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# EEC- and ADULT-Associated *TP63* Mutations Exhibit Functional Heterogeneity Toward P63 Responsive Sequences

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**ABSTRACT:** *TP63* germ-line mutations are responsible for a group of human ectodermal dysplasia syndromes, underlining the key role of P63 in the development of ectoderm-derived tissues. Here, we report the identification of two *TP63* alleles, G134V (p.Gly173Val) and insR155 (p.Thr193_Tyr194insArg), associated to ADULT and EEC syndromes, respectively. These alleles, along with previously identified G134D (p.Gly173Asp) and R204W (p.Arg243Trp), were functionally characterized in yeast, studied in a mammalian cell line and modeled based on the crystal structure of the P63 DNA-binding domain. Although the p.Arg243Trp mutant showed both complete loss of transactivation function and ability to interfere over wild-type P63, the impact of p.Gly173Asp, p.Gly173Val, and p.Thr193_Tyr194insArg varied depending on the response element (RE) tested. Interestingly, p.Gly173Asp and p.Gly173Val mutants were characterized by a severe defect in transactivation along with interfering ability on two DN-P63$\alpha$-specific REs derived from genes closely related to the clinical manifestations of the *TP63*-associated syndromes, namely PERP and COL18A1. The modeling of the mutations supported the distinct functional effect of each mutant. The present results highlight the importance of integrating different functional endpoints that take in account the features of P63 proteins' target sequences to examine the impact of *TP63* mutations and the associated clinical variability.**
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**KEY WORDS:** *TP63*; ectodermal dysplasia; transactivation; yeast

## Introduction

*TP63* (MIM #603273) is a member of the *TP53* gene family [Yang et al., 1998]. As *TP53* and *TP73*, it encodes a sequence-specific transcription factor that activates target genes involved in different cellular pathways [van Bokhoven et al., 2011]. Alternative promoter usage and differential C-terminal splicing result in different P63 isoforms [Mangiulli et al., 2009]. The TA isoforms (TA-P63$\alpha$, $\beta$, $\gamma$, $\delta$, and $\varepsilon$) contain the N-terminal transactivation domain (TA1), whereas the DN isoforms (DN-P63$\alpha$, $\beta$, $\gamma$, $\delta$, and $\varepsilon$) are transcribed from an internal promoter (P2) and, although lacking the TA1 domain, they still retain specific transactivation properties [Dohn et al., 2001]. A second C-terminal transactivation domain (TA2) is exclusively present in P63$\alpha$ and $\beta$ isoforms (TA and DN) [Ghioni et al., 2002]. In addition, TA*-P63 isoforms, which are encoded by the TA transcripts and characterized by the presence of a 39 amino acid N-terminal extension, have been described [Yang et al., 1998].

A tissue-specific expression of P63 isoforms has been highlighted. Although the DN-P63$\alpha$ is predominantly expressed in basal epithelial cells of skin, breast, prostate, and urothelia and its expression decreases with increasing cell differentiation, TA-P63 isoforms are barely detectable in the same tissues [Candi et al., 2008; Mckeon, 2004]. Conversely, the expression of TA-P63 isoforms has been described in female and male germ lines, where, because of their

Additional Supporting Information may be found in the online version of this article.

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proapoptotic function, they play a key role in the cellular response to DNA damage [Beyer et al., 2011; Levine et al., 2011; Petre-Lazar et al., 2007; Suh et al., 2006].

Germ-line heterozygous mutations in the *TP63* gene underlie the molecular basis of a subset of human ectodermal dysplasia syndromes (EDs). These include EEC (ectrodactyly, ED, clefting; MIM #604292), AEC (ankyloblepharon, ED, clefting; MIM #106260), ADULT (acro, dermato, ungual, lacrimal, tooth; MIM #103285), Rapp–Hodgkin (RHS; MIM #129400), and limb-mammary (LMS; MIM #603543) syndromes. In various combinations, all these clinical conditions share anomalies in hair, teeth, nail, and sweat gland functions. The anomalies affecting the epidermis and the epidermal appendages are extremely variable and some syndromes are also associated with malformations in specific organs and systems [Rinne et al., 2007]. Nonsyndromic split hand and foot malformation (SHFM4; MIM #605289) is also caused by mutations in the *TP63* gene.

Several reports suggest genotype–phenotype correlations linked to the type and localization of inherited mutations in the P63 protein. The EEC syndrome, the prototype of the *TP63*-associated syndromes, is mainly caused by missense mutations in the DNA Binding Domain (DBD) of the P63 protein, particularly in residues that interact with DNA. There are five frequently mutated arginine residues that account for nearly 90% of the reported EEC cases [Rinne et al., 2007]. Also, the majority of individuals with ADULT syndrome harbor a mutation within the DBD that, in contrast to EEC-associated mutations, is distant from the P63 protein–DNA interaction surface. Apparently, this mutation confers a gain of transactivation activity to a specific P63 isoform (DN-P63$\gamma$) [Duijf et al., 2002]. Conversely, AEC and RHS are caused by mutations that target the C-terminus of the P63 protein and include either point mutations in the SAM (sterile-alpha motif) domain or deletions in the SAM or transactivation inhibitory (TI) domains [Sawardekar and Zaenglein, 2011]. In LMS, in addition to C-terminal mutations that result in truncation of the P63$\alpha$ isoform, mutations at the N-terminus of the *TP63* gene have been observed [Rinne et al., 2007]. Despite the described genotype–phenotype correlations, the variability among the *TP63*-linked EDs, even within a single clinically defined disease, is considerable. Such variability might be ascribed to a differential transactivation potential of P63 mutant proteins toward target promoters [Khokhar et al., 2008; Lo Iacono et al., 2006, 2008] and/or to combinations of genetic modifier or epigenetic factors that only recently have been considered [Gritli-Linde, 2010].

Here, we report the identification of two *TP63* alleles (G134V and insR155, corresponding to p.Gly173Val, c.518G>T and p.Thr193_Tyr194insArg, c.580–2A>G) found in two patients affected by ADULT and EEC syndromes, respectively. These alleles along with G134D (p.Gly173Asp, c.518G>A) and R204W (p.Arg243Trp, c.727C>T) previously identified in EDs were functionally characterized in yeast, studied in a mammalian cell line (as DN-P63$\alpha$ variants) and modeled based on the crystal structure of the P63 DBD. The results revealed functional heterogeneity of P63 mutants in term of transactivation ability, potential to inhibit the wild-type P63 when heterozygous (defined as interfering ability) and temperature sensitivity on a subset of P63 responsive sequences. The protein modeling revealed structural distortions that are compatible with the observed functional heterogeneity.

Considering the clinical variability of *TP63*-associated syndromes, our data dissect the functional complexity produced by mutations on *TP63* gene and highlight the importance of integrating clinical classification with parameters that takes into account the functional effect of P63 mutant proteins on distinct target sequences.

## Materials and Methods

### Nomenclature

The P63 variants reported in the present study are indicated following the systematic nomenclature approved by Human Genome Variation Society (HGVS; www.hgvs.org/mutnomen). Nucleotide numbering starts from the A (+1) of the translation initiation ATG codon of the cDNA sequence RefSeq NM_003722.4. The amino acid residues are numbered according to the NP_001108450.1 and NP_003713.3 sequences containing an additional 39 N-terminal codons that were not present in the original P63 protein sequence NP_003713.1 [Osada et al., 1998]. The systematic nomenclature differs from the traditional nomenclature, found in some published literature, which is based on amino acid numbering of the original P63 protein sequence. A summary of variant descriptions using both nomenclature systems is reported in Table 1, with the single-letter amino acid code being used for the traditional names. The *TP63* variants identified in present article were submitted to the locus-specific database of P63 protein (www.lovd.nl/TP63).

### Yeast Strains and Media

The available *Saccharomyces cerevisiae* haploid yeast strains yLFM-response elements (REs) (P21–5′, PUMA, MDM2, BAX) [Inga et al., 2002] were used. The new yeast strains yLFM-PERP and yLFM-COL18A1 were generated using the *delitto perfetto* approach [Storici and Resnick, 2003] by genomic cloning

**Table 1. *TP63* Alleles Under Study and their Nomenclature**

| Traditional mutation nomenclature | HGVS nomenclature | | | |
|---|---|---|---|---|
| References | Ref Seq Acc N° | Mutation | Protein RefSeq | Mutation (protein) |
| G134D | NM_003722.4 | c.518G>A | NP_003713.3 | p.Gly173Asp |
| Slavotinek et al. (2005) | | | | |
| Vera-Carbonell et al. (2012) | | | | |
| G134V | NM_003722.4 | c.518G>T | NP_003713.3 | p.Gly173Val |
| Prontera et al. (2011) | | | | |
| This work | | | | |
| insR155 | NG_007550.1 | g.237804A>G | NP_003713.3 | p.Thr193_Tyr194insArg |
| This work | NM_003722.4 | c.580–2A>G | | |
| R204W | NM_003722.4 | c.727C>T | NP_003713.3 | p.Arg243Trp |
| Celli et al. (1999) | | | | |
| Avitan-Hersh et al. (2010) | | | | |

Nucleotide numbering according to HGVS, with +1 corresponding to the A of the ATG translation start codon in the indicated RefSeq cDNA.

of the human *PERP* intronic RE (+3,431 bp from the transcription start site [TSS]: AGGCAAGCTC-CAGCTTGTTC) [Ihrie et al., 2005] and of the human *COL18A1* promoter *RE* [Shalom-Feuerstein et al., 2011] (–1,555 bp from the TSS: AGACATGCAC-ACACATGCAC, identified by us through the free bioinformatic tool TFBind, http://tfbind.hgc.jp/) [Tsunoda and Takagi, 1999]. The oligonucleotides (TIB MOLBIOL, Berlin, Germany) for genomic cloning contained, besides RE sequence, a 5′ and a 3′ region of homology with yeast genome: 5′-*gcggaattgacttttcttgaataatacat*-RE-gcagatccgccaggcgtgtatatagcgtgg-3′. The haploid strain yIG397 (3XRGC::pCYC1::*ADE2*) was used for the gap repair assay [Flaman et al., 1995]. Cells were grown in 1% yeast extract, 2% peptone, 2% dextrose with the addition of 200 mg/l adenine (YPDA medium) or in selective medium containing dextrose or raffinose as carbon source and adenine (200 mg/l) but lacking tryptophan and/or leucine (Sigma-Aldrich, Sant Louis, Missouri, USA; BiokarDiagnostics, Allonne, France). Galactose (Sigma-Aldrich, Sant Louis, Missouri, USA) was added to the medium to modulate P63 (or P53) expression under the inducible *GAL1,10* promoter. Plates containing 5-fluoroorotic acid (5-FOA) (Toronto Research Chemicals, Toronto, Canada) and Geneticin (G418) (Gibco, Life Technologies, Carlsbad, California, USA) were used for the generation of the new yLFM-PERP and yLFM-COL18A1 yeast strains.

## Cell Line and Culture Conditions

Human colon cancer HCT116 *TP53*$^{-/-}$ cell line was a gift of Dr. B. Vogelstein. Cells were grown in RPMI 1640 (Gibco-Invitrogen, Life Technologies, Carlsbad, California, USA) supplemented with 10% fetal bovine serum and maintained at 37°C in 5% $CO_2$ (100% humidity).

## Yeast Expression Vectors

For constitutive expression of P63 proteins ($\alpha$ isoform) under the *ADH1* promoter, pTS-based yeast expression vectors (*TRP1* as selection marker) [Inga et al., 1997] were constructed using a PCR-based approach followed by a gap repair assay [Monti et al., 2011]. The vectors were generated starting from available pcDNA3.1-*TP63α* plasmids (Supporting Information). Yeast vectors expressing wild-type and mutant proteins (DN-P63$\alpha$ isoform) under the inducible *GAL1,10* promoter (pTSG-based) were constructed by double digesting the pTS-based expression vectors with the *Xho*I and *Not*I restriction enzymes (New England Biolabs, Ipswich, Massachusetts, USA). The DNA fragment containing the DN-*TP63α* coding sequence (cDNA) was then cloned in an identical double digested pTSG-based vector [Inga et al., 2001]. Similarly, the pTSG-based plasmid expressing TA-P63$\alpha$ protein was constructed. For the experiments regarding the interfering ability, a new pLS-based vector (*LEU2* as selection marker) was constructed by ligation of the 5.5 kb *Pvu*I fragment containing the *pADH1* wild-type DN-*TP63α* cDNA from the pTS vector to the 4.2 kb *Pvu*I fragment containing the CEN/ARS *LEU2* region from pLS76 [Inga et al., 1997]. Plasmids pRS314 (*TRP1*) and pRS315 (*LEU2*) were used as empty vectors. The pTSG-P53 plasmid, harboring the human wild-type *TP53* cDNA under the inducible *GAL1,10* promoter, was already available [Inga et al., 2001].

## Mammalian Expression Vectors and Reporters

The mammalian pCI-neo plasmids expressing the wild-type and mutant *TP63* alleles (DN-P63$\alpha$ isoform) were obtained from *Xho*I/*Not*I double digestion of pTS-DN vectors along with empty pCI-neo backbone. The fragment from pTS-based vector corresponding to DN-*TP63α* cDNA was ligated to the 5.4 kb fragment from pCI-neo plasmid. The pCI-neo vector expressing the wild-type TA-P63$\alpha$ protein was similarly generated. Plasmid pCI-neo was used as empty vector. The pGL3–1138 (P21: 2.3 kb promoter fragment, containing both the *P21–5′* and the *P21–3′* REs), pGL3-MDM2 (MDM2: 350 bp region with both P53 REs present in intron 1 of the *MDM2* gene) and pGL3–1012 (BAX: 400 bp region of intron 1 of the *BAX* gene) reporter vectors (gift of Dr. M. Oren) were used for mammalian transient transfection experiments. pGL3-P21–5′ (P21–5′ RE) reporter vector (gift of Dr. Menendez) that contained only the distal P53 RE from the *P21* gene and corresponding to the RE of yLFM-P21–5′ strain, was also tested. The pRL-SV40 plasmid, harboring the luciferase gene from *Renilla reniformis* under the control of a constitutive promoter, was used to normalize for transfection efficiency.

## Quantitative Evaluation of P63 Transactivation and Interfering Ability in Yeast

The analysis of transactivation ability of P63 mutants was carried out by transforming (LiAc method) yLFM-REs strains with the P63 wild-type and mutant protein expression vectors (pTS- or pTSG-based) along with the empty vector pRS314. P53 family members (P53, TA-P63$\alpha$, and DN-P63$\alpha$ wild-type) transactivation ability was compared using pTSG-based vectors. Colonies, when needed, were incubated at different temperatures (24, 30, and 36°C). The luciferase assay was conducted according to the miniaturized protocol we recently developed [Andreotti et al., 2011](Supporting Information). The transactivation ability of wild-type and mutant P63 proteins was expressed as relative light unit (RLU); fold of induction over empty vector (pRS314) were also calculated and used to determine the percentage of luciferase activity of P63 mutants with respect to *wild-type*.

The analysis of interfering ability was carried out in the same yeast strains and based on a constitutive expression of wild-type DN-P63$\alpha$ (pLS-based, *LEU2*) and on increasing expression of wild-type or mutants (pTSG-based, *TRP1*) through an 8 hr culture in media containing different amounts of galactose inducer (0.016% and 0.128%) of double Leu+Trp+ transformants. The interfering ability of mutant P63s was expressed as RLU; fold of induction over empty vectors (pRS314 plus pRS315) were also calculated and used to determine the percentage of luciferase activity obtained when P63 mutant and wild-type are coexpressed compared with the activity of a single wild-type P63 protein.

## Quantitative Evaluation of the P63 Mutants Transactivation Ability in the Mammalian HCT116 *TP53*$^{-/-}$ Cell Line

The day before transfection HCT116 *TP53*$^{-/-}$ were seeded in 24-well plates ($0.8 \times 10^5$/well). Transfections were performed at 70%–80% confluence using the *Trans*IT-LT1 transfection reagent according to the manufacturer's protocol (Mirus Bio LLC, Madison, USA). The total plasmid DNA amount per well was kept constant at 500 ng by adding 250 ng of pGL3 promoter-derived P53 reporter plasmids, 200 ng of the expression or empty vectors and 50 ng of the pRL-SV40 plasmid. Cells were harvested 24 hr after the transfection and luciferase assays were conducted as detailed in Supporting Information. The transactivation ability of P63 mutants was expressed as RLU.

### Protein Modeling

To model the structure of P63 DBD mutants, the crystal structure of P63 DBD bound to a 10 bp oligonucleotide was used as a starting model (PDB ID: 3qym); the numbering of amino acid positions refers to the P63 protein structure that lacks N-terminal additional 39 codons. The mutation p.Gly173Asp (indicated in the structure as G134D), p.Gly173Val (indicated in the structure as G134V), and p.ArgR243Trp (indicated in the structure as R204W) were modeled by substituting the side chain of the wild-type amino acids by mutant using the program SCWLR4 [Krivov et al., 2009] and clashes were relieved by 100 steps with a step length of 0.02 Å steepest descent energy minimization followed by 100 steps of conjugated gradient with the same step length. The Amber ff99 force field parameters were used and minimization was carried as implemented in the Chimera molecular graphics program. To model p.Thr193_Tyr194insArg (indicated in the structure as insR155), the Phyre2 protein recognition program was used [Kelley and Sternberg 2009]. The Phyre2 model was energy minimized in the same manner that the other mutants. The side chain of K149 (as indicated in the structure, and corresponding to K188) that was not present in the 3qym crystal structure was modeled with the same protocol.

## Results

### Identification of *TP63* Mutations in ADULT and EEC Patients

The screening of the *TP63* gene in the ADULT or EEC patients (Supporting Information) revealed the presence of: (1) an heterozygous substitution in exon 4, the c.518G>T, causing the change of the Glycine 173 into a Valine (p.Gly173Val) (ADULT) and (2) a heterozygous intronic substitution at the canonical acceptor splice site between intron 4 and exon 5 (c.580–2A>G) (EEC). This latter mutation leads to the activation of an adjacent cryptic splice site causing the in-frame insertion of an Arginine residue after Threonine 193 (p.Thr193_Tyr194insArg). This mutation has neither been reported in the single nucleotide polymorphism (dbSNP at www.ncbi.nlm.nih.gov/snp) nor in the Ensemble databases (http://www.ensembl.org). The traditional nomenclature of *TP63* alleles is reported in Table 1.

### Wild-Type P63$\alpha$ Isoforms can Act as Transcription Factors in Yeast

To study the functionality of P63 mutants in yeast, we first tested the transactivation ability of different wild-type P63$\alpha$ isoforms. Vectors expressing TA*, TA, and DN isoforms under a moderate promoter (*ADH1*) were transformed in four isogenic reporter strains containing the *P21–5′, MDM2, PUMA, or BAX* P53 REs upstream of the luciferase cDNA [Inga et al., 2002]. *P21*, *MDM2*, *PUMA*, and *BAX* are well-known P53 family target genes involved in the regulation of cell cycle, protein stability, and apoptosis. These genes participate also to epithelial morphogenesis and homeostasis through P63-dependent regulation [Candi et al., 2006; Pyati et al., 2011; Westfall et al., 2003]. We found that P63$\alpha$ isoforms were able to transactivate the *P21–5′, MDM2, PUMA*, or *BAX* REs but, within the same RE, they were characterized by different transactivation potential: in fact, whereas the TA variant was the most active on every RE tested (being 1.5–3-fold more potent than DN), the TA* isoform was always the least active (Fig. 1). This last result is consistent with data obtained in mammalian cells [Yang et al., 1998], suggesting that the N-terminal extension of 39 amino acids negatively influences the transactivation ability of this isoform. Our evidences confirm and extend previous findings obtained in yeast [Shimada et al., 1999], further validating the yeast assay as useful tool for functional studies of P63 protein isoforms.

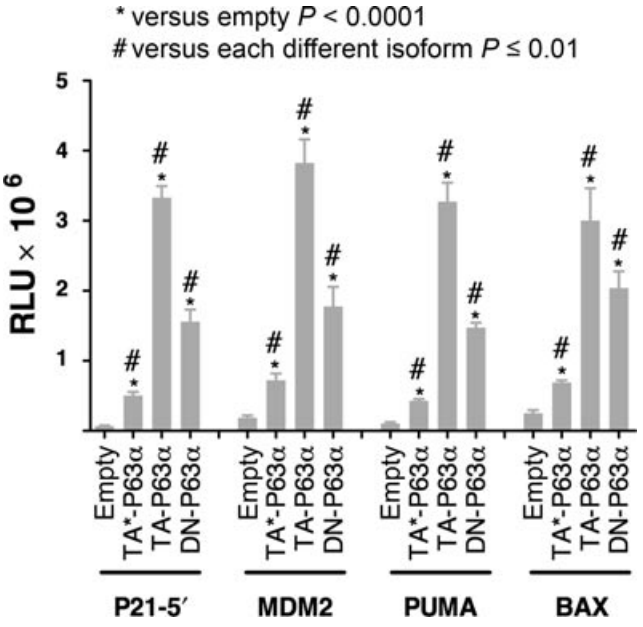


**Figure 1.** Transactivation ability of P63$\alpha$ isoforms (TA*, TA, and DN) in yLFM-P21–5′, yLFM-MDM2, yLFM-PUMA, and yLFM-BAX yeast strains. The transactivation ability was determined using a constitutive expression of P63 proteins (ADH *1* promoter). Presented are the average relative light units (RLU) and standard deviations of four biological replicates. RLU were obtained upon normalization for cell number (OD at 600 nm). Statistical analysis was performed within the single RE. TA*-P63$\alpha$, TA-P63$\alpha$, and DN-P63$\alpha$-fold of induction over empty vector (pRS314) are the following: $7.3 \pm 0.7$, $47.8 \pm 2.4$, and $22.4 \pm 2.5$, respectively on *P21–5′* RE; $3.8 \pm 0.5$, $20.2 \pm 1.8$, and $9.4 \pm 1.5$, respectively on *MDM2* RE; $4.0 \pm 0.2$, $30.7 \pm 2.5$, and $13.8 \pm 0.7$, respectively on *PUMA* RE; $2.7 \pm 0.1$, $11.8 \pm 1.8$, and $8.0 \pm 0.9$, respectively on *BAX* RE.

### The P63 p.Gly173Val and p.Thr193_Tyr194insArg Mutants Reveal Residual and Target-Dependent Transactivation Activity on *P21, MDM2, PUMA*, and *BAX* REs

Because DN-P63$\alpha$ protein is the main isoform expressed at the basal layer of epidermal tissues and mutant proteins accumulate in the skin of EEC and AEC syndrome patients [Browne et al., 2011], the functional studies were focused on the DN-P63$\alpha$ isoform. The transactivation potential of the p.Gly173Val and p.Thr193_Tyr194insArg mutants was measured upon their expression at moderate levels (*ADH1* promoter) using the P21–5′, MDM2, PUMA, or BAX reporter strains. The previously identified p.Gly173Asp (G134D) and p.Arg243Trp (R204W) mutant alleles were also considered (Table 1) and the corresponding proteins characterized to verify the effect of different amino acid substitutions at the same codon, and to confirm the reported loss of transactivation function for the latter mutant [Celli et al., 1999]. The results showed a reduction with respect to wild-type, but not a complete loss of transactivation ability especially for p.Gly173Asp, p.Gly173Val, and p.Thr193_Tyr194insArg mutants in every yeast reporter strain (Fig. 2; Supp. Table S1). The p.Thr193_Tyr194insArg mutant was

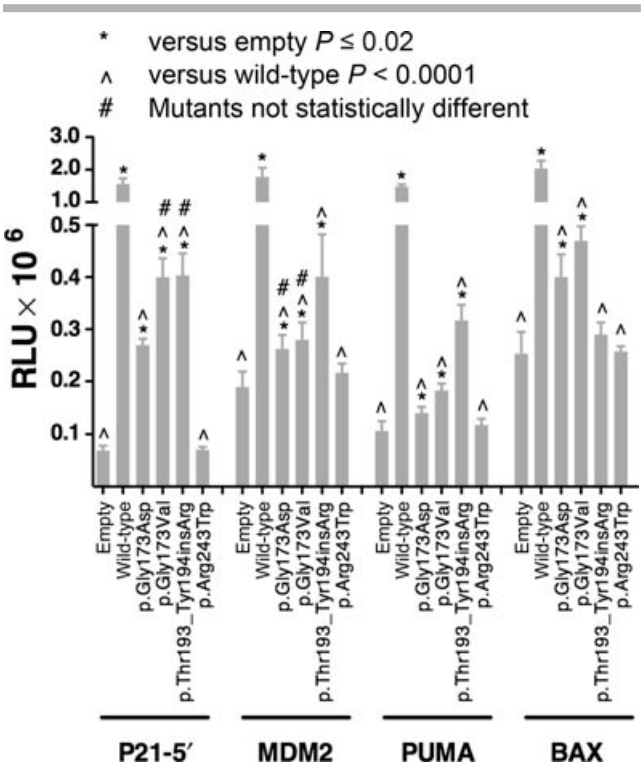


**Figure 2.** Transactivation ability of wild-type and mutant DN-P63$\alpha$ proteins in yLFM-P21–5′, yLFM-MDM2, yLFM-PUMA, and yLFM-BAX yeast strains. The transactivation ability was determined using a constitutive expression of P63 proteins (*ADH1* promoter). Presented are the average RLU and standard deviations measured as in Figure 1. Statistical analysis was performed within the single RE. P63 mutants showed a significantly different activity among each other ($P \leq 0.03$) with the exceptions indicated in the figure. Wild-type and mutant DN-P63$\alpha$-fold of induction over empty vector (pRS314) are reported in Supp. Table S1 and used to calculate the relative transactivation ability of DN-P63$\alpha$ mutants with respect to wild-type.

more active than p.Gly173Asp and p.Gly173Val in MDM2 and PUMA reporter strains, whereas its activity was comparable to p.Gly173Val on the *P21–5′* RE, and lower on *BAX* RE (Fig. 2). Moreover, p.Gly173Asp was generally less active than p.Gly173Val. The p.Arg243Trp showed, as expected, the highest loss of transactivation ability on every RE.

Previous studies on P53 demonstrated that the use of the inducible *GAL1,10* promoter resulting in variable (galactose-dependent) expression of the protein in yeast, provided for a more sensitive screening tool for the functional characterization of *TP53* mutations [Resnick and Inga, 2003]. To better characterize the transactivation potential of P63 mutant protein, vectors expressing wild-type and mutant proteins under the *GAL1,10* promoter were constructed and transformed in yeast. A galactose concentration-dependent increase of transactivation ability by wild-type DN-P63$\alpha$ protein was observed (Supp. Fig. S1). At relatively high galactose level (0.128%), the p.Gly173Asp, p.Gly173Val, and p.Thr193_Tyr194insArg mutants confirmed the differential and residual transactivation potential that in the case of *P21–5′* RE was only 20% lower than wild-type P63 (Supp. Table S2).

To rule out that the observed heterogeneity in transactivation could be because of the differences in protein steady-state levels, Western blot analysis was performed in one strain, that is, yLFM-PUMA, as all strains are isogenic and differ only for the RE sequence (20 bp) upstream the luciferase reporter gene. The Western blot showed that the p.Gly173Asp, p.Gly173Val, p.Thr193_Tyr194insArg, and p.Arg243Trp proteins (DN-P63$\alpha$) are equally expressed and at comparable levels with the wild-type (Supp. Fig. S2). Thus, the heterogeneity in transactivation of P63 mutant proteins is not because of the differences in protein steady state levels.

## The P63 p.Gly173Val and p.Thr193_Tyr194insArg Mutants Show Transactivation Potential in the Mammalian HCT116 *TP53*$^{-/-}$ Cell Line

To determine whether the functional data obtained in yeast were representative of what occurs in mammalian cells, P63 mutants (DN-P63$\alpha$ isoform) were transiently expressed in the human epithelial colon cancer-derived HCT116 *TP53*$^{-/-}$ cell line along with different reporter vectors that contained either a *P21* RE or promoter fragments derived from the *P21*, *BAX*, and *MDM2* genes. This cell line does not contain detectable level of endogenous DN-P63$\alpha$ and TA-P63$\alpha$ (Supp. Fig. S3) as well as other P63 isoforms; also the basal expression of P73 is low [Vilgelm et al., 2010]. Thus, this cell line appeared to be suitable for the study of the transactivation specificity of each DN-P63$\alpha$ mutant. The p.Arg243Trp mutant showed a loss of transactivation ability with all reporters. The other mutants appeared to be more active than the wild-type toward the MDM2 reporter plasmid. The p.Thr193_Tyr194insArg was also more active toward a fragment of the *P21* promoter (containing both the *P21–5′* and the *P21–3′* REs) than with the reporter containing only the P21–5′ RE (Fig. 3), suggesting that the *P21–3′* RE may contribute to the modulation of its activity. The p.Gly173Asp and p.Gly173Val showed only a modest defect in transactivation with P21 and BAX reporters compared with wild-type P63. All together these results showed the transactivation potential associated with p.Gly173Asp, p.Gly173Val, and p.Thr193_Tyr194insArg mutants and the complete loss of function of p.Arg243Trp.

## The P63 p.Gly173Val Mutant Shows Loss of Activity on *PERP* and *COL18A*1 DN-P63$\alpha$-Specific REs, whereas the p.Thr193_Tyr194insArg Retains Partial Activity Exclusively on *COL18A1* RE

Even though *P21*, *MDM2*, *PUMA*, and *BAX* are transactivated by P63, they represent neither P63-specific targets nor genes closely related to the clinical manifestations of the *TP63*-associated syndromes. Based on literature, *PERP* and *COL18A1* were considered representative of the latter genes. *PERP* is a well-known P63-regulated gene during epithelial development that encodes a tetraspan membrane protein involved in cell–cell interactions [Ihrie et al., 2005], whereas *COL18A1* is a newly identified P63 target gene related to skin biology [Shalom-Feuerstein et al., 2011] that encodes the alpha chain of type XVIII collagen, a main component of basal laminae of the epidermis. The REs responsible for P63 responsiveness were selected (section *Materials and Methods*) and the new yLFM-PERP and yLFM-COL18A1 yeast strains were constructed. To determine the relative transactivation ability of P63 (per se and with respect to P53), vectors expressing wild-type P63 (DN- and TA-P63$\alpha$ isoforms) or P53 proteins under the *GAL1,10* promoter were transformed in yLFM-PERP and yLFM-COL18A1 strains along with the previously used yLFM-P21–5′, yLFM-MDM2, yLFM-PUMA, and yLFM-BAX strains. The results showed that in the latter four strains P53 was clearly a more potent transcription

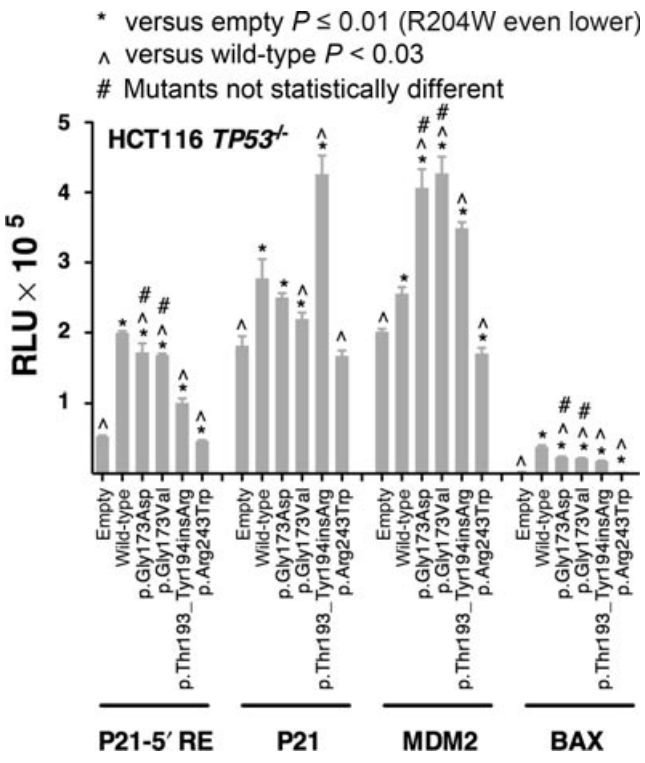


**Figure 3.** Transactivation ability of wild-type and mutant DN-P63$\alpha$ proteins in mammalian HCT116 *TP53*$^{-/-}$ cells. The assays were performed using the P21–5′, P21, MDM2, and BAX reporter vectors. Presented are the means and the standard deviations of at least three biological replicates. Average RLU were obtained upon normalization to Renilla luciferase. Statistical analysis was performed within the single reporter. P63 mutants showed a significantly different activity among each other ($P \leq 0.03$) with the exceptions indicated in the figure. Wild-type, p.Gly173Asp, p.Gly173Val, p.Thr193_Tyr194insArg, and p.Arg243Trp (DN-P63$\alpha$) fold of induction over empty vector (pCI-neo) are the following: $3.8 \pm 0.0$, $3.2 \pm 0.2$, $3.2 \pm 0.0$, $1.9 \pm 0.1$, and $0.9 \pm 0.0$, respectively on P21–5′; $1.5 \pm 0.2$, $1.4 \pm 0.0$, $1.2 \pm 0.0$, $2.3 \pm 0.1$, and $0.9 \pm 0.0$, respectively on P21; $1.3 \pm 0.0$, $2.0 \pm 0.1$, $2.1 \pm 0.1$, $1.7 \pm 0.0$, and $0.8 \pm 0.0$, respectively on MDM2; $17.4 \pm 1.0$, $10.6 \pm 0.4$, $10.0 \pm 0.2$, $8.1 \pm 0.3$, and $1.2 \pm 0.0$, respectively on BAX. As positive control, P53 activity (pC53-SN3 plasmid) was evaluated on the same reporters (fold: $4.6 \pm 0.2$, $8.7 \pm 0.1$, $19.0 \pm 0.2$, and $170.5 \pm 3.8$, respectively).

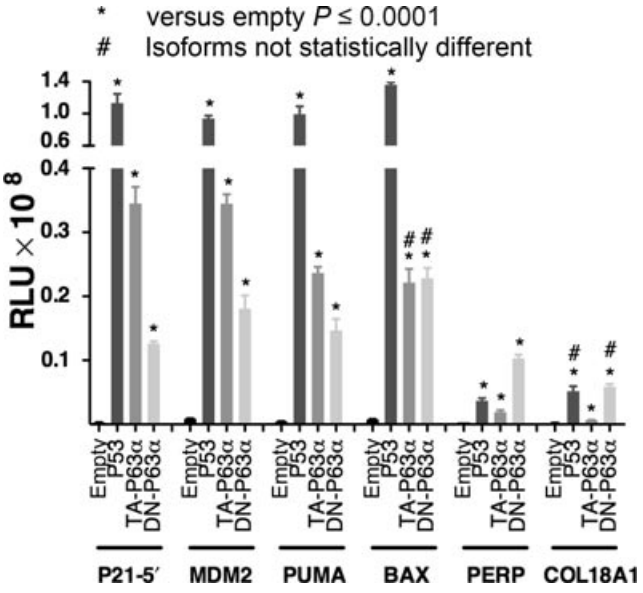


**Figure 4.** Transactivation ability of wild-type P53 (dark gray) and P63$\alpha$ isoforms (TA and DN, gray and light gray, respectively) in yLFM-P21–5′, yLFM-MDM2, yLFM-PUMA, yLFM-BAX, yLFM-PERP, and yLFM-COL18A1 yeast strains. The transactivation ability was determined using an inducible expression of the proteins under the *GAL1,10* promoter (8 hr in 0.128% galactose). Presented are the average RLU and standard deviations measured as in Figure 1. Statistical analysis was performed within the single RE. All isoforms were statistically different among each other ($P \leq 0.005$) with the exceptions indicated in the figure. P53, TA-P63$\alpha$, and DN-P63$\alpha$-fold of induction over empty vector (pRS314) are the following: $378.5 \pm 38.2$, $115.2 \pm 8.6$, and $42.1 \pm 1.2$, respectively on *P21–5′* RE; $104.0 \pm 4.1$, $38.2 \pm 1.6$, and $20.0 \pm 2.3$, respectively on *MDM2* RE; $225.5 \pm 2.1$, $53.6 \pm 2.1$, and $33.3 \pm 4.1$, respectively on *PUMA* RE; $179.5 \pm 4.2$, $29.2 \pm 2.8$, and $30.1 \pm 2.1$, respectively on *BAX* RE; $11.4 \pm 1.3$, $5.9 \pm 0.9$, and $31.8 \pm 1.7$, respectively on *PERP* RE; $22.6 \pm 3.5$, $2.9 \pm 0.2$, and $25.8 \pm 1.7$, respectively on *COL18A1* RE.

factor than DN-P63$\alpha$ (fivefold to ninefold) and that TA-P63$\alpha$ worked equally or better than DN-P63$\alpha$ (TA-P63/DN-P63 = onefold to threefold). In the yLFM-PERP and yLFM-COL18A1 strains instead P53 showed lower (~0.3-fold) or equal (~0.9-fold) transactivation ability than that of DN-P63$\alpha$, respectively. Furthermore, in the same strains, DN-P63$\alpha$ worked better than TA-P63$\alpha$ (DN-P63/TA-P63 = fivefold to 10-fold) (Fig. 4). The data indicated that the REs of *PERP* and *COL18A1* genes are more DN-P63$\alpha$ specific than those of *P21*, *MDM2*, *PUMA*, and *BAX*, which are more P53 and TA-P63$\alpha$ responsive. Based on those results, it was therefore very interesting to analyze the transactivation activity of the mutants as DN-P63$\alpha$ variant (p.Gly173Asp, p.Gly173Val, p.Thr193_Tyr194insArg, and p.Arg243Trp) in yLFM-PERP and yLFM-COL18A1 strains. Although overexpressed (0.128% galactose), all alleles showed a drastic loss of transactivation activity with respect to wild-type on these REs. Only the p.Thr193_Tyr194insArg mutant retains some residual activity on *COL18A1* target sequence (24% of wild-type) (Fig. 5; Supp. Table S3).

## The P63 p.Gly173Val Mutant, but Not the p.Thr193_Tyr194insArg, Exhibits Interfering Ability over Wild-Type P63 in Yeast

Because P63 is a tetrameric transcription factor and because in the case of heterozygous germ-line mutations, both alleles are expected to be equally expressed, heterozygous mutant cells should contain wild-type, mixed, and mutant tetramers, provided that the mutant protein is able to form oligomers with the wild-type. The extent to which P63 mutants may interfere with wild-type P63 function in the heterozygous state has not been deeply investigated.

To assess the potential for mutant DN-P63$\alpha$ to inhibit wild-type DN-P63$\alpha$-mediated transactivation (interfering ability), the yeast reporter strains were cotransformed with the wild-type allele expressed under an *ADH1* constitutive promoter, and the mutant allele expressed under an inducible *GAL1,10* promoter, so that the expression levels of mutant P63 could be increased using high galactose concentration. With this experimental set-up, we wanted to mimic the heterozygous state and mutant overexpression occurring in the *TP63*-associated syndromes [Browne et al., 2011]. Coexpression of two wild-type alleles in the same experimental condition was also evaluated and the net transactivation determined. No significant effect was evident at low level of induction (0.016% galactose) (Supp. Fig. S4). Conversely, at relatively high induction (0.128% galactose) the p.Arg243Trp mutant was considered interfering, that is, it reduced the wild-type DN-P63$\alpha$ activity more than 40% in all

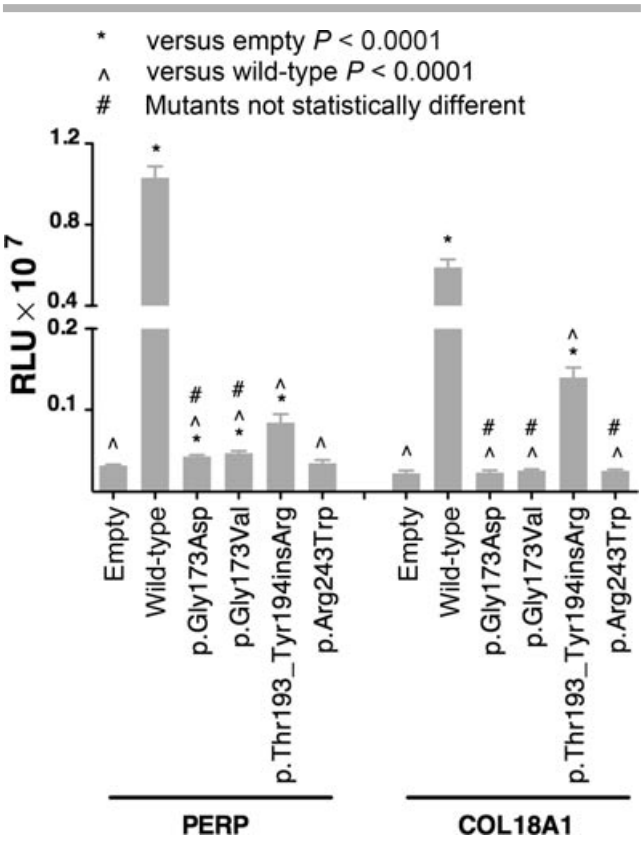


**Figure 5.** Transactivation ability of wild-type and mutant DN-P63$\alpha$ proteins expressed under the inducible *GAL1,10* promoter in the yLFM-PERP and yLFM-COL18A1 reporter strains. The transactivation ability was determined using an inducible expression of P63 proteins under the *GAL1,10* promoter (8 hr in 0.128% galactose). Presented are the average RLU and standard deviations measured as in Figure 1. Statistical analysis was performed within the single RE. P63 mutants showed a significantly different activity among each other ($P \leq 0.01$) with the exceptions indicated in the figure. Wild-type and mutant DN-P63$\alpha$ fold of induction over empty vector (pRS314) are reported in Supp. Table S3 and used to calculate the relative transactivation ability of DN-P63$\alpha$ mutants with respect to wild-type.

tested strains (Fig. 6 and Supp. Table S4). The other P63 mutants (p.Gly173Asp, p.Gly173Val, and p.Thr193_Tyr194insArg) showed no interfering ability on *P21, MDM2, PUMA*, and *BAX* REs and generally an increase in transactivation was observed when coexpressed with the wild-type. On the contrary, p.Gly173Asp and p.Gly173Val were clearly interfering on *PERP* and *COL18A1* REs, whereas the p.Thr193_Tyr194insArg did not. Thus, these results indicate that some P63 mutants are able to interfere in a RE dependent manner.

### The P63 p.Thr193_Tyr194insArg Mutant Shows a Temperature Sensitive Phenotype

Temperature sensitivity, indicative of a conformational flexibility at the protein level, was also investigated. To this aim yeast transformants (pTS-DN wild-type and mutant) were grown at 24, 30, and 36°C before the transactivation measurements. Changing the temperature caused differences in transactivation profile of the wild-type DN-P63$\alpha$ isoform that seemed to be more active at lower temperature, that is, it showed a temperature sensitive (TS) phenotype. With regard to the mutants, p.Thr193_Tyr194insArg was the only one showing a clear TS phenotype on four strains (yLFM-P21–5′, yLFM-MDM2, yLFM-PUMA, and yLFM-BAX) (Supp. Fig. S5).

### The Structural Modeling of P63 p.Gly173Val and p.Thr193_Tyr194insArg Mutants Correlates with their Functional Features

Structure–function relationships were investigated by modeling the *TP63* mutations under study using the recently published P63 DBD structure in complex with 10 bp DNA (PDB ID: 3qym) [Chen et al., 2011]. Taking into account crystal packing, a tetramer of P63 DBD bound to a complete 20 bp RE was observed. The mutated amino acids are at different positions of the P63 structure (Supp. Fig. S6A). R204 (corresponding to R243) is located 13 Å from the DNA at the beginning of Helix-1 (Supp. Fig. S6B); T154 (corresponding to T193) that precedes the observed insertion is in the middle of Sheet-2 ($\beta$2) at 18 Å from the DNA (Supp. Fig. S6C), whereas G134 (corresponding to G173) is in the N-terminus of the DBD, 34 Å apart from the DNA (Supp. Fig. S6D). Moreover, R204 (corresponding to R243) residue is completely buried in the structure with 0 Å$^2$ exposed to water. For this reason, any mutation in that position, including the p.Arg243Trp (R204W) here studied, would require a complete arrangement of the atoms around the new side chain. Arginine 204 (corresponding to R243) is also adjacent to Cysteine 205 (corresponding to C244) that coordinates the zinc atom required for the proper DNA binding by the P63 protein, thus distortions in Helix-1 will affect the zinc-binding site and both residues S272 (corresponding to S311) and R279 corresponding to R318) that directly contact the DNA. On the other hand, mutations at position G134 (G134D: p.Gly173Asp; G134V: p.Gly173Val) and the Arginine insertion between T154 and Y155 residue (insR155: p.Thr193_Tyr194insArg) are expected to be less deleterious because both regions are exposed to solvent, with 96 and 210 Å$^2$, respectively. For example, changes at position 134 are likely to be accommodated with some displacement of Strand-10 ($\beta$10), but without major distortions to the DBD fold. Despite such flexibility, the introduction of a charge, as in the mutant p.Gly173Asp, is likely to have a greater effect than a substitution that preserves the hydrophobic environment, as the mutant p.Gly173Val does. In a similar manner, the insertion of an Arginine between T154 and Y155 (insR155: p.Thr193_Tyr194insArg) is likely to be accommodated in the loop between Sheets $\beta$2 and $\beta$2' without affecting the main fold of the DBD, although the position of K149 (corresponding to K188) that contacts the DNA phosphate backbone might be disturbed. Taken together, the structural data that concern the identified P63 mutants point toward a distinct functional effect for each mutant, as observed in functional assays.

## Discussion

P63 plays key roles in epithelial morphogenesis and cancer progression, with the latter function becoming evident only in adulthood [Melino, 2011]. Pathogenic *TP63* mutations can cause at least five different syndromes (EEC, AEC, ADULT, LMS, and RHS) that exhibit developmental defects [Rinne et al., 2007]. Distinct *TP63* mutational patterns are observed in each syndrome, indicating possible genotype–phenotype correlations, especially in EEC and AEC/RHS syndromes, where mutations are clustered in the DBD, SAM, or TI domains, respectively. Nevertheless, there are examples of the same mutation associated to different clinical syndromes [Avitan-Hersh et al., 2010; Rinne et al., 2007] or intermediate phenotype [Celik et al., 2011; Kier-Swiatecka et al., 2007; Prontera et al.,

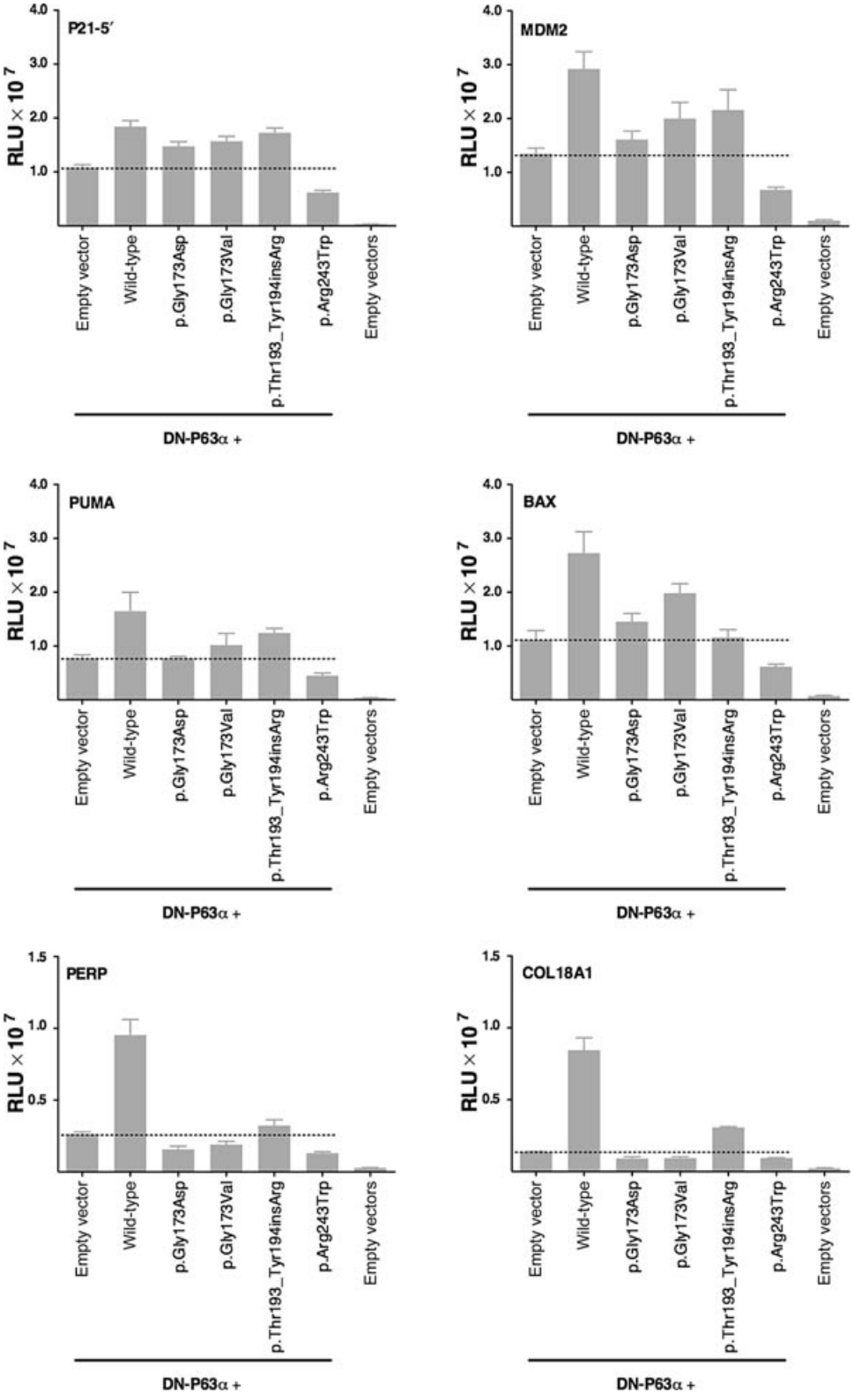


**Figure 6.** Interfering ability of mutant DN-P63$\alpha$ protein over wild-type DN-P63$\alpha$. Wild-type DN-P63$\alpha$ was constitutively expressed under the *ADH1* promoter in yLFM-P21–5′, yLFM-MDM2, yLFM-PUMA, yLFM-BAX, yLFM-PERP, and yLFM-COL18A1 reporter strains, whereas mutant proteins were expressed under the inducible *GAL1,10* promoter. Coexpression of two wild-type alleles in the same experimental condition was also evaluated. High level of mutant P63 protein expression was achieved by adding galactose in the media (8 hr at 0.128% galactose). Interfering mutant proteins (when coexpressed with wild-type) caused a significant reduction in luciferase activity compared with that measured with a single wild-type protein (below dashed line). Presented are the average RLU and standard deviations measured as in the Figure 1. p.Arg243Trp showed interfering ability on all REs ($P < 0.0001$), whereas p.Gly173Asp and p.Gly173Val were interfering only on PERP and COL18A1 ($P < 0.0001$).

2008; Serra et al., 2011]. These findings highlight the complexity of genotype–phenotype correlations across the various *TP63* syndromes, suggesting that different factors could influence the final clinical manifestations associated with a specific *TP63* mutations.

Potential *TP63* modifiers have been identified in genes that predispose to nonsyndromic cleft palate such as *IRF6*, *DLX5*, and *DLX6* [Gritli-Linde, 2010; Kouwenhoven et al., 2010]. Very recently, a differential altered stability of DN-P63 mutants was observed in distinct EDs [Browne et al., 2011], suggesting that factors involved in the modulation of P63 protein stability can also be considered as potential modifiers. The SATB2 protein is another example of modifiers as it is the first P63 binding partner that was demonstrated to differentially influence mutant proteins functionality [Chung et al., 2011]. A possible factor contributing to the variability among the *TP63*-linked EDs might be also a differential transactivation potential of P63 mutant proteins toward target promoters. In this light, a more complete functional analysis of P63 mutants is expected to improve the understanding of the molecular and cellular pathways involved in the *TP63*-associated syndromes. This type of approach was previously adopted for the search of correlations between *TP53* germ-line mutations functionality and clinical manifestations in the Li-Fraumeni syndrome. Using a well-established yeast-based system we showed, for the first time, that functional heterogeneity of P53 germ-line mutant proteins can correlate with clinical variables in associated familial tumors [Monti et al., 2007, 2011].

Here, we described the identification of two *TP63* alleles (p.Gly173Val and p.Thr193_Tyr194insArg) found in association with ADULT and EEC syndromes, respectively, and the functional characterization of the corresponding mutant proteins. The p.Gly173Val amino acid substitution has been also identified in another patient with ADULT phenotype associated with cleft palate [Prontera et al., 2011]. The p.Thr193_Tyr194insArg is a novel EEC-associated *TP63* mutation. Functional analysis was extended to p.Gly173Asp, already reported in ADULT, LMS, and RHS patients, and to p.Arg243Trp, previously identified in patients affected with EEC and ADULT syndromes (Table 1). Extensive functional characterization was achieved using the newly developed format of yeast functional assay [Andreotti et al., 2011], by analyzing transactivation ability and interfering ability of P63 mutants as DN-P63$\alpha$ variant on different P63 REs (associated with *P21*, *MDM2*, *PUMA*, *BAX*, *PERP*, and *COL18A1* genes).

Our results clearly showed heterogeneity in transactivation potential of the different P63 mutant proteins. Moreover, individual mutants have lost the ability to transactivate with some REs, still retaining partial activity with others. For example, the p.Gly173Asp and p.Gly173Val mutants show high residual transactivation potential on *P21, MDM2, PUMA*, and *BAX* targets but complete loss of function on *PERP* and *COL18A1* REs (Figs. 2 and 5, and Supp. Tables S2, S3, and S4). Conversely, the EEC-associated p.Thr193_Tyr194insArg showed a change in transactivation specificity with respect to p.Gly173Asp and p.Gly173Val mutants, being particularly defective toward the *BAX* and the *PERP* REs. A different *TP63* allele corresponding to p.Thr193_Tyr194insPro was previously reported in association with nonsyndromic SHFM4 [van Bokhoven et al., 2001]. Since *BAX* gene expression was reported to be functionally important in cell cycle and apoptosis especially during embryogenesis [Candi et al., 2006], it is tempting to speculate that the clinical feature of hand and foot malformation, common to SHFM4 and our EEC patient, might be associated with a lack or reduction of transactivation ability on specific target genes, including *BAX*. The p.Arg243Trp mutant, on the contrary, was the only one analyzed in this work to show a severe loss of transactivation ability on all REs. The inability of p.Arg243Trp to induce common P63 and P53 targets (*P21*, *MDM2*, and *MASPIN*) as well as some specific P63 targets (*SHH*, *VDR*, and *DHRS3*) was previously observed [Khokhar et al., 2008; Kirschner et al., 2010].

Regarding the interfering ability, the p.Arg243Trp mutant showed also the capacity to interfere over wild-type on all REs (Fig. 6), a feature that can influence the clinical manifestations associated with P63 mutant protein, as *TP63*-associated syndromes are autosomal dominant disorders. p.Arg243Trp appears to have a dual negative effect on the expression of target genes: it is transcriptionally inactive per se but it also inhibits the wild-type protein. Conversely, the p.Gly173Asp and p.Gly173Val mutants are clearly interfering only on *PERP* and *COL18A1* targets. The p.Thr193_Tyr194insArg was the only mutant with no interfering ability.

Consistent with results obtained in yeast, the p.Gly173Asp, p.Gly173Val, and p.Thr193_Tyr194insArg P63 mutants showed transactivation potential also in mammalian cells, whereas p.Arg243Trp did not. Moreover, the p.Gly173Asp, p.Gly173Val, and p.Thr193_Tyr194insArg exhibited a higher transactivation activity with respect to wild-type on some mammalian reporters (MDM2 and P21) (Fig. 3). A similar effect was previously described and associated with the ADULT p.Arg337Gly (R298G) and p.Arg337Gln (R298Q) mutations (DN-P63$\gamma$ isoform) [Rinne et al., 2006]. This feature was not highlighted by the yeast assay, possibly because of the different organization of the target sequence in yeast (RE) with respect to the mammalian reporter, and/or to the existence of modulators that could influence the functions of mutant P63 proteins in mammalian cells.

Can the functional results be interpreted in light of the structural localization and the type of modification (amino acid substitution or insertion) that affect P63 protein? The differences in the solvent accessible surface area observed in the crystal structure of P63 DBD bound to DNA for each of the mutated positions provide the main structural explanation to the functional effects described in this study (Supp. Fig. S6). As modeled, modifications (substitution or insertion) at 134 (corresponding to codon 173) and 155 (corresponding to codon 193) positions are more likely expected to protrude toward the surface of the protein without affecting the structure as much as the mutation in the buried residue R204 (corresponding to codon 243). The loss of transactivation observed for amino acid substitutions at position 134 (corresponding to codon 173) might be because of its closeness to the N-terminus of DBD that is involved in the assembly of the tetramerization interface and helps to recruit other proteins of the transcription machinery [Ethayathulla et al., 2012]. Furthermore, position 134 (corresponding to codon 173) is located at the C-terminus of the Proline-rich domain, whose structural integrity is important to transcriptional and apoptosis-inducing function of P63 protein [Helton et al., 2008].

In the absence of experimental results on mutant functionality and protein crystal structure, software, comprising the POLYPHEN-2 (http://genetics.bwh.harvard.edu/pph2/), is often used to predict the effect of a single amino acid substitution on the function of the protein under study. Using this tool, we observed that the p.Gly173Asp, p.Gly173Val, and p.Arg243Trp mutations were all indistinctly predicted to be "probably damaging" with an equal score of 1. Our results clearly showed the functional heterogeneity of the P63 mutants, which may be underestimated using this predictive tool.

Taken together, our results show that the P63 p.Gly173Val and p.Thr193_Tyr194insArg mutants, found in patients with ADULT and EEC phenotype, respectively, have different residual transactivation potential and interfering ability as a function of RE sequences. Thus, difference in clinical manifestations might be linked to a

different effect of these mutant proteins on the transactivation of P63 target genes.

EEC and ADULT syndromes following clinical guidelines have overlapping features including ED, limb abnormalities and dental changes but differentiating signs such as orofacial clefting, which are considered absent in ADULT syndrome. In particular, cleft lip and/or palate are critical clues for differential diagnosis. In this light it was unexpected to find a clinical report of a patient carrier of the p.Gly173Val germ-line mutation that was classified as affected by ADULT syndrome but showing cleft palate [Prontera et al., 2011]. Interestingly, also the ADULT patient identified in the present study with p.Gly173Val mutation showed narrow palate with bifid uvula as a mild clinical manifestation of the orofacial-clefting. On the other hand, the p.Arg243Trp mutation, previously considered mainly EEC-specific [Rinne et al., 2007], was recently found also in association with an ADULT patient [Avitan-Hersh et al., 2010]. These observations point to the lack of unequivocal clinical criteria that clearly distinguish these syndromes from each other, suggesting the requirement of combining these conditions in a unique clinical entity [Prontera et al., 2011].

The considerable overlap in the clinical features among *TP63*-associated syndrome, suggests the need for an integration of the clinical classification with some functional parameters regarding P63 mutant proteins. The functional classification might take into account different features of the P63 mutant such as: (1) transactivation ability on P63 target genes that are involved in specific developmental pathways, (2) interfering ability that involve the interaction among normal and mutated P63 isoforms, and (3) mechanisms of gain of functions. These features could be evaluated with our genetically well-defined yeast-based assay, being adaptable to high-throughput screening on a matrix of factors that can influence the P63 network including P63 protein levels, *TP63* mutations, nature of the REs and presence of P63-interacting proteins.

In this work, we demonstrated for the first time that the REs associated with *PERP* and *COL18A1* genes (Fig. 4) are transactivated better by DN-P63$\alpha$ than TA-P63$\alpha$. The results obtained with *PERP* are in apparent contrast with data by Ihrie et al. (2005) that show a preferential activation by TA-P63$\alpha$. This might be because of the fact that the reporter used for the cell-based assay contains, besides the RE found in the first intron of *PERP* gene and cloned in yeast, three additional p53 REs belonging to the promoter region. It is tempting to speculate that DN-P63$\alpha$ acts as transcriptional modulator of *PERP* gene exclusively through the intronic RE we studied. Interestingly, DN-P63$\alpha$ was even more active than P53 with this RE. Considering also the higher P53 protein steady-state levels in yeast with respect to P63 protein and, conversely, the comparable level of DN-P63$\alpha$ and TA-P63$\alpha$ isoforms (Supp. Fig. S2), the *PERP* and *COL18A1* RE sequences we studied can be considered as DN-P63$\alpha$-specific targets even thought they represent only two of several P63 target genes required for the development or maintenance of ectoderm-derived tissues. In this light it is interesting to note that *Perp*$^{-/-}$ mice display some features of human EDs syndromes, including inflamed skin, fur with a patchy appearance and broken or absent nails but not defects concerning craniofacial and limb development [Ihrie et al., 2006]. Also the absence of type XVIII collagen in mice (encoded by *COL18A1* gene) results in a variety of eyes, skin, heart and kidney abnormalities [Utriainen et al., 2004] that might be related to the malformations in specific organs and systems observed in EDs patients. Interestingly, the p.Gly173Asp and p.Gly173Val mutants seemed to be as much defective as the p.Arg243Trp toward REs derived from *PERP* and *COL18A1*, genes closely related to the clinical manifestations of the *TP63*-associated syndromes.

Lastly, no specific cures are available for *TP63*-associated EDs but recent studies are exploring the potential to pharmacologically or genetically modulate P63 protein in tissues [Rökaeus et al., 2010], which could have important therapeutic implications to reduce the burden of progressive symptoms associated with *TP63*-linked EDs, including skin erosion in AEC patients. In this perspective, our preliminary data in yeast-based functional assay underscoring the TS phenotype of P63 mutants (e.g., p.Thr193_Tyr194insArg) (Supp. Fig. S5) could be useful for the future design and testing of compounds able to reactivate P63 mutant in patients that harbor that specific allele.

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*Disclosure statement*: The authors declare no conflict of interest.

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