1; OMIM 225060). We have recently demonstrated that heterozygous mutations in the *p63* gene are the major cause of EEC syndrome. Linkage studies suggest that the related LMS and ADULT syndromes are also caused by mutations in the *p63* gene. Thus, it appears that *p63* gene mutations have highly pleiotropic effects. We have analysed p63 in AEC syndrome patients and identified missense mutations in eight families. All mutations give rise to amino acid substitutions in the sterile alpha motif (SAM) domain, and are predicted to affect protein–protein interactions. In contrast, the vast majority of the mutations found in EEC syndrome are amino acid substitutions in the DNA-binding domain. Thus, a clear genotype–phenotype correlation can be recognized for EEC and AEC syndromes.

INTRODUCTION

The particular association of ectodermal dysplasia with cleft lip/palate was reported initially in 1961 by Roselli and Gulienetti (1) as an autosomal recessive trait and subsequently as a clinical entity in other recessive and dominant disorders. Examples of these include Rapp–Hodgkin syndrome, Hay–Wells [ankyloblepharon–ectodermal dysplasia-clefting (AEC)] syndrome (OMIM 106260), Bowen–Armstrong syndrome, ectrodactyly–ectodermal dysplasia–cleft lip/palate (EEC) syndrome (OMIM 604292), acro-dermato-ungual-lacrimal-tooth (ADULT) syndrome (OMIM 103285) and recessive cleft lip/palate–ectodermal dysplasia (CLPED1; OMIM 225060). The additional feature of ectrodactyly is observed in a subset of these multiple congenital malformation syndromes, such as EEC syndrome, ADULT syndrome, lacrimo-auricular-dental-digital (LADD) syndrome (OMIM 149730), and limb–mammary (LMS) syndrome (OMIM 603543). All these syndromes show an autosomal dominant inheritance and, except for LMS, a high incidence of sporadic cases. Clinical distinction among these syndromes is sustained both by the degree of expressivity of each disorder and by the occurrence of unique characteristics. However, the strong similarities between these conditions may indicate an involvement of the same gene. The localization of

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one of the above syndromes, LMS, to chromosome 3q27 has allowed testing of this hypothesis (2). Limited linkage analyses with markers from 3q27 indeed suggested that the LMS, EEC and ADULT syndromes are allelic disorders (2–5). Heterozygous mutations were subsequently identified in the *p63* gene in EEC syndrome patients (3) and further mutation analyses in LMS, LADD and ADULT syndrome patients should provide conclusive evidence for involvement of the *p63* gene in these syndromes. Recently, causative mutations have been identified in the *p63* gene of patients with split hand/split foot malformation (SHFM), a non-syndromic form of ectrodactyly (6; H. van Bokhoven *et al.*, unpublished data).

The *p63* gene, also known as *p51* or *KET*, is a homologue of the archetypal tumour suppressor gene *p53* (7–11). The *p63* gene uses two different transcription initiation sites and is subject to extensive alternative splicing, which gives rise to at least six p63 isotypes (7). These isotypes have different and even opposing activities. The three protein motifs of the p53 protein are also found in p63: a transactivation domain (TA), a DNA-binding domain and a tetramerization domain. Recently, a fourth domain was identified, the sterile alpha motif (SAM) domain, which is present only in the α-isotypes of p63 (12–14). This SAM domain is also present in another p53 homologue, p73, but not in p53 itself (13). SAM domains are protein–protein interaction modules that are found in >40 proteins involved in developmental regulation, such as the receptor tyrosine kinases EphB2 and Eph4A (15–18). SAM domains generally consist of five helices, i.e. four α helices and a small 3^{10} helix, which are packed into a compact globular domain (12,13,15). SAM domains are able to form homo- and heterodimers, but neither p63 nor p73 is able to form homo- or heterodimers through their SAM domains. It is likely that the SAM domains of p63 and p73 interact with the SAM domain of other proteins or with proteins that do not have a SAM domain at all.

Clinical variability is one of the hallmarks of EEC syndrome. Most patients present with a generalized ectodermal dysplasia, which manifests as sparse hair, dry skin, pilosebaceous gland dysplasia, lacrimal duct obstruction and oligodontia. Cleft lip with or without cleft palate occurs in the majority of EEC syndrome patients. The greatest variability is observed for abnormalities of hands and feet, ranging from ectrodactyly, a severe defect of the central rays also known as split hand/split foot malformation (SHFM), at one end of the spectrum to no apparent limb defect at all at the other end of the spectrum. So far, the mutations found in EEC syndrome give rise either to amino acid substitutions in the DNA-binding domain or to shifts of the reading frame, which specifically truncate the α-isotypes of p63 (3,6; H. van Bokhoven *et al.*, unpublished data). Given the large variation of the phenotypic outcome of p63 mutations in EEC syndrome and probably also in the ADULT and LMS syndromes it seems very possible that p63 mutations also underlie other ectodermal dysplasia syndromes. In the present study we report the identification of mutations in AEC/Hay–Wells syndrome, which does not comprise ectrodactyly or other major limb defects, but instead has ankyloblepharon (fused eyelids) and severe scalp dermatitis as distinguishing features (19–23).

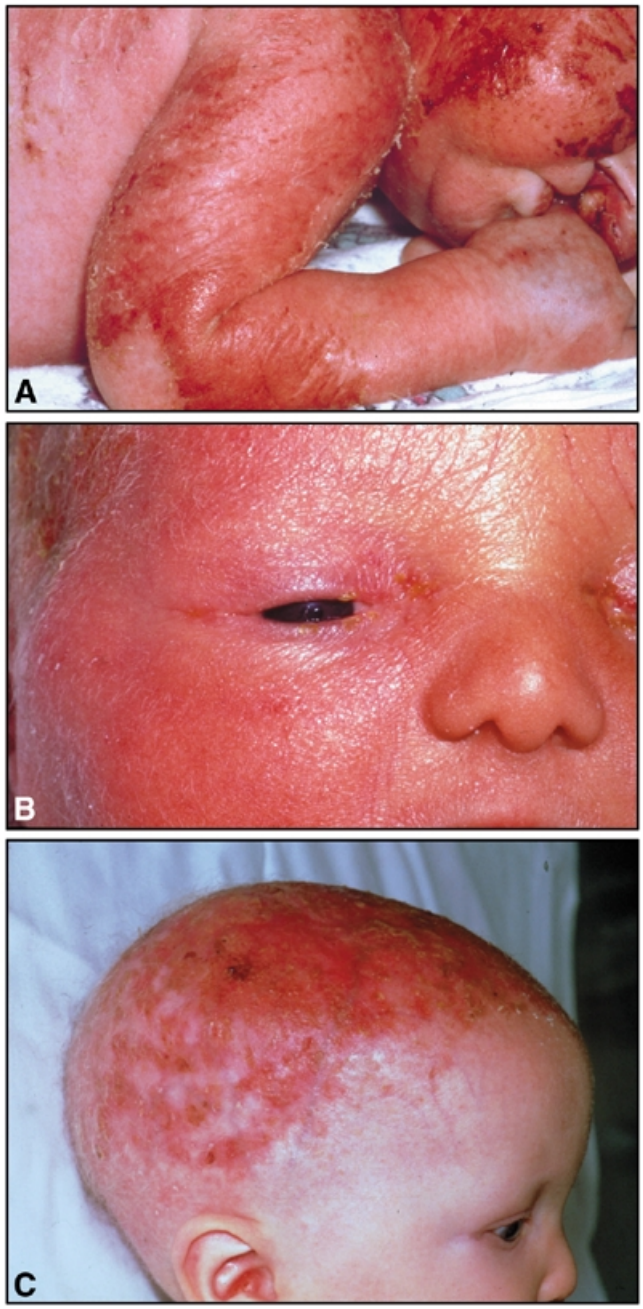

Figure 1. Phenotypic features of patients with AEC syndrome. (**A**) A neonate with skin erythema, erosions and cleft lip/palate. (**B**) A newborn with partial fusion of the eyelids (ankyloblepharon). (**C**) An 18-month-old child with extensive erosive scalp dermatitis and alopecia.

RESULTS

Eight families with the typical clinical features of AEC syndrome were available for mutation analysis (Fig. 1). Individual exons and flanking intron sequences of the *p63* gene were amplified from DNA of an affected individual from each family. In each individual, single nucleotide transitions were identified in exon 13, which are predicted to result in amino

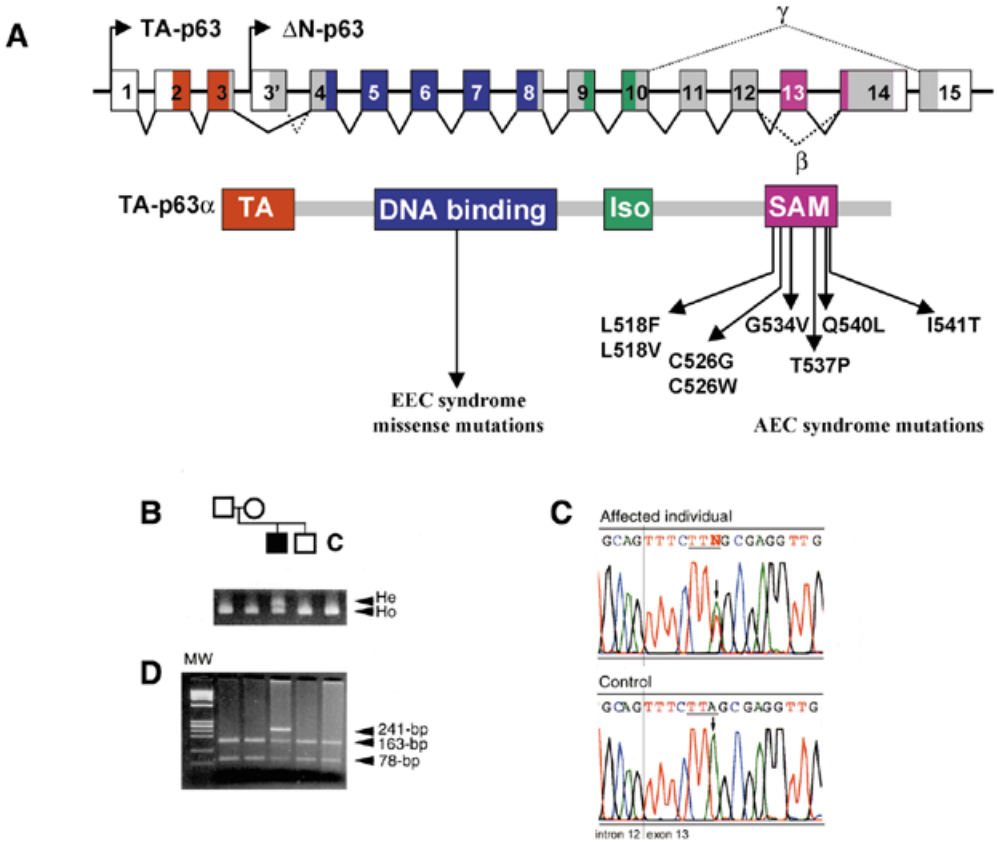

Figure 2. p63 mutations in the AEC syndrome cluster in the SAM domain. (**A**) Intron–exon structure of the *p63* gene showing the two transcriptional start sites, which give rise to p63 isotypes with (TA-p63) or without (ΔN-p63) the transactivation (TA) domain. The α, β and γ isotypes of p63 are the result of alternative splicing routes: splicing events that produce the α isotypes are indicated with solid lines; splicing events that produce the β and γ isotypes are indicated with dotted lines. Conserved protein domains are shown for TA-p63α: red, TA domain; blue, DNA-binding domain; green, isomerization domain; purple, SAM domain. Heterozygous missense mutations identified in AEC syndrome are indicated: L518V (1540T→G), L518F (1542A→T), C526G (1564T→G), C526W (1566T→G), G534V (1589G→T), T537P (1597A→C), Q540L (1607A→T) and I541T (1610T→C). All AEC mutations are located in the SAM domain, whereas all missense mutations in EEC syndrome are in the DNA-binding domain. The SAM domain mutations are encoded by exon 13 and are predicted to affect only the α isotypes, whereas the β and γ isotypes are left unaffected. (**B**) Delineation of a *de novo* mutation, L518F, in a patient with AEC syndrome. Heteroduplex analysis of PCR products spanning exon 13 and flanking introns of *p63* reveals a heteroduplex bandshift (He) in the affected individual but only homoduplex bands (Ho) in the other family members. (**C**) Nucleotide sequencing of the corresponding PCR products shows a heterozygous A→T substitution that converts a leucine residue (TTA) to a phenylalanine (TTT), designated L518F. (**D**) The nucleotide change results in a loss of a recognition site for the endonuclease *Dde*I. All PCR products, except that of the affected individual, are digested into products of 163 and 78 bp in size. Loss of the cut site in the patient results in an additional band of 241 bp.

acid substitutions in the SAM domain (Fig. 2A). Each mutation was unique, but two amino acids were substituted twice: L518V and L518F, and C526G and C526W. The other predicted changes were G534V, T537P, Q540L and I541T. Missense mutations affecting the SAM domain have not been identified previously in EEC syndrome or in related disorders (3; H. van Bokhoven *et al.*, unpublished data). Several of the mutations were *de novo*, which is strong evidence for their causative role in AEC syndrome. In addition, none of the above mutations was detected in 300 control chromosomes.

The SAM domain is a highly conserved structural motif found in p63 and p73, but not in p53. The solution structure of the SAM domain of p73 has been resolved and consists of five α helices (13) (Fig. 3A). The primary structure of the SAM domains of p63 and p73 are highly homologous. Based on the p73 SAM domain structure we have built a homology model of the p63 SAM domain to determine the position of the mutated amino acids (Fig. 3B). In this model structure the mutations can be separated into two different subgroups. The first subgroup contains those mutations that affect amino acids that are predicted to be buried inside the protein and have a small solvent accessible surface. This includes L518 (1.3% of its surface accessible to solvent), I541(1%) and C526 (6.2%). The second subgroup contains all other amino acids that have a larger solvent accessible surface: G534 (26.2%), T537 (47.1%) and Q540 (37.4%). Mutation of the first subgroup of amino acids is likely to affect the overall structure and stability of the protein by altering the packing of the helices. In contrast, the second subgroup of mutations is not predicted to cause gross conformational changes, which was confirmed by circular dichroism analysis for SAM mutant Q540L (data not shown). Interestingly, the three mutated amino acids that are predicted to have a high solvent accessible surface area are clustered in a small region around helix 3, which could indicate that this region is involved in binding of the SAM domain to its interaction partner.

The p63 SAM domain is contained only in the α-isotypes of p63, which in contrast to the β- and γ-isotypes do not have

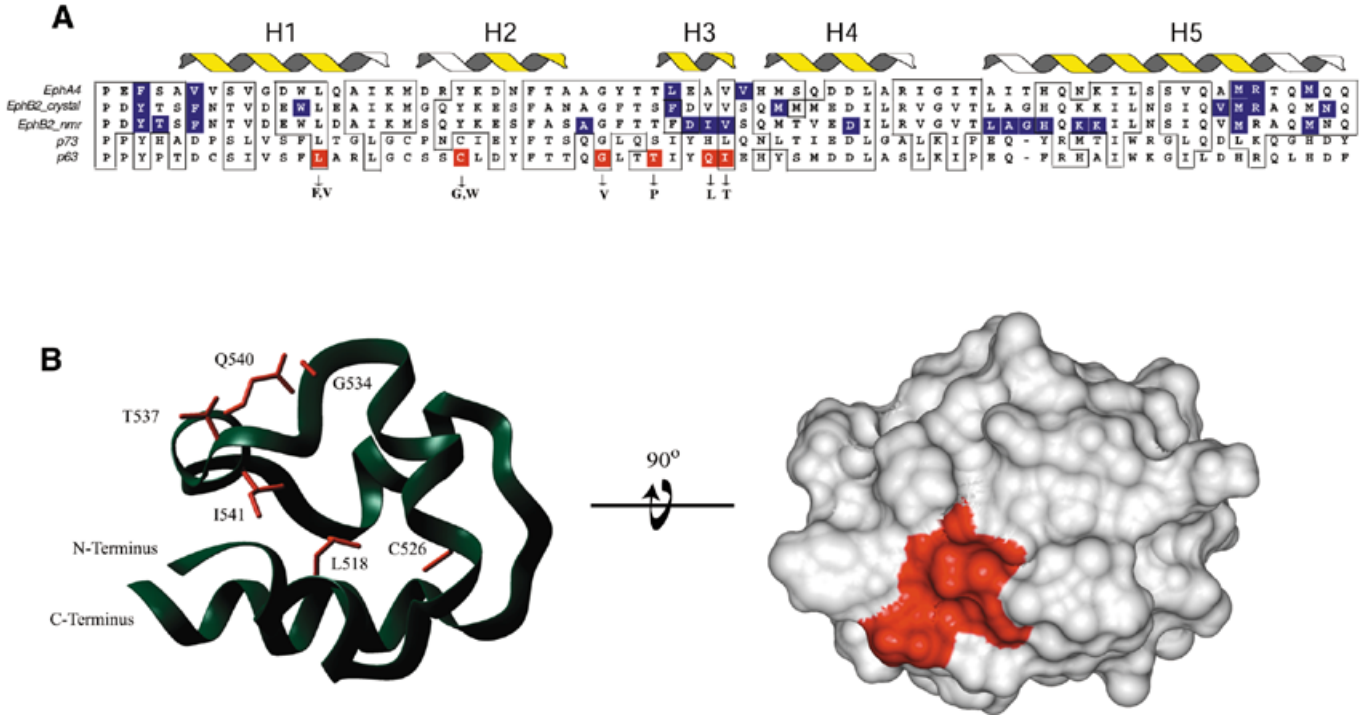

Figure 3. Position of missense mutations in the p63 SAM domain in AEC syndrome. (**A**) Multiple sequence alignment of SAM domains of p63 with SAM domains whose structures have been solved (13,16–18). The five helices are indicated as H1–H5. The white helices are the helices in the Eph receptors and the yellow parts are the helices in p73. Identical and similar amino acids are depicted in white boxes. Residues that are involved in dimerization are highlighted in blue. The mutated amino acids in p63 are indicated with red boxes. (**B**) Homology model of the p63 SAM domain. A similar five-helical fold is predicted as for p73 and the other SAM domains with known structure. The position of mutated amino acids is indicated in red. On the right is a space-filling representation, showing the clustering of amino acids glycine 534, threonine 537 and glutamine 540 on the surface of the p63 SAM domain. The left panel is a ribbon model turned by 90° that shows the position of all mutated amino acids. Amino acids leucine 518, cysteine 526 and isoleucine 541 are buried inside the SAM domain and are likely to be involved in the proper packing of the α helices.

transactivation activity. Instead, the ΔNp63α isotype exhibits dominant-negative activity towards transactivation by TA-p63γ (7). A protein domain contained in the α-tail probably inhibits p63-mediated transactivation, either directly or indirectly. Since the SAM domain is a protein–protein interaction module that is uniquely contained in the α-tail, it is possible that the SAM domain itself is responsible for this inhibition of transactivation. Indeed, frameshift mutations found in EEC syndrome patients that remove the SAM domain and C-terminal sequences give rise to truncated proteins that have lost their inhibitory effect and are instead capable of transactivation (3). We have investigated the possibility that the missense mutations in the SAM domain also give rise to this reversal of transactivation properties. Expression vectors for four of the missense mutations, L518F, G534V, T537P and Q540L, were transfected into Saos-2 cells and transactivation assays were conducted as described (3,7). In this system, wild-type p53 and TA-p63γ are clearly capable of driving transcription of a β-galactosidase reporter gene (Fig. 4A). In contrast, neither the wild-type TA-p63α nor any of the AEC mutant TA-p63α proteins showed any detectable transactivation activity. Thus, the missense mutations in the SAM domain do not lead to a gain of transactivation activity. We then examined whether the SAM domain mutation could influence the dominant-negative properties of ΔN-p63α towards p53- and p63-mediated transactivation. Wild-type ΔN-p63α clearly inhibited p53-mediated expression, which is likely to be caused, at least in part, by an effective competition for the DNA-binding site (3,7). In contrast, none of the SAM-domain mutant ΔN-p63α isotypes had any inhibitory effect on transactivation by p53 (Fig. 4B), suggesting that these mutant proteins have lost their DNA-binding capacity. The p63 mutants even seem to stimulate p53-mediated transactivation by 20–50%. This observation may be explained by scavenging of inhibitory components, e.g. endogenous ΔN-p63α. The effects towards p63-mediated transactivation showed a similar pattern: whereas wild-type ΔN-p63α brought about a 2-fold reduction in transactivation by TA-p63γ, no such effect was seen for any of the SAM domain mutants (Fig. 4C). This effect on TA-p63γ cannot be explained by a loss of DNA-binding capacity alone. Mutations in the DNA-binding domain that disrupt the p63 DNA binding capacity are unable to suppress transactivation by p53 but, in contrast to the SAM-domain mutations, these DNA-binding domain mutations have no apparent effect on p63-mediated transactivation (3) (P.H.G. Duijf, unpublished data). Hence, the lack of transdominant activity for the SAM domain ΔN-p63α mutant isotypes suggests additional effects for these SAM domain mutations. Disruption of protein–protein interactions through the p63 SAM domain may be responsible.

The severe skin anomalies in AEC syndrome, particularly in neonates and infants, prompted us to examine skin sections from these patients using immunohistochemistry (Fig. 5). In agreement with previous findings (7,24,25), nuclear p63-staining was observed in the basal keratinocyte layer in normal

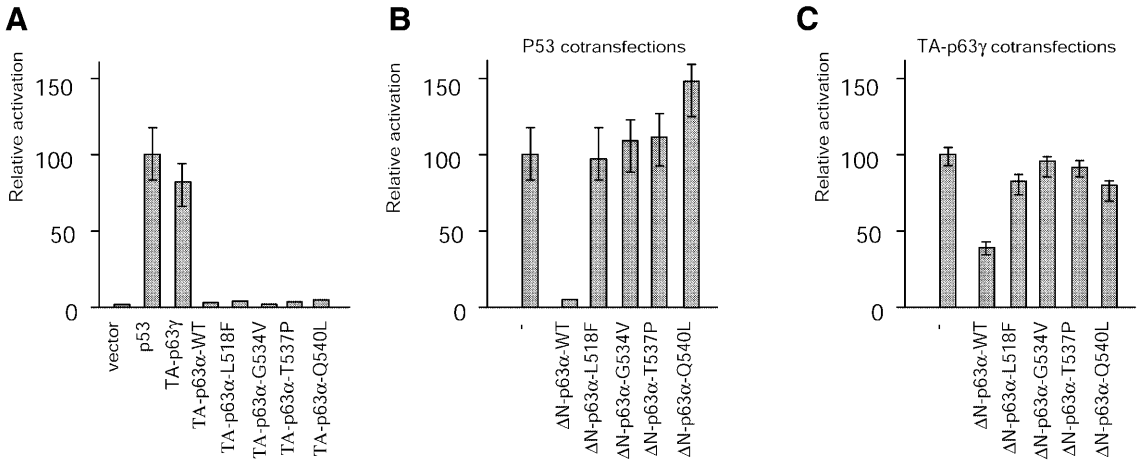

Figure 4. Transactivation of a β-galactosidase reporter gene by wild-type and mutant p63 isotypes. (**A**) Human Saos-2 cells were transfected as indicated and assayed for transactivation using a β-galactosidase reporter gene containing p53-binding sites. The relative transactivation activity is given with respect to p53 expression vector. TA-p63γ also exhibits transactivation activity, but neither wild-type nor mutant TA-p63α shows any activity above background (vector alone). (**B**) Cotransfection of p53 expression vector with wild-type and mutant ΔN-p63α isotypes, as indicated. Wild-type ΔN-p63α, but none of the mutants, shows suppression of p53-mediated transactivation. (**C**) Cotransfection of TA-p63γ expression vector with wild-type and mutant ΔN-p63α isotypes, as indicated. Suppression of p63-mediated transactivation was only observed for wild-type ΔN-p63α. The other SAM domain mutants have lost their repressive activity towards p63-mediated transactivation.

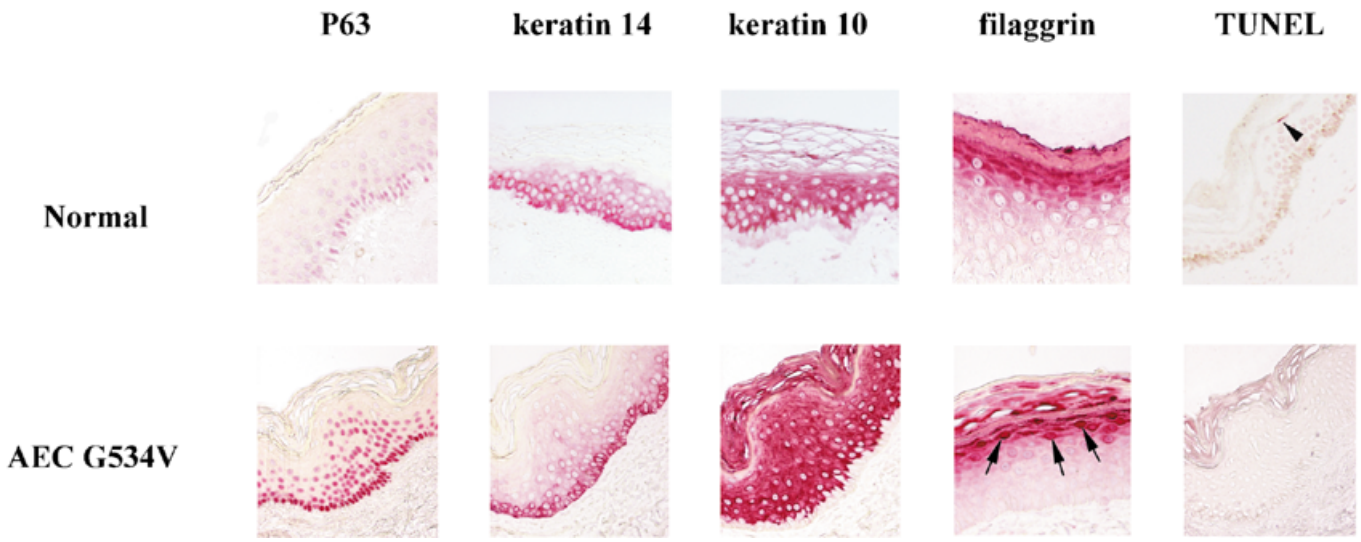

Figure 5. Immunohistochemical analysis of p63 and epidermal differentiation markers in skin tissue of a normal individual and from an AEC syndrome patient carrying the G534V mutation. A normal skin biopsy shows nuclear p63 staining in the basal cell layer. The intensity of staining is much stronger in skin from the AEC syndrome patient, and not restricted to the basal layer. Aberrant nuclear staining (indicated by arrows) is observed for filaggrin in the patient, but not in the normal skin. Keratin 10 and 14 immunolabelling patterns are similar in both biopsies. TUNEL staining does not reveal an increased number of cells showing DNA damage for the AEC syndrome patient. The arrowhead indicates an apoptotic cell.

controls. In contrast, an AEC syndrome patient with the G534V mutation not only showed prominent nuclear staining in the basal layer, but also in differentiating cells in the suprabasal layers (Fig. 5). Thus, in contrast to normal human epidermis, p63 in AEC syndrome is no longer confined to cells with high proliferative potential, but is also present in cells that normally are undergoing terminal differentiation. We next performed an immunohistochemical analysis of several epidermal structural proteins and differentiation markers. Most of these proteins, including keratin 10 and 14, showed the expected staining

patterns both in control and patient skin (Fig. 5). The only exception was anti-filaggrin, a marker for intermediate filaments in terminally differentiating epidermis. Anti-filaggrin showed both cytoplasmic and nuclear staining in cells of the AEC syndrome patient, instead of only cytoplasmic staining in the control. Normally, profilaggrin is transiently localized in the apoptotic nuclei of cells that are about to become part of the enucleated stratum corneum (26). Skin biopsies from patients with autosomal dominant forms of keratoderma, involving mutations in the loricrin gene (27,28), also show aberrant extensive nuclear localization of filaggrin (26,29), which is thought to disrupt the normal apoptotic machinery (26,30). Such an apoptotic defect does not appear to be involved in AEC syndrome since no increase in TUNEL-positive cells was observed (Fig. 5).

DISCUSSION

A clear genotype–phenotype correlation can be recognized for EEC and AEC syndromes. In EEC syndrome the vast majority of the mutations are amino acid substitutions in the DNA-binding domain (3,6; H. van Bokhoven *et al.*, unpublished data). This domain is contained in all p63 isotypes. Here we show that AEC syndrome is caused by missense mutations in the p63 SAM domain. The SAM domain is contained only in those p63 isotypes that have dominant-negative properties. Structural and functional analyses suggest that disruption of the interaction between the SAM domain and other proteins causes AEC syndrome. The interacting protein may be an important modulator for p63 transcriptional activity. Possibly, the unknown interacting protein is involved in the selection of specific p63 target genes. Such a role would be in accordance with the specific phenotype of AEC syndrome, which in contrast to EEC syndrome, does not include ectrodactyly, but instead presents with ankyloblepharon and severe scalp dermatitis.

In normal skin, p63 is restricted to the basal cells, the keratinocytes, which have high proliferative potential, and *p63* expression is rapidly lost when cells start to differentiate (7,31,32). The terminal differentiation of epidermal cells is characterized by the transient nuclear localization of filaggrin in apoptotic cells (26,30). Immunohistochemical examination of a skin section from an AEC syndrome patient revealed an aberrant localization of both p63 and filaggrin. It was recently demonstrated that p63 is able to transactivate the loricrin and involucrin gene promoters (32). Interestingly, aberrant extensive nuclear localization of filaggrin in skin cells has previously been reported for patients with loricrin gene mutations (27,28). The epidermal cells of these patients contain nuclear aggregates of profilaggrin, (mutant) loricrin and fragmented DNA (26,29). It was rather unexpected, therefore, that the TUNEL staining did not disclose any evidence for fragmented DNA in cells from the AEC syndrome patient. An explanation for this may lie in the multitude of existing p63 isotypes. The predominant p63 isotype in keratinocytes, ΔN-p63α, has dominant-negative activities and is unable to induce apoptosis in transfected cells (7). Possibly, SAM domain mutations in this p63 isotype do affect some of the dominant-negative effects in transactivation—for example, towards loricrin expression—but do not induce or even prevent the cells from undergoing apoptosis.

Interestingly, the three mutated amino acids that are predicted to have a high solvent-accessible surface area are clustered in a small region around helix 3, which could indicate that this region is involved in binding of the SAM domain to its interaction partner. In contrast, none of the residues that are involved in dimerization or oligomerization in crystal structures of other SAM domains have so far been found to be mutated in p63. This observation suggests that the SAM domain of p63 is capable of interaction with a non-SAM domain protein. Disruption of this interaction then gives rise to the typical developmental defects of AEC syndrome.

The various p63 isotypes have different and even opposing activities. For example, TA-p63γ exhibits transactivation activity on various p53-responsive promoters, whereas ΔN-p63α isotypes do not have this activity but, in contrast, inhibit transactivation by TA-p63γ. It has been proposed that a protein domain contained in the α-tail inhibits p63-mediated transactivation, either directly or indirectly through association with an unknown factor (3,7). Missense mutations found in EEC syndrome result in a loss of the DNA-binding capacity of all isotypes of p63 and, consequently, a loss of transactivation activity (3). The results of the transactivation assays with constructs harbouring AEC mutations were less straightforward. Introduction of the SAM domain mutations into ΔN-p63α gave rise to a loss of repressive activity towards TA-p63γ. The same loss of repression was previously demonstrated for wild-type ΔN-p63γ and a ΔN-p63α mutant isotype (ΔN-p63αFS) mimicking the exon 13 frameshift mutation from an EEC syndrome patient (3). On the other hand, both the TA-p63γ and the TA-p63αFS isotypes exhibit transactivation activity themselves, which is not the case for TA-p63α isotypes carrying the AEC mutations. Also, co-expression of p53 with mutant ΔN-p63α revealed an apparent loss of DNA-binding capacity due to the SAM domain missense mutations. We propose a model that accounts for all the findings of the transactivation assays. The crucial feature of this model is that the p63 SAM domain interacts with an as yet unknown protein(s) provisionally referred to as factor-X, which fulfils a dual role. The first role of factor-X binding would be that it confers DNA-binding capacity to p63 isotypes carrying the α-tail. Thus, in the absence of factor-X, the α-isotypes are proposed to be unable to bind to the DNA recognition sites. The second role for factor-X binding would be the formation of an inhibitory complex with transactivating isotypes of p63. The overall result of factor-X would be the formation of inhibitory complexes on the target DNA. The inhibition of transactivation may be brought about by factor-X itself, by the SAM domain or by sequences from the α-tail surrounding the SAM domain.

The above model is based on the results of transactivation assays using a p53 minimal responsive element in Saos-2 cells. It is well known that p63-mediated transactivation is affected both by the choice of the reporter system and by the cell system that is used for the assays (33–35). The regulation of p63-mediated transactivation by a protein interacting with the SAM domain may well be responsible for these variable results. Finding the proteins that interact with the SAM domain of p63 will help to better explain the results of the transactivation assays. Moreover, knowledge of these factors may provide a molecular explanation for the clinical heterogeneity observed for p63 mutations and uncover further genes underlying human multiple congenital anomaly syndromes that have ectodermal defects.

Another point of interest would be the identification of target genes for p63. One strategy to accomplish this is by studying differential gene expression with cDNA microarrays in cells expressing either wild-type or mutant p63 isotypes. The other approach would be to directly test whether p63 is able to drive the expression of a number of potential target genes. Examples of these are genes that were found to be mutated in human ectodermal dysplasias, such as *EDA1*, *Plakophilin-1*, *Downless*, *PVRL1* and *GJB6* (36–40), genes involved in human facial clefting syndromes, such as *MSX1* (41) and *PVRL1* (39), or genes involved in ectrodactyly such as the human counterpart of the murine *Dactylin* gene (42). The target genes for p63 are likely candidates for involvement in human developmental disorders encompassing either limb defects, ectodermal defects or facial clefting. In addition, it can be anticipated that p63 target genes as well as p63-interacting proteins are modifiers of the phenotypes seen for p63 mutations.

SUBJECTS AND METHODS

Subjects

Families were ascertained through clinical dermatology and genetics services. Patient 1 (L518F; 1542A→T) is a 6-year-old boy who had extensive cutaneous erosions with markedly fragile skin at birth. The erosions were most pronounced on the scalp and trunk. Scalp, eyebrow and eyelash hair failed to develop. Additional features noted were cleft lip and palate, mid-face hypoplasia, ankyloblepharon, lacrimal duct atresia, a ventral hypospadia and dystrophic nails. Detailed ophthalmological examination revealed persistent blepharitis and corneal scarring with punctate keratopathy. The external auditory meati developed poorly and repeated infections along with recurrent skin shedding resulted in a conductive deafness. There was no evidence of limb defects. Patient 2 (G534V; 1589G→T) is a 7-year-old girl who had a similar presentation at birth with extensive skin erosions, cleft lip and palate, ankyloblepharon and mid-face hypoplasia. The cutaneous erosions developed similarly as in the above case. At the age of 4 years, other features became apparent, including hypodontia, corneal scarring and conductive deafness. She also had mild syndactyly of the third and fourth toes bilaterally. Patient 3 (C526G; 1564T→G) is a 10-month-old baby. Congenital abnormalities in this child have comprised skin fragility, cleft lip and palate, partial ankyloblepharon, nipple hypoplasia, hypospadias and aplastic external auditory meati. There is a marked erosive scalp dermatitis. Patient 4 (Q540L; 1607A→T) is a 58-year-old woman first reported as case no. 5 in the original description by Hay and Wells (19). Patient 5 (C526W; 1566T→G) is an 8-year-old girl. The clinical history and features are very similar to those of patient 2. Patient 6 (T537P; 1597A→C) is a 19-day-old boy presenting with a median cleft palate, ankyloblepharon and features of ectodermal dysplasia, but no skin erosions. At age 12 he is developing alopecia. His mother had a similar clinical history, but in addition had a syndactyly of the second and third toes, supernumerary nipples and an absence of one parotid gland and duct. Patient 7 (L518V; 1540T→G) has similar clinical features to those of patient 1. At the age of 2.5 years, conductive hearing loss from obstructive webs in both external ear canals was demonstrated. The clinical features of patient 8 (I541T; 1610T→C) are also similar to those of patient 1. Neither parent nor any other family members are clinically affected for patients 1–3, 5, 7 and 8. Patient 4 has a similarly affected 23-year-old daughter.

Mutation analysis

Primers were designed for amplification of all exons of the *p63* gene (3). Primers used for exon 13 were 13F, 5′-CTT ATC TCG CCA ATG CAG TTG G-3′, and 13R, 5′-AAC TAC AAG GCG GTT GTC ATC AG-3′. Mutations were screened using conformation-sensitive gel-electrophoresis (43) or by direct sequencing with Big Dye labelling in an ABI310/ABI3700 genetic analyser (PE Biosystems). Mutations were verified by direct sequencing with forward and reverse primers or by restriction endonuclease digestion, according to the manufacturer's recommendations (New England Biolabs). Direct sequencing of exon 13 in 150 ethnically matched control individuals (300 chromosomes) failed to disclose the presence of any of the sequence variants detected nor any other common or rare polymorphisms in exon 13 or flanking introns. The position of mutations is given according to the original published TA-p63α sequence (7) (GenBank accession no. AF075430), which does not encode the 39 additional amino acids at the N-terminus as reported by Hagiwara *et al.* (44) (GenBank accession no. AF091627).

SAM domain modelling

The initial molecular model of the p63 SAM domain was developed based on sequence alignments between the p63 and the p73 SAM domains and the NMR structure of p73 SAM using the Homology program (Biosym/MSI). To refine this molecular model, molecular dynamics (MD) simulations and energy minimization were carried out with the CVFF91 force field within the Discover program (Biosym/MSI). The corresponding AEC mutations were introduced into the p63 SAM model and submitted to another round of dynamics/minimization. All molecular modelling was carried out with the Insight II program (Biosym/MSI).

Immunohistochemical analysis of skin biopsies

Skin biopsies from AEC patients and unaffected individuals were fixed in 4% buffered formaldehyde solution. Paraffin sections were pre-incubated in 20% normal horse serum, and incubated for 1 h with anti-p63 mAb 4A4 (17), diluted 1:100. Subsequently, the sections were incubated with a biotinylated affinity-purified anti-mouse IgG antibody (30 min, 1:200), followed by alkaline phosphatase–biotin avidin complex (ABC kit for mouse IgG, Vectastain; Vector Laboratories) and developed with 0.4 mg/ml amino-9-ethylcarbazole (Aldrich).

Transactivation assays

Plasmid DNA from mammalian expression vectors containing the murine *p63* sequence under control of a CMV promoter (7) was used as template for site-directed mutagenesis using the QuikChange procedure (Stratagene). The following oligonucleotides and their reverse complements were used to create the mutations:
L518F: 5′-GCA TTG TCA GTT TCT TT**G** CAA GGT TGG GC-3′;
Q540L: 5′-GAC CAC CAT CTA TC**T** GAT TGA GCA TTA CTC-3′;
T537P: 5′-CCA GGG GCT GAC C**C**C CAT CTA TCA GAT T-3′;

G534V: 5′-TTT CAC GAC CCA GG**T** GCT GAC CAC CAT CT-3′. To exclude unwanted nucleotide changes the entire open reading frame of each clone was sequenced.

For transactivation assays, human Saos-2 cells were transfected, lysed in detergent lysis buffer 36–48 h after transfection, and assayed for transactivation using a β-galactosidase reporter gene containing p53-binding sites (PG13-βgal) as described previously (7). A constitutive luciferase expression vector (PGL3; Promega) was used in all samples to normalize for transfection efficiency and sample preparation. All experiments were performed in triplicate.

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REFERENCES

1. Rosselli, D. and Gulienetti, R. (1961) Ectodermal dysplasia. *Br. J. Plast. Surg.*, **14**, 190–204.
2. van Bokhoven, H., Jung, M., Smits, A.P., van Beersum, S., Schendorf, R., van Steensel, M., Veenstra, M., Tuerlings, J.H., Mariman, E.C., Brunner, H.G. *et al.* (1999) Limb mammary syndrome: a new genetic disorder with mammary hypoplasia, ectrodactyly, and other hand/foot anomalies maps to human chromosome 3q27. *Am. J. Hum. Genet.*, **64**, 538–546.
3. Celli, J., Duijf, P., Hamel, B.C., Bamshad, M., Kramer, B., Smits, A.P., Newbury-Ecob, R., Hennekam, R.C., Van Buggenhout, G., van Haeringen, A. *et al.* (1999) Heterozygous germline mutations in the p53 homolog p63 are the cause of EEC syndrome. *Cell*, **99**, 143–153.
4. Propping, P., Friedl, W., Wienker, T.F., Uhlhaas, S. and Zerres, K. (2000) ADULT syndrome allelic to limb mammary syndrome (LMS)?. *Am. J. Med. Genet.*, **90**, 179–182.
5. Bamshad, M., Jorde, L.B. and Carey, J.C. (2000) Getting a LEAD on EEC. *Am. J. Med. Genet.*, **90**, 183–184.
6. Ianakiev, P., Kilpatrick, M.W., Toudjarska, I., Basel, D., Beighton, P. and Tsipouras, P. (2000) Split-hand/split-foot malformation is caused by mutations in the p63 gene on 3q27. *Am. J. Hum. Genet.*, **67**, 59–66.
7. Yang, A., Kaghad, M., Wang, Y., Gillett, E., Fleming, M.D., Dotsch, V., Andrews, N.C., Caput, D. and McKeon, F. (1998) p63, a p53 homolog at 3q27–29, encodes multiple products with transactivating, death-inducing and dominant-negative activities. *Mol. Cell*, **2**, 305–316.
8. Osada, M., Ohba, M., Kawahara, C., Ishioka, C., Kanamaru, R., Katoh, I., Ikawa, Y., Nimura, Y., Nakagawara, A., Obinata, M. *et al.* (1998) Cloning and functional analysis of human p51, which structurally and functionally resembles p53. *Nature Med.*, **4**, 839–843.
9. Senoo, M., Seki, N., Ohira, M., Sugano, S., Watanabe, M., Inuzuka, S., Okamoto, T., Tachibana, M., Tanaka, T., Shinkai, Y. *et al.* (1998) A second p53-related protein, p73L, with high homology to p73. *Biochem. Biophys. Res. Commun.*, **248**, 603–607.
10. Trink, B., Okami, K., Wu, L., Sriuranpong, V., Jen, J. and Sidransky, D. (1998) A new human p53 homologue. *Nature Med.*, **4**, 747–748.
11. Schmale, H. and Bamberger, C. (1997) A novel protein with strong homology to the tumor suppressor p53. *Oncogene*, **15**, 1363–1367.
12. Bork, P. and Koonin, E.V. (1998) Predicting functions from protein sequences—where are the bottlenecks? *Nature Genet.*, **18**, 313–318.
13. Chi, S.W., Ayed, A. and Arrowsmith, C.H. (1999) Solution structure of a conserved C-terminal domain of p73 with structural homology to the SAM domain. *EMBO J.*, **18**, 4438–4445.
14. Thanos, C.D. and Bowie, J.U. (1999) p53 Family members p63 and p73 are SAM domain-containing proteins. *Protein Sci.*, **8**, 1708–1710.
15. Schultz, J., Ponting, C.P., Hofmann, K. and Bork, P. (1997) SAM as a protein interaction domain involved in developmental regulation. *Protein Sci.*, **6**, 249–253.
16. Smalla, M., Schmieder, P., Kelly, M., Ter Laak, A., Krause, G., Ball, L., Wahl, M., Bork, P. and Oschkinat, H. (1999) Solution structure of the receptor tyrosine kinase EphB2 SAM domain and identification of two distinct homotypic interaction sites. *Protein Sci.*, **8**, 1954–1961.
17. Stapleton, D., Balan, I., Pawson, T. and Sicheri, F. (1999) The crystal structure of an Eph receptor SAM domain reveals a mechanism for modular dimerization. *Nature Struct. Biol.*, **6**, 44–49.
18. Thanos, C.D., Goodwill, K.E. and Bowie, J.U. (1999) Oligomeric structure of the human EphB2 receptor SAM domain. *Science*, **283**, 833–836.
19. Hay, R.J. and Wells, R.S. (1976) The syndrome of ankyloblepharon, ectodermal defects and cleft lip and palate: an autosomal dominant condition. *Br. J. Dermatol.*, **94**, 277–289.
20. Greene, S.L., Michels, V.V. and Doyle, J.A. (1987) Variable expression in ankyloblepharon-ectodermal defects-cleft lip and palate syndrome. *Am. J. Med. Genet.*, **27**, 207–212.
21. Spiegel, J. and Colton, A. (1985) AEC syndrome: ankyloblepharon, ectodermal defects, and cleft lip and palate. Report of two cases. *J. Am. Acad. Dermatol.*, **12**, 810–815.
22. Weiss, A.H., Riscile, G. and Kousseff, B.G. (1992) Ankyloblepharon filiforme adnatum. *Am. J. Med. Genet.*, **42**, 369–373.
23. Fosko, S.W., Stenn, K.S. and Bolognia, J.L. (1992) Ectodermal dysplasias associated with clefting: significance of scalp dermatitis. *J. Am. Acad. Dermatol.*, **27**, 249–256.
24. Yang, A., Schweitzer, R., Sun, D., Kaghad, M., Walker, N., Bronson, R.T., Tabin, C., Sharpe, A., Caput, D., Crum, C. *et al.* (1999) p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. *Nature*, **398**, 714–718.
25. Mills, A.A., Zheng, B., Wang, X.J., Vogel, H., Roop, D.R. and Bradley, A. (1999) p63 is a p53 homologue required for limb and epidermal morphogenesis. *Nature*, **398**, 708–713.
26. Ishida-Yamamoto, A., Takahashi, H., Presland, R.B., Dale, B.A. and Iizuka, H. (1998) Translocation of profilaggrin N-terminal domain into keratinocyte nuclei with fragmented DNA in normal human skin and loricrin keratoderma. *Lab. Invest.*, **78**, 1245–1253.
27. Maestrini, E., Monaco, A.P., McGrath, J.A., Ishida-Yamamoto, A., Camisa, C., Hovnanian, A., Weeks, D.E., Lathrop, M., Uitto, J. and Christiano, A.M. (1996) A molecular defect in loricrin, the major component of the cornified cell envelope, underlies Vohwinkel's syndrome. *Nature Genet.*, **13**, 70–77.
28. Ishida-Yamamoto, A., McGrath, J.A., Lam, H., Iizuka, H., Friedman, R.A. and Christiano, A.M. (1997) The molecular pathology of progressive symmetric erythrokeratoderma: a frameshift mutation in the loricrin gene and perturbations in the cornified cell envelope. *Am. J. Hum. Genet.*, **61**, 581–589.
29. Ishida-Yamamoto, A., Tanaka, H., Nakane, H., Takahashi, H., Hashimoto, Y. and Iizuka, H. (1999) Programmed cell death in normal epidermis and loricrin keratoderma. Multiple functions of profilaggrin in keratinization. *J. Invest. Dermatol. Symp. Proc.*, **4**, 145–149.
30. Kuechle, M.K., Presland, R.B., Lewis, S.P., Fleckman, P. and Dale, B.A. (2000) Inducible expression of filaggrin increases keratinocyte susceptibility to apoptotic cell death. *Cell Death Differ.*, **7**, 566–573.
31. Parsa, R., Yang, A., McKeon, F. and Green, H. (1999) Association of p63 with proliferative potential in normal and neoplastic human keratinocytes. *J. Invest. Dermatol.*, **113**, 1099–1105.
32. De Laurenzi, V., Rossi, A., Terrinoni, A., Barcaroli, D., Levrero, M., Costanzo, A., Knight, R.A., Guerrieri, P. and Melino, G. (2000) p63 and p73 transactivate differentiation gene promoters in human keratinocytes. *Biochem. Biophys. Res. Commun.*, **273**, 342–346.
33. Shimada, A., Kato, S., Enjo, K., Osada, M., Ikawa, Y., Kohno, K., Obinata, M., Kanamaru, R., Ikawa, S. and Ishioka, C. (1999) The transcriptional activities of p53 and its homologue p51/p63: similarities and differences. *Cancer Res.*, **59**, 2781–2786.
34. Kato, S., Shimada, A., Osada, M., Ikawa, S., Obinata, M., Nakagawara, A., Kanamaru, R. and Ishioka, C. (1999) Effects of p51/p63 missense mutations on transcriptional activities of p53 downstream gene promoters. *Cancer Res.*, **59**, 5908–5911.
35. Ikawa, S., Nakagawara, A. and Ikawa, Y. (1999) p53 family genes: structural comparison, expression and mutation. *Cell Death Differ.*, **6**, 1154–1161.
36. Kere, J., Srivastava, A.K., Montonen, O., Zonana, J., Thomas, N., Ferguson, B., Munoz, F., Morgan, D., Clarke, A., Baybayan, P. *et al.* (1996) X-linked anhidrotic (hypohidrotic) ectodermal dysplasia is caused by mutation in a novel transmembrane protein. *Nature Genet.*, **13**, 409–416.
37. McGrath, J.A., McMillan, J.R., Shemanko, C.S., Runswick, S.K., Leigh, I.M., Lane, E.B., Garrod, D.R. and Eady, R.A. (1997) Mutations in the

plakophilin 1 gene result in ectodermal dysplasia/skin fragility syndrome. *Nature Genet.*, **17**, 240–244.

38. Monreal, A.W., Ferguson, B.M., Headon, D.J., Street, S.L., Overbeek, P.A. and Zonana, J. (1999) Mutations in the human homologue of mouse dl cause autosomal recessive and dominant hypohidrotic ectodermal dysplasia. *Nature Genet.*, **22**, 366–369.
39. Suzuki, K., Hu, D., Bustos, T., Zlotogora, J., Richieri-Costa, A., Helms, J.A. and Spritz, R.A. (2000) Mutations of PVRL1, encoding a cell-cell adhesion molecule/herpesvirus receptor, in cleft lip/palate-ectodermal dysplasia. *Nature Genet.*, **25**, 427–430.
40. Lamartine, J., Munhoz, E.G., Kibar, Z., Lanneluc, I., Callouet, E., Laoudj, D., Lemaitre, G., Hand, C., Hayflick, S.J., Zonana, J. *et al.* (2000) Mutations in GJB6 cause hidrotic ectodermal dysplasia. *Nature Genet.*, **26**, 142–144.
41. van den Boogaard, M.J., Dorland, M., Beemer, F.A. and van Amstel, H.K. (2000) MSX1 mutation is associated with orofacial clefting and tooth agenesis in humans. *Nature Genet.*, **24**, 342–343.
42. Sidow, A., Bulotsky, M.S., Kerrebrock, A.W., Birren, B.W., Altshuler, D., Jaenisch, R., Johnson, K.R. and Lander, E.S. (1999) A novel member of the F-box/WD40 gene family, encoding dactylin, is disrupted in the mouse dactylaplasia mutant. *Nature Genet.*, **23**, 104–107.
43. Ganguly, A., Rock, M.J. and Prockop, D.J. (1993) Conformation-sensitive gel electrophoresis for rapid detection of single-base differences in double-stranded PCR products and DNA fragments: evidence for solvent-induced bends in DNA heteroduplexes. *Proc. Natl Acad. Sci. USA*, **90**, 10325–10329.
44. Hagiwara, K., McMenamin, M.G., Miura, K. and Harris, C.C. (1999) Mutational analysis of the p63/p73L/p51/p40/CUSP/KET gene in human cancer cell lines using intronic primers. *Cancer Res.*, **59**, 4165–4169.