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Gain-of-function mutation in ADULT syndrome reveals the presence of a second transactivation domain in p63

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The transcriptional co-activator p63 is of crucial importance for correct development of the limbs, ectodermal appendages (skin, nails, teeth, hair, glands), lip and palate. Mutations in the *p63* gene are found in a number of human syndromes, including ectrodactyly-ectodermal dysplasia-cleft lip/palate (EEC) syndrome, limb-mammary syndrome (LMS), Hay–Wells syndrome and in non-syndromic split-hand/split-foot malformation (SHFM). Each syndrome has a specific pattern of mutations with different functional effects in *in vitro* functional assays. We report a mutation R298Q in acro-dermato-ungual-lacrimal-tooth (ADULT) syndrome, another EEC-like condition. The mutation is located in the DNA binding domain of p63, which harbors almost all EEC associated mutations. However, unlike mutations in EEC syndrome, the R298Q ADULT syndrome mutation does not impair DNA binding. Rather, the mutation confers novel transcription activation capacity on the ΔN-p63γ isoform, which normally does not possess such activity. These results confirm that ADULT syndrome is a clinically as well as molecularly distinct member of the expanding p63 mutation family of human malformation syndromes. Our results further show that p63 contains a second transactivation domain which is normally repressed and can become activated by mutations in the DNA binding domain of p63.

INTRODUCTION

The *p63* gene is a homologue of the *p53* tumor suppressor gene. Like p53, p63 contains a transactivation (TA) domain to induce transcription of target genes, a DNA binding domain, and an oligomerization domain (OD), used to form tetramers (1). In contrast to p53, multiple protein products are produced from the *p63* gene. Two promoters are present at the 5′ end of the gene. Transcription from the TA-promoter produces TA-p63 proteins, while transcription from the ΔN-promoter creates ΔN-p63 products lacking the TA domain. In addition, three alternative splicing routes at the 3′ end generate proteins with different C-termini, denoted p63α, p63β and p63γ. A sterile alpha motif (SAM) domain and a transactivation inhibitory (TI) domain are contained only in the α-isoforms. The SAM domain, which is absent in p53, is a protein–protein interaction domain also found in other developmentally important proteins, such as another p53 homologue, p73, and several Eph receptor tyrosine kinases (2,3). The TI domain represses transactivation by the TA domain *in cis* and *in trans* (1; Z.Serber, H.Lai, A.Yang, H.Ou, A.Kelly, B.Darimont, P.Duijf, H.van Bokhoven, F.McKeon and V.Dötsch, manuscript in preparation). Hence, except for TA-p63α, which contains a TI domain in addition to a TA domain, the TA-p63 isoforms possess transactivation activity. Consequently, these isoforms can induce expression of p63 target genes. The ΔN-p63 isoforms lack transactivation activity and ΔN-p63α can repress the transactivation activity of transactivating p63 isotypes as well as of p53. This occurs by competition for p53/p63 DNA binding sites, or by forming transcriptionally inactive hetero-complexes (1). P63 is highly expressed in the basal cells of the epidermis and in the apical ectodermal ridge, a specified epithelium significant for proper limb outgrowth and morphogenesis (1). In both these differentiating tissues, p63 is crucial for maintaining the stem cell identity of the basal cells and hence indispensable for correct development of the skin as well as the limbs (4,5).

Ectrodactyly or split-hand/split-foot malformation (SHFM) (MIM 183600, 313350, 600095, 605289) is a developmental limb defect that is characterized by the absence of the median digital rays of the hands and/or feet and syndactyly of the remaining digits (6,7). SHFM may occur as an isolated entity, but is often part of a syndrome. A well known example is ectrodactyly-ectodermal dysplasia-cleft lip/palate (EEC) syndrome (MIM 604292), which, in addition to ectrodactyly, is characterized by ectodermal dysplasia and facial clefting (8). Ectodermal dysplasia affects the skin and other ectodermal

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derivatives such as teeth, hair and nails. Clefting affects the lip, sometimes in combination with the palate. We have shown previously that most cases of EEC syndrome are caused by missense mutations in the DNA binding domain of p63 (9,10). Hence, the loss of p63 DNA binding activity seems to be the major molecular cause of EEC syndrome.

Recent genetic studies have revealed that other EEC-like disorders are also caused by mutations in p63. Ankyloblepharon-ectodermal dysplasia-cleft lip/palate (AEC) syndrome (MIM 106260), also known as Hay–Wells syndrome, is caused by missense mutations in the SAM domain, which are predicted to disrupt the interaction between the SAM domain and interacting proteins (11). In two families with limb-mammary syndrome (LMS), frameshift mutations in and after the SAM domain have been detected, respectively (10). Finally, some cases of isolated SHFM are caused by p63 mutations (10,12). SHFM involves different types of mutations that are found throughout the protein. Two missense mutations and a splice site mutation have been found in the DNA binding domain and a nonsense mutation has been detected close to the C-terminal end of the p63α-isotypes. These studies establish a clear genotype–phenotype correlation (10).

In 1993, Propping and Zerres (13) described a family with an autosomal dominant disorder that resembles EEC syndrome. They named this disorder acro-dermato-ungual-lacrimal-tooth (ADULT) syndrome (MIM 103285). ADULT syndrome is characterized by ectrodactyly and ectodermal abnormalities such as nail dysplasia, hypodontia, lacrimal duct obstruction, sparse hair and a thin skin. ADULT syndrome is differentiated from EEC, LMS and AEC syndrome by the absence of facial clefts and by the presence of excessive freckling and neurodermitic signs. As in LMS, hypoplastic nipples/breasts are frequently observed (13,14). ADULT syndrome was mapped to chromosome 3q27 in which the *p63* gene is also located (15), suggesting that ADULT syndrome may also be caused by a mutation in the *p63* gene. Recently, a p63 mutation affecting only ΔN-p63 was described in an isolated patient with phenotypic features of both LMS and ADULT syndrome (16).

Here, we show that the ADULT syndrome family originally described by Propping and Zerres (13), carries a heterozygous missense mutation in the DNA binding domain of p63. In contrast to EEC mutations found in the DNA binding domain, this ADULT mutation is not located in close proximity to the DNA and hence does not result in a loss of DNA binding activity. Instead, the ADULT syndrome mutation confers a gain-of-function effect that has not been observed previously for other p63 mutations. Thus, the phenotypic separation between the EEC and ADULT syndromes is reinforced by the demonstration that they involve *p63* gene mutations with very different functional consequences.

RESULTS

ADULT syndrome is caused by a missense mutation in the DNA binding domain of p63

To test the hypothesis that ADULT syndrome is allelic with EEC syndrome we sequenced all exons of the *p63* gene in a patient from the ADULT syndrome family that was initially described by Propping and Zerres (13). A heterozygous G→A transition was found in exon 8 at nucleotide position 1010, changing codon 298 from CGA to CAA (Fig. 1A). The predicted alteration in amino acid sequence is a conversion of arginine to glutamine. To examine whether this R298Q mutation co-segregates within the ADULT syndrome family, an allele-specific oligonucleotide (ASO) hybridization was performed. Using primers specific for introns 7 and 8, and genomic DNA from patient II:7 (15) and several of his family members, genomic fragments containing exon 8 were PCR amplified. Southern blots with these PCR products were hybridized with a wild-type and a mutant probe, respectively. Unaffected members in the family possess only wild-type alleles, whereas in all affected individuals the mutation is heterozygously present (Fig. 1B), indicating that the R298Q mutation co-segregates with affected individuals within the family. To exclude the possibility that R298Q is a common polymorphism, we also tested 50 control individuals for this sequence deviation. None of these were positive (data not shown). In addition, the R298Q mutation was never detected in over 150 patients tested for p63 mutations. Taken together, our data indicate that the ADULT syndrome phenotype in this family is caused by the R298Q mutation in the DNA binding domain of p63.

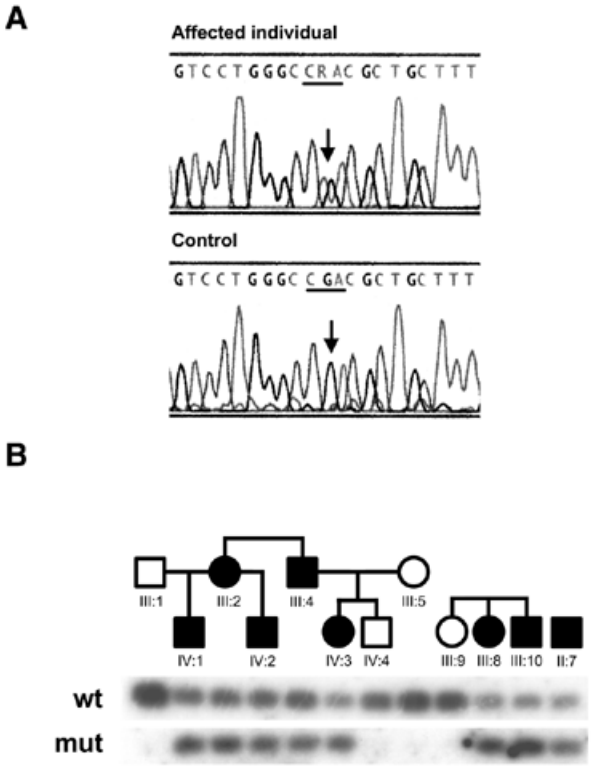

Figure 1. Segregation of the R298Q mutation within the ADULT syndrome family. (**A**) Using intron-specific primers and genomic DNA from patient II:7 as a template, exon 8 was PCR amplified. Nucleotide sequencing of this PCR product revealed a heterozygous G→A transition, changing codon 298 from CGA to CAA, so that the predicted amino acid change is arginine to glutamine. (**B**) From patient II:7 and several of his family members, genomic DNA containing exon 8 was PCR amplified as described in (A). An ASO hybridization was performed as described. The mutant probe (mut) hybridizes only with PCR fragments derived from the R298Q allele in affected individuals, but not in unaffected family members. The wild-type probe (wt) hybridizes with the wild-type alleles. Numbers below individuals correspond to those indicated in the pedigree described by Propping *et al.* (15).

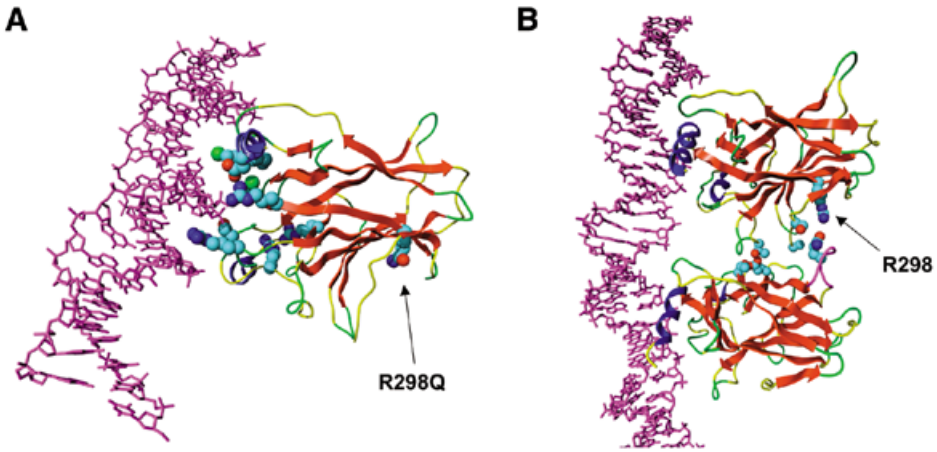

Figure 2. Model of the DNA binding domain of p63. (**A**) EEC syndrome mutations are found in the region of the DNA binding domain that is in close proximity to the DNA. The amino acids involved in EEC syndrome are R204, R227, C269, S272, R279, R280, R304, C306, C308 and P309 (10) (highlighted). In contrast, the R298 residue, mutated to Q in ADULT syndrome, is found at the outside, in the last β-strand of the DNA binding domain. Hence, R298 is unlikely to be of direct importance for DNA binding. (**B**) Within a p63 tetramer, two dimers interact with each other via their DNA binding domains to confer maximum DNA binding affinity. Modelled are two p63 monomers that are each part of a different dimer. Based on p53 data (17), the amino acids most important for this interaction in p63 are probably T116, S121 and T199 in the upper monomer, and T169, E229, G230 and Q255 in the lower monomer. R298 in the upper monomer could contribute to stabilization of this interaction by forming hydrogen bonds with Q255 in the lower monomer. Alternatively, R298 could be involved in interactions with other proteins. Highlighted amino acids are (from left to right): T169, E229, T199, A125, S128, Q255 and R298. Residues T169, E229 and Q255 are located in the lower monomer, the others in the upper monomer.

Protein modelling predicts that the R298Q mutation does not affect DNA binding

We previously demonstrated that EEC syndrome is mostly due to missense mutations in the DNA binding domain of p63 (9,10). Protein modelling indicated that such mutations cause a loss of DNA binding activity. Indeed, functional assays with mutant p63 confirmed that the EEC mutations in the DNA binding domain confer a loss of transactivation activity (9). The ADULT syndrome mutation R298Q is also located in the DNA binding domain. As the EEC and ADULT syndromes show a different pattern of clinical features, the ADULT syndrome mutation may be structurally different from those found in EEC syndrome. To investigate this possibility, we built a model of the p63 DNA binding domain. According to this model, the residues mutated in EEC syndrome are all involved in DNA binding. These either directly interact with the DNA or are important for maintaining the proper structure of the DNA binding domain (Fig. 2A) (9,10). In contrast, the R298 residue is not located in close proximity to the DNA and the exposed location of this residue suggests that it does not provoke major structural changes (Fig. 2A). Hence, substitution of this amino acid by glutamine is unlikely to result in a loss of DNA binding. We envisage two possible effects of the R298Q mutation. First, the arginine might be involved in p63 dimer–dimer interactions, which, like in p53 (17), stabilize the binding of p63 to the DNA as a tetramer. The R298 residue of one monomer might contribute to stabilizing this interaction by forming hydrogen bonds with residues in the other monomer (Fig. 2B). Alternatively, R298 may be involved in interactions with other proteins that control expression of target genes.

R298Q is a gain-of-function mutation

In order to discriminate between the two aforementioned possibilities, we studied the functional consequences of the R298Q mutation. We introduced the mutation in a mammalian expression vector that contained the murine cDNA of TA-p63α, TA-p63γ, ΔN-p63α or ΔN-p63γ, under control of a CMV promoter. Each of these vectors was co-transfected with a construct, which contains a p53 responsive element controlling the expression of the β-galactosidase reporter gene.

The mutant protein TA-p63γ^{R298Q} can induce transcription of the reporter gene to levels comparable to wild-type TA-p63γ (Fig. 3A). Thus, in accordance with our structure model, the R298Q mutation does not affect the DNA binding capacity of p63, in contrast to the DNA binding domain missense mutations that are found in EEC syndrome (9). This observation also indicates that the R298Q mutation does not affect p63 homo-dimerization. We next examined the effect of the R298Q mutation on transactivational properties of TA-p63α, ΔN-p63α and ΔN-p63γ, which normally do not possess such activity. TA-p63α^{R298Q} and ΔN-p63α^{R298Q} do not drive transcription and thus behave like the respective wild-type molecules. Wild-type ΔN-p63γ also does not possess transactivation activity in this assay. In sharp contrast, ΔN-p63γ^{R298Q} shows very high transactivation activity in this assay (Fig. 3A). This activity is ~25% higher than that of TA-p63γ and even higher than the activity of wild-type p53. This finding, which was highly reproducible in independent assays, was unexpected as the ΔN-p63γ isotype lacks the canonical TA domain. It is unlikely that destabilization of a dimer–dimer interaction (Fig. 2B) would impose such a gain-of-function effect. Thus, it is more likely that the R298Q mutation disrupts the interaction with another protein.

Wild-type ΔN-p63α has a dominant-negative effect towards transcription induced by TA-p63γ due to a repressive domain in the α-C-terminus (1; Z.Serber, H.Lai, A.Yang, H.Ou, A.Kelly, B.Darimont, P.Duijf, H.van Bokhoven, F.McKeon and V.Dötsch, manuscript in preparation). Hence, TA-p63γ-mediated transcriptional activation is significantly reduced upon co-expression with ΔN-p63α (Fig. 3B) (1). To see whether transcription induced by TA-p63γ^{R298Q} can be repressed in a

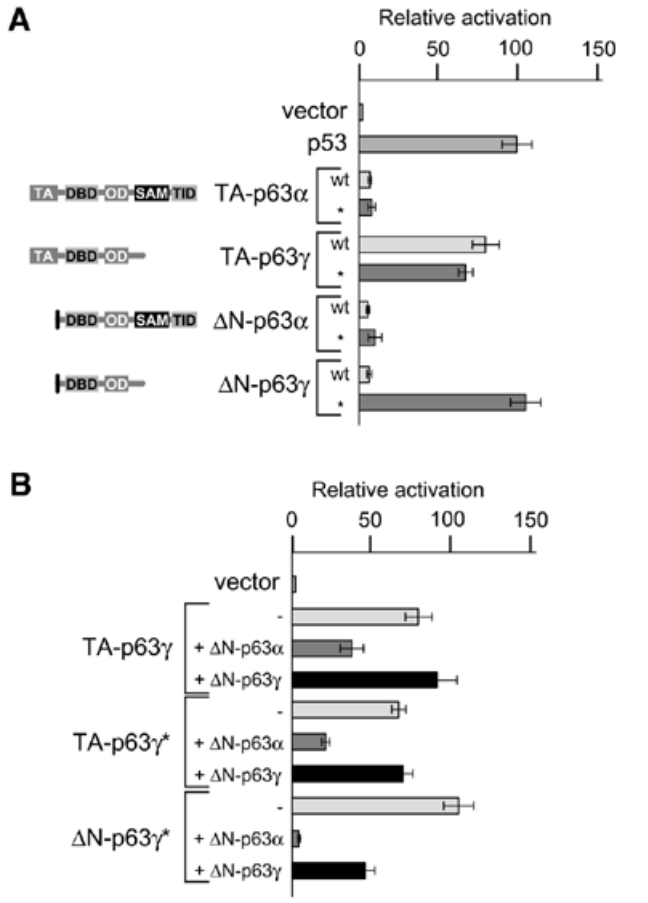

Figure 3. Transactivation of a reporter gene by wild-type and R298Q p63 isotypes. (**A**) Human Saos-2 cells were transfected with wild-type (wt; light grey) or R298Q (asterisk; dark grey) vector as indicated, and assayed for transactivation of a β-galactosidase reporter gene containing a p53 responsive element. The relative transactivation activity is given with respect to the p53 expression vector. TA-p63α, TA-p63γ and ΔN-p63α containing the ADULT syndrome mutation behave like their respective wild-type counterparts, whereas ΔN-p63γ^{R298Q} gains transactivation activity. Of each p63 isoform tested, a schematic representation is shown, indicating the presence of the functional domains. TA, transactivation domain; DBD, DNA binding domain; OD, oligomerization domain; SAM, sterile alpha motif; TID, transactivation inhibitory domain. (**B**) Transfections of TA-p63γ, TA-p63γ^{R298Q} and ΔN-p63γ^{R298Q} alone (light grey), or together with ΔN-p63α (dark grey) or ΔN-p63γ (black), as indicated. Like transactivation induced by wild-type TA-p63γ, transactivation by TA-p63γ^{R298Q} can be repressed by ΔN-p63α, but not by ΔN-p63γ. Reporter gene expression induced by ΔN-p63γ^{R298Q} is completely repressed by ΔN-p63α, but only with ~60% by ΔN-p63γ.

similar fashion, TA-p63γ^{R298Q} and ΔN-p63α were co-expressed in Saos-2 cells and assayed for reporter gene activity. Like wild-type TA-p63γ, TA-p63γ^{R298Q}-mediated transactivation can be reduced by ΔN-p63α with ≥50% (Fig. 3B).

In contrast to ΔN-p63α, ΔN-p63γ is unable to repress transactivation brought about by TA-p63γ. It rather appears to stabilize TA-p63γ-mediated transactivation (Fig. 3B) (1). Co-expression of TA-p63γ^{R298Q} and ΔN-p63γ shows a similar effect (Fig. 3B). These results indicate that TA-p63γ^{R298Q} can still bind to p53 DNA consensus sequences and that in this assay, it behaves like wild-type TA-p63γ.

To investigate whether the transactivation activity of ΔN-p63γ^{R298Q} could be repressed by the ΔN-p63 isotypes, this molecule was co-expressed with ΔN-p63α and ΔN-p63γ, respectively. Interestingly, transactivation by ΔN-p63γ^{R298Q} was repressed almost completely by ΔN-p63α, but only with ~60% by ΔN-p63γ (Fig. 3B). It thus appears that the α-tail is a potent inhibitor of the transactivation activity of ΔN-p63γ^{R298Q}.

In conclusion, the R298Q mutation has no measurable effect on DNA binding in these assays. The only direct result of the R298Q mutation is a novel transactivation capacity for the ΔN-p63γ isotype. This activity can be effectively repressed by ΔN-p63α.

DISCUSSION

Earlier genetic studies have demonstrated a clear genotype–phenotype correlation for EEC and EEC-like syndromes. The majority of EEC syndrome cases is due to missense mutations in the DNA binding domain (9,10). Hay–Wells syndrome is caused by missense mutations in the SAM domain (11), some cases of SHFM by different types of mutations at various locations in p63 (10,12) and LMS is caused by frameshift mutations in the C-terminus of p63 (10). Our results extend this genotype–phenotype correlation by showing that ADULT syndrome is caused by a mutation in the DNA binding domain of p63 (R298Q) that, in sharp contrast to EEC mutations, does not result in a loss of DNA binding activity. TA-p63γ^{R298Q} can drive transcription of a reporter gene similar to wild-type TA-p63γ. In both cases, the transcriptional activity is inhibited upon co-expression with ΔN-p63α, and stabilized upon co-expression with ΔN-p63γ. This has been explained previously by the presence and absence of a TI domain in ΔN-p63α and ΔN-p63γ, respectively (1; Z.Serber, H.Lai, A.Yang, H.Ou, A.Kelly, B.Darimont, P.Duijf, H.van Bokhoven, F.McKeon and V.Dötsch, manuscript in preparation). Interestingly, the R298Q mutation confers a gain of transactivation activity on ΔN-p63γ, even though this isoform lacks the N-terminal TA domain. To explain this phenomenon, we envisage two possibilities. First, the R298Q mutation could be a true gain-of-function mutation: it could provide p63^{R298Q} with a binding site for a co-activator molecule. This co-activator could then recruit other necessary transcription factors to initiate transcription of a target gene. Second, the R298Q mutation could expose a second transactivation domain (i.e. a TA-domain different from the canonical N-terminal TA-domain in TA-p63) within ΔN-p63 to become operational and induce transcription of a target gene. In wild-type ΔN-p63 such second transactivation domain would be kept in an inactive state either by the binding of a co-repressor or by intramolecular shielding. Several recent observations support this second possibility. In line with our findings, Dohn *et al.* (18) provided indirect evidence of the presence of a second p63 transactivation domain in the N-terminus of ΔN-p63. Overexpression of ΔN-p63α induces expression of GADD45, a growth arrest and DNA damage-inducible p53 target gene. Subsequently, transfections with truncated ΔN-p63α constructs indicated that the increase of GADD45 expression was not observed when the N-terminal 26 amino acids were lacking. This suggests the presence of a second transactivation (TA2) domain in ΔN-p63α (18). This TA2 domain is also present in ΔN-p63γ, suggesting that this TA2 domain might be activated by the R298Q mutation. Interestingly, the recently reported N6H mutation in a patient with an ADULT-like phenotype is located within the TA2 domain of ΔN-p63 (16). This mutation may also activate the normally

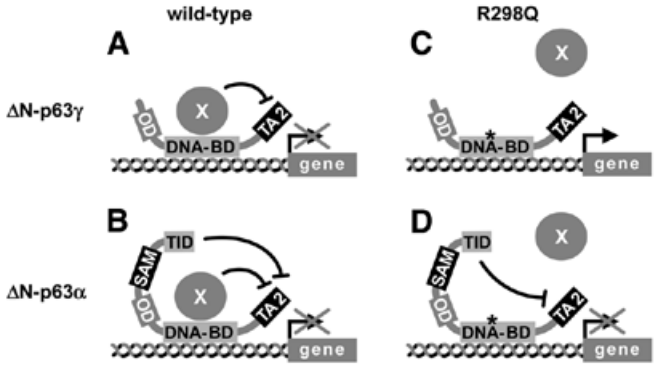

Figure 4. Model for transactivational properties of wild-type ΔN-p63 and ΔN-p63^{R298Q}. In contrast to p63 containing an EEC mutation in the DNA binding domain (not shown), wild-type ΔN-p63 (**A** and **B**) and ΔN-p63^{R298Q} (**C** and **D**) can bind to the DNA. In wild-type p63, a potential second transactivation (TA2) domain, present only in ΔN-p63, may be kept transcriptionally inactive (arrows) by a transcriptional repressor protein (X) that binds to the DNA binding domain (A and B). In addition, the α-C-terminus, possibly the TI domain, can also repress TA2 activity (B). If, due to the R298Q mutation (asterisk), binding of repressor X is lost (C and D), this would explain why ΔN-p63γ^{R298Q} (C), but not ΔN-p63α^{R298Q} (D), gains transactivation activity (arrows). Alternatively, TA2 itself may fold back and bind to the DNA binding domain, rendering it transcriptionally inactive. The R298Q mutation could prevent TA2 from binding to the DNA binding domain, resulting in a gain of transactivation (not shown). Shown are the second transactivation domain (TA2), DNA binding domain (DNA-BD), oligomerization domain (OD), SAM domain (SAM) and transactivation inhibitory domain (TID).

silenced TA2 domain, providing a possible functional explanation for the two different mutations both causing a very similar ADULT syndrome phenotype.

Taken together, we propose a model that could explain why the R298Q mutation causes an ADULT syndrome phenotype, as well as why R298Q is a gain-of-function mutation. We suggest that a co-repressor molecule normally binds to the p63 DNA binding domain and thereby represses transactivation activity of the TA2 domain in ΔN-p63 (Fig. 4A and B). The R298Q mutation abolishes binding of this co-repressor. As a consequence, ΔN-p63γ^{R298Q} gains transactivation activity (Fig. 4C). The absence of transactivation activity in ΔN-p63α^{R298Q} (Fig. 3A) may be explained by the presence of a TI domain in the α-C-terminus (Fig. 4D). Evidence for this has been described previously (11; Z.Serber, H.Lai, A.Yang, H.Ou, A.Kelly, B.Darimont, P.Duijf, H.van Bokhoven, F.McKeon and V.Dötsch, manuscript in preparation) and some of our results support this finding. ΔN-p63γ can repress the transactivation activity of ΔN-p63γ^{R298Q} by ~60%, whereas ΔN-p63α is able to completely repress this activity (Fig. 3B). Assuming equal DNA binding kinetics for ΔN-p63α and ΔN-p63γ, this cannot be explained by competition for DNA consensus sequences. Rather, this effect should be attributed to the distinct C-termini in ΔN-p63α and ΔN-p63γ. Of note, there is an alternative possibility in the presented model (Fig. 4). Transactivation activity of the TA2 domain may not be repressed by a co-repressor X that binds to the DNA binding domain, but instead, the TA2 domain itself may bind to the DNA binding domain, thereby losing its transactivation activity. The R298Q mutation could disrupt this TA2–DNA binding domain interaction.

We conclude that the R298Q ADULT syndrome mutation not only causes a phenotype distinct from EEC syndrome, it is also located in a different region of the p63 DNA binding domain, and acts through a different mechanism. As p63^{R298Q} can still bind to the DNA and confers transactivation activity in ΔN-p63γ^{R298Q}, it is a gain-of-function instead of a loss-of-function mutation, as observed in EEC syndrome. Our data strongly suggest the presence of a second transactivation (TA2) domain, which is constitutively activated by the R298Q mutation. In our model we propose that the R298Q mutation either abolishes the binding to a co-repressor or disrupts the interaction of the DNA binding domain with the TA2 domain. Like the canonical TA domain, this TA2 domain can be effectively repressed by the α-tail of p63 both *in cis* and *in trans*. In addition to the existence of multiple p63 isoforms, the presence of two TA domains in p63 adds to the functional complexity of this intriguing gene that fulfils a crucial role during vertebrate development.

MATERIALS AND METHODS

Patients

The ADULT syndrome family members used in this study have been clinically described previously (13).

Mutation analysis

Genomic DNA from each individual investigated was isolated from peripheral blood lymphocytes as described (19). Using genomic DNA from patient II:7 (15) as a template and primers and PCR conditions as previously described (10), all exons and intron–exon boundaries of the *p63* gene were PCR amplified. Primers used for exon 8 were 8F, 5′-GTA GAT CTT CAG GGG ACT TTC-3′; and 8R, 5′-CCA ACA TCA GGA GAA GGA TTC-3′. PCR reactions for exon 8 were performed in 25 µl volume containing 100 ng genomic DNA, 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 100 ng of each primer and 1 U *Taq* polymerase (Gibco BRL), using the following parameters: 1 min at 94°C, 2 min at 54°C and 1 min at 72°C for 35 cycles. PCR fragments were size-fractionated by electrophoresis in 1.5% agarose gels and visualized by staining with ethidium bromide. PCR products were excised from the gel and purified according to the Qiaquick gel extraction protocol (Qiagen). Purified amplicons were screened for mutations by direct sequencing with the BigDye Terminator chemistry (PE Applied Biosystems) using intron-specific primers. Electrophoresis and analysis were performed on an ABI Prism 3700 genetic analyzer (PE Applied Biosystems).

Within the family, segregation of the mutation with the phenotype was verified and the sequence alteration was checked in 50 control individuals by allele-specific oligonucleotide (ASO) hybridization as described by Shuber *et al.* (20). Primers used are wild-type: 5′-CTG GGC CGA CGC TGC-3′, and mutant: 5′-CTG GGC CAA CGC TGC-3′. The position of the mutation corresponds to the original published TA-p63α sequence (GenBank accession no. AF075430) (1), which does not include the 39 additional codons at the N-terminal end that were reported later by Hagiwara *et al.* (21) (GenBank accession no. AF091627).

Protein modelling

The model of a dimeric p63 DNA binding domain (Fig. 2B) was built by exploiting the sequence similarity to the known 2.2 Å crystal structure of the corresponding p53 domain (PDB entry 1TSR) (17). In addition to the high level of sequence identity (62%), the model is supported by the fact that both proteins, p53 and p63, recognize the same DNA sequence. The modelling was done with WHAT IF (22), following a protocol described by Chinea *et al.* (23). Then the model was minimized with the YASARA NOVA force field, that was parameterized for the refinement of homology models (www.yasara.com/servers). Validation of the model with WHAT_CHECK (24) revealed no large problems. The model coordinates are available at www.cmbi.kun.nl/gv/service/p63dimer/.

Transactivation assays

Plasmid DNA from mammalian expression vectors containing the murine p63 cDNA sequence under control of a CMV promoter (1) was used as a template for site-directed mutagenesis using the QuickChange procedure (Stratagene). Oligonucleotide 5′-GCA AGTCCT GGG CCA ACG GTG CTT TGA GG-3′ and its reverse complement were used to create the R298Q mutation. The obtained clones were selected for the presence of the R298Q mutation by direct sequencing (ABI3700 genetic analyzer; see above). The remainder of the open reading frame was also sequenced to prevent unwanted nucleotide changes.

Transactivation assays were carried out as described previously (11). In short, human Saos-2 cells were transfected with wild-type and/or mutant p63 expression vectors, and a β-galactosidase reporter construct containing p53-binding sites [PG13-β-gal (1)]. Cells were lysed in detergent lysis buffer 36–48 h after transfection, and assayed for β-galactosidase activity. A second reporter construct (PGL3; Promega), from which luciferase was constitutively expressed, was used in all samples to compensate for transfection efficiency and sample preparation. All experiments were performed in triplicate.

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