

The C-terminus of p63 contains multiple regulatory elements with different functions

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The transcription factor p63 is expressed as at least six different isoforms, of which two have been assigned critical biological roles within ectodermal development and skin stem cell biology on the one hand and supervision of the genetic stability of oocytes on the other hand. These two isoforms contain a C-terminal inhibitory domain that negatively regulates their transcriptional activity. This inhibitory domain contains two individual components: one that uses an internal binding mechanism to interact with and mask the transactivation domain and one that is based on sumoylation. We have carried out an extensive alanine scanning study to identify critical regions within the inhibitory domain. These experiments show that a stretch of ~13 amino acids is crucial for the binding function. Further, investigation of transcriptional activity and the intracellular level of mutants that cannot be sumoylated suggests that sumoylation reduces the concentration of p63. We therefore propose that the inhibitory function of the C-terminal domain is in part due to direct inhibition of the transcriptional activity of the protein and in part due to indirect inhibition by controlling the concentration of p63.

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The transcription factor p63 shows high sequence homology to the tumor suppressor p53.^{1–4} Although the potential role of p63 in tumor suppression is still debated,^{5–8} it has been assigned crucial roles in epithelial stem cell biology and in surveillance of the genetic stability of oocytes.^{9–14} These two distinct biological functions are executed by the two isoforms of p63 that possess a non-truncated form of the C-terminus. TAp63 α , which represents the full gene product, is expressed in oocytes,¹² while the Δ Np63 α isoform, which lacks the N-terminal transactivation domain, is highly expressed in the basal layer of epithelial tissues.⁹ The four additional isoforms of p63, which have not been ascribed specific functions to date, are created through the combination of the two different N-termini (TA and Δ N) with additional C-terminal splice variants consummating the six combinations: TAp63 α , TAp63 β , TAp63 γ , Δ Np63 α , Δ Np63 β , and Δ Np63 γ .² The six isoforms show very different transcriptional activities in cell culture experiments.^{2,15} Owing to the presence of the TA domain, TAp63 β and TAp63 γ are constitutively active on prototypical p53 promoters such as *p21*, *mdm2*, and *bax*, but TAp63 α is rendered inactive by an auto-inhibitory sequence

located within the α C-terminus.^{2,15,16} This same C-terminal inhibitory element endows the Δ Np63 α isoform with a strong dominant-negative ability against TAp63 γ .²

In a previous study, we had mapped the auto-inhibitory domain (transcriptional inhibitory domain (TID)) in TAp63 α to the last 70 amino acids.¹⁵ Further biochemical analysis suggested that the TID interacts with the N-terminal transactivation domain, thus forming a closed and inactive conformation of the transcription factor. These experiments also revealed that the TID contains two different subdomains, both of which differentially contribute to the observed inhibition. Whereas the 45 N-terminal amino acids of the TID are involved in binding and masking of the TA domain, the 25 C-terminal amino acids do not contribute to binding, but are responsible for ~50% of the overall inhibitory effect of the TID. Sequence analysis showed that this C-terminal subdomain contains a classical sumoylation sequence and sumoylation in the C-terminus was shown for both TAp63 α ^{17,18} and the highly homologous protein TAp73 α .¹⁹

To gain further insight into the different mechanisms of auto-inhibition and of potential relief of auto-inhibition within

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Abbreviations: TID, transcriptional inhibitory domain; TA, transactivation; SAM, sterile alpha motif; SAOS, sarcoma osteogenic; GST, Glutathione S-Transferase; GAPDH, Glyceraldehyde 3 phosphate dehydrogenase; SUMO, Small Ubiquitin-like Modifier

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the p63 C-terminus, we have carried out an extensive mutational analysis of the TID using biochemical experiments. The results presented here show that a stretch of ~13 highly conserved amino acids is responsible for the auto-inhibiting intramolecular interaction. In addition, our results suggest that sumoylation inhibits the transcriptional activity of TAp63 α indirectly by reducing its intracellular concentration.

Results

The TAp63 α C-terminus contains two distinct inhibitory elements. Using C-terminal deletion mutagenesis we had previously shown that the intramolecular inhibition in TAp63 α can be disrupted by removing the last 35 amino acids.¹⁵ The aim of this study was to use site-directed mutagenesis between the last helix of the SAM domain and the C-terminus of TAp63 α (amino acids 563–641) to identify amino acids that are important for the inhibitory mechanism. In our previous study, we had shown a correlation between the binding of deletion mutants to an external TI domain in GST pulldown assays and the transcriptional activity of these mutants on the p21 promoter in SAOS2 cells.¹⁵ Here we use the same assays in combination with investigation of the intracellular concentration to obtain a more detailed picture of the function of the TI domain.

To identify crucial amino acids within the inhibitory domain, we generated a library of 16 mutant TAp63 α clones in which

Table 1 Alanine scanning library covering from the end of the SAM domain to the C-terminus of TAp63 α

Clone	Amino acids	Abbreviation
1	W559A/K560A/G561A	WKG > AAA
2	H565A/R566A/Q567A	HRQ > AAA
3	D560A/F561A/S562A	DFS > AAA
4	H576A/L577A/L578A	HLL > AAA
5	S582A/G583A	SG > AA
6	S588A/V589A/G590A	SVG > AAA
7	S592A/E593A/T594A	SET > AAA
8	G596A/E597A/R598A	GER > AAA
9	I600A/D601	ID > AAA
10	F605A/T606A/L607A	FTL > AAA
11	T610A/I611A/S612A	TIS > AAA
12	S612A/F613A/P614A	SFP > AAA
13	P614A/P615A/R616A	PPR > AAA
14	E618A/W619A/N620A	EWN > AAA
15	F624A/D625A/M626A	FDM > AAA
16	S628A/R629A/R630A	SRR > AAA
17	Q634A/R635A/I636A	QRI > AAA
18	K637L	K > L
19	W559A/K560A/G561A/K637L	WKG > AAA/K > L
20	H565A/R566A/Q567A/K637L	HRQ > AAA/K > L
21	D560A/F561A/S562A/K637L	DFS > AAA/K > L
22	H576A/L577A/L578A/K637L	HLL > AAA/K > L
23	S582A/G583A/K637L	SG > AA/K > L
24	S588A/V589A/G590A/K637L	SVG > AAA/K > L
25	S592A/E593A/T594A/K637L	SET > AAA/K > L
26	G596A/E597A/R598A/K637L	GER > AAA/K > L
27	I600A/D601/K637L	ID > AAA/K > L
28	F605A/T606A/L607A/K637L	FTL > AAA/K > L
29	T610A/I611A/S612A/K637L	TIS > AAA/K > L
30	S612A/F613A/P614A/K637L	SFP > AAA/K > L
31	P614A/P615A/R616A/K637L	PPR > AAA/K > L
32	E618A/W619A/N620A/K637L	EWN > AAA/K > L
33	F624A/D625A/M626A/K637L	FDM > AAA/K > L
34	S628A/R629A/R630A/K637L	SRR > AAA/K > L

three consecutive amino acids were simultaneously mutated to alanine (Table 1, clones 1–17). The mutant clones will be referenced according to the abbreviations listed in Table 1; mutant clone 1, which corresponds to the triple mutant W559A/K560A/G561A, will be referred to as TAp63 α ^{WKG > AAA}. Once constructed, the library of mutants was assayed for transcriptional activity on the p21 promoter (Figure 1a).

An increase to 30–40% of the activity of the fully active isoform TAp63 γ was observed for mutants between amino acids F605 and R616 as well as for mutant TAp63 α ^{QRI > AAA}. This last mutant affects the sumoylation site in p63 and it had been shown that its removal increases the transcriptional activity to 40–50% of TAp63 γ .^{15,18} Previously reported pull-down experiments had demonstrated that the last 25 amino acids, including the sumoylation site, are not important for binding of the TA domain but act independently.¹⁵ Owing to the increase in activity both by mutations in the region 605–616 and in the sumoylation site, a second library was constructed by adding a K637L mutation to each clone that removes the acceptor lysine of the sumoylation site (Table 1, clones 18–34). In this second library the mutants TAp63 α ^{FTL > AAA} and TAp63 α ^{TIS > AAA}, which previously increased moderately, increased to an excess of 100% activity. In addition, TAp63 α ^{SFP > AAA} and TAp63 α ^{PPR > AAA} showed a significantly increased activity. Outside of these clones, the

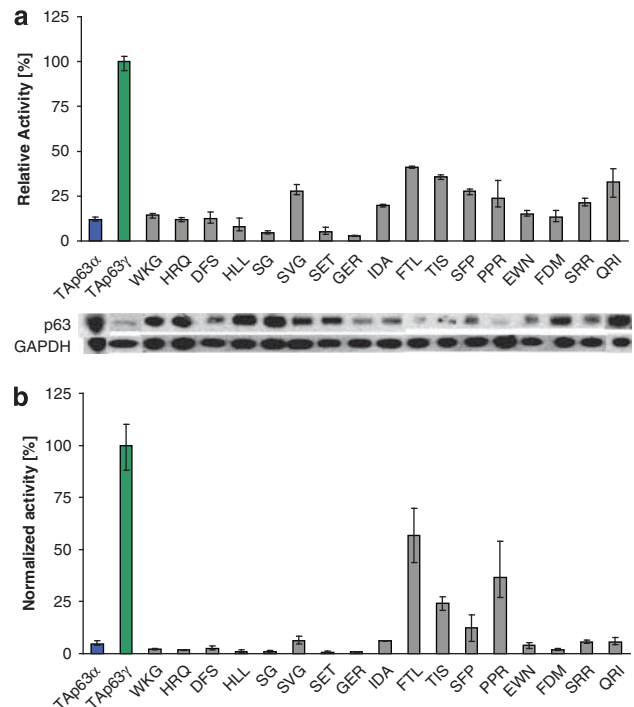


Figure 1 Investigation of the relative transcriptional activity of several triple-alanine mutants of TAp63 α in SAOS-2 cells on a p21 promoter. (a) Each measurement was carried out in triplicate. The activity of the wild-type TAp63 α and TAp63 γ is shown in blue and green, respectively. The activity of TAp63 α is set to 100%. (b) Determination of the intracellular protein level of the different p63 mutants using an N-terminal myc-tag. For standardization western blots of GAPDH are used. The transcriptional activity measured in (a) is normalized with the western blots to eliminate the influence of the intracellular protein level. Only mutants in a stretch between F605 and R616 show an increased activity

activity of most of the remaining constructs bearing the K637L mutation increased to approximately 30–40% of the activity level of TAp63 γ , which is an effect attributed to the mutation in the sumoylation site (Figure 2a), as a similar increase in

activity was also observed when K637L was the only mutation in TAp63 α .

Similar results can be obtained with mutant TAp63 α forms lacking a stretch of amino acids between Q402–T495 (called QP domain owing to its high content of glutamines and prolines, and which harbors a helix that interacts with the oligomerization domain²⁰). Although TAp63 α Δ QP is even less active than TAp63 α , removal of the sumoylation site leads to a slight increase and mutation of F605/T606/L607 to AAA leads to a strong increase in transcriptional activity, suggesting that the entire QP domain is not necessary for the inhibitory mechanism (Supplementary Figure S1).

An alignment of TAp63 α proteins from diverse species revealed several conserved amino acids in the C-terminus, with the most significant cluster spanning 10 amino acids. With only slight exceptions, the amino acids traversed by the two active TI mutants (TAp63 α ^{FTL>AAA}, TAp63 α ^{TIS>AAA}) project directly onto this conserved 10-amino-acid sequence (Figure 3). The mutagenesis data, supported by sequence alignments, thus define the core of the TI domain as the 10-amino-acid stretch from R604 to F613 (RFTLRQTISF), with some less conserved C-terminal flanking regions (PPR in human p63) as additionally functionally important.

The results reported above provide further evidence that the TID contains two independent regulatory functions, of which one is based on sumoylation in the C-terminus of the TID and the other presumably on binding and masking of the TA domain, and each of these being responsible for approximately 50% of the observed inhibition within TAp63 α . The separate nature of these two functions is further demonstrated by sequence comparison of the TI domains of vertebrates and invertebrates (Figure 3): In addition to the amino acids constituting the core of the TID, the IKEE sumoylation site at the C-terminus is highly conserved in vertebrate sequences. Surprisingly, however, the sumoylation site is missing in invertebrate sequences. Further sequence analysis revealed an (I/V)KEE sequence N-terminal to the SAM domain in all sequences that miss the C-terminal sumoylation site. Investigation of the corresponding TAp63 α protein of *Mytilus trossulus* (mt-TAp63 α) suggested that the elements that control the transcriptional activity in mammalian

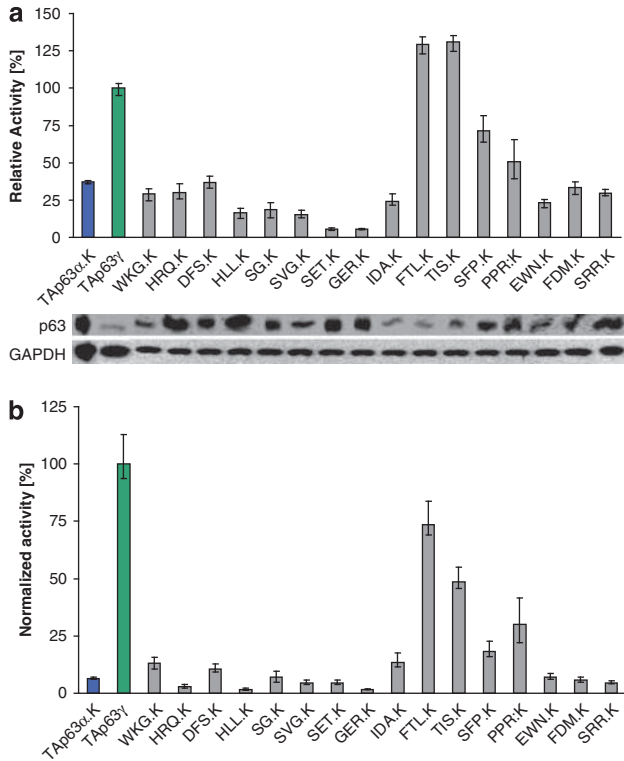


Figure 2 (a) The measurement of the transcriptional activity as shown in Figure 1 was repeated with all triple alanine mutants carrying an additional K637L mutation to remove the sumoylation site. Each measurement was carried out in triplicate. (b) The intracellular protein level was determined by western blot analysis of p63 and normalized using western blots of GAPDH. The transcriptional activity of the different mutants was then normalized for intracellular concentration. The results are very similar to those obtained in the series with an intact sumoylation site (Figure 1), which suggests that the sumoylation is not directly involved in controlling the activity of p63, but acts indirectly by influencing the intracellular concentration

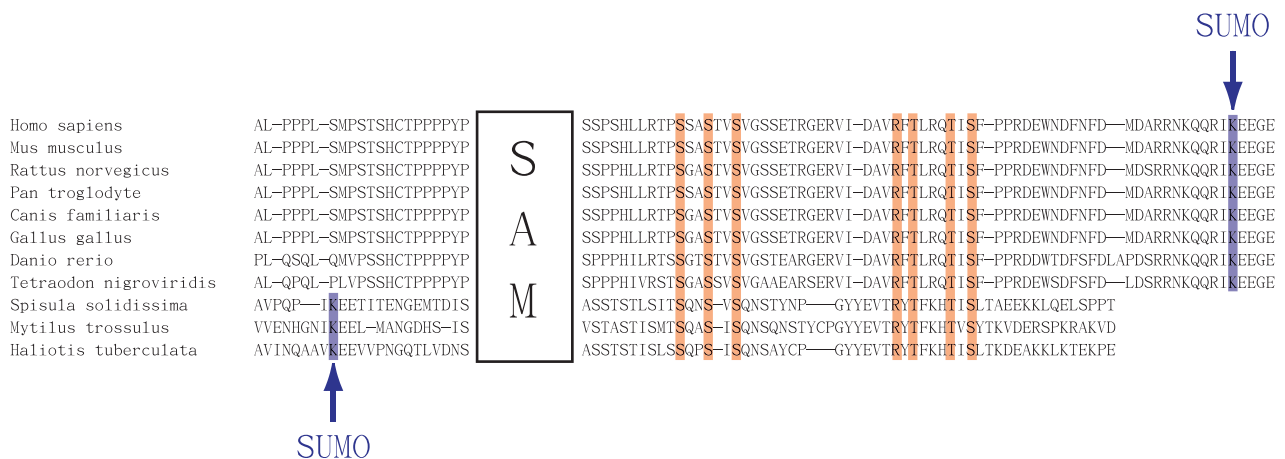


Figure 3 Sequence alignment of C-terminal p63 sequences of various vertebrate and invertebrate species. Sequences N-terminal to the SAM domain to the end of the protein are shown. The sequences of the SAM domains themselves are not shown. Strictly conserved amino acids are labeled red. The conserved KEE sumoylation motif is labeled in blue. This sumoylation sequence is located N-terminal to the SAM domain in invertebrate species

TAp63 α are indeed conserved in this invertebrate protein (Supplementary Figure S2).

Sumoylation regulates the intracellular concentration.

Sumoylation can have different effects on proteins and can influence stabilization, destabilization, or intracellular localization.^{21–25} In the case of p63, sumoylation has been reported to destabilize the protein.¹⁸ To investigate whether the increase in transcriptional activity of the p63 mutants with a mutated sumoylation site might reflect changes in concentration rather than release of an inhibitory effect, we determined the concentration of the different p63 mutant forms in the lysates of the samples tested for transcriptional activity by western blotting. Most alanine scanning mutants showed a high abundance similar to wild-type TAp63 α . Only mutations in the conserved 10-amino-acid stretch and in the sumoylation site significantly altered the observed protein concentration. The two mutants TAp63 α ^{F_{TL}>AAA} and TAp63 α ^{T_{IS}>AAA}, and TAp63 α ^{PPR>AAA}, a mutant directly C-terminal to the conserved stretch, decreased in concentration, whereas the sumoylation mutant TAp63 α ^{QRI>AAA} increased in concentration (Figure 1a). This observation is consistent with earlier investigations that had revealed that transcriptionally active TAp63 γ shows a significantly reduced intracellular concentration compared with transcriptionally impaired mutants.²⁶

As further analysis, the concentrations of TAp63 α were quantified by densitometry to produce a protein concentration-based normalization factor for the transcriptional activity of each clone. The activity data for the experimental series with both mutant libraries were re-processed with these normalization factors, thus removing a concentration-dependent influence on the measured activity (Figures 1b and 2b, Supplementary Figures S3 and S4). For TAp63 α the activity increase observed with disruption of the sumoylation site was directly proportional to the increase in protein concentration, resulting in no net increase in activity. Normalization of the activities for all mutants in both series produced similar results, showing that mutations in a stretch between F605 and R616 led to significantly increased transcriptional activity that reaches ~70% of the activity of TAp63 γ . In contrast, the activity for all other mutants remained low, with some showing a slight increase relative to the activity of wild-type TAp63 α . Sumoylation, therefore, affects the intracellular protein concentration, but does not decrease the intrinsic transactivation potential of wild-type TAp63 α or truly ‘inhibit’ the transcription factor.

As a control experiment to investigate the relationship between sumoylation and intracellular protein concentration, we used siRNAs to knock down the SUMO-E2 enzyme Ubc9. This protein is a key enzyme for sumoylation reactions^{27–29} and knockdown of Ubc9 was shown to abolish sumoylation of p63.¹⁷ Knockdown of Ubc9 using siRNAs led to an increase in the transcriptional activity of TAp63 α by 60% as compared with cells transfected with mock siRNAs (Supplementary Figure S5). No effect could be observed with TAp63 γ . Western blot analysis confirmed that the increase in the transcriptional activity of TAp63 α was proportional to an increase in the intracellular concentration of the protein. After normalization with respect to protein concentration, both

siRNA- and mock siRNA-treated cells showed nearly the same transcriptional activity (Supplementary Figure S5). The effect of knocking down Ubc9 on the p63 concentration and activity was less than the effect of mutating the SUMO acceptor site. This difference can be explained with the only partial knockdown of Ubc9 (approximately 70%). Nevertheless, the overall trend of these experiments confirms our interpretation that sumoylation influences the intracellular concentration.

The amino acids of the TI core are solely responsible for a sequence-specific intramolecular interaction.

Previous experiments had suggested that the last 25 amino acids of the TI domain, containing the sumoylation site, are not involved in binding and masking of the TA domain.¹⁵ Contribution to binding had been evaluated by pulldown assays with an external GST–TID fusion protein. In this assay, isoforms with an unmasked TA domain bind to this external TID. The TA domain of wild-type TAp63 α possesses an intact intramolecular interaction with its TI domain and therefore does not bind efficiently to the external GST–TID (Figure 4). TAp63 γ , in contrast, possesses an available TA domain with which it binds efficiently to the GST–TID resin.

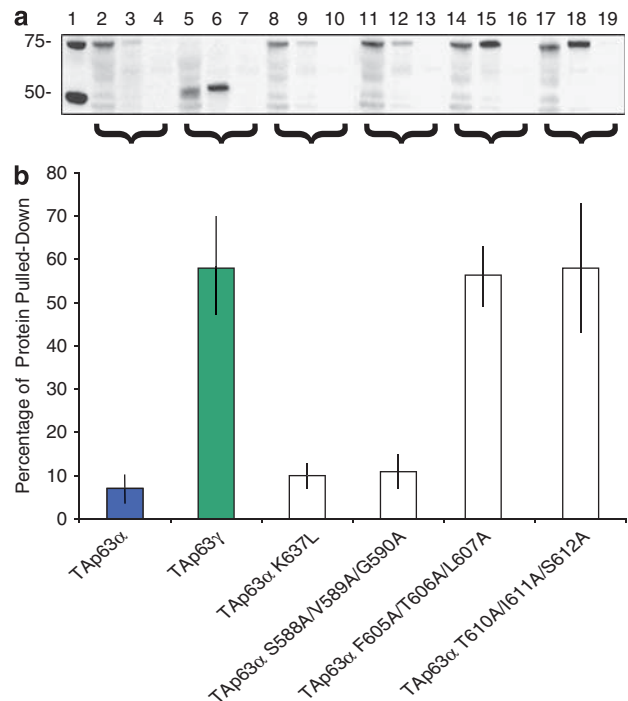


Figure 4 Pulldown assays of different p63 forms with GST–TID fusion protein bound to a glutathione column. An antibody against the N-terminal myc-tag of the constructs was used for the experiments. (a) Raw data with the first lane showing the amount of protein used for the pulldown experiments, second lane the amount of protein bound to the column and third lane the amount of protein bound to a column loaded with a mutated form of the GST–TID fusion protein (GST–TID^{mut} (F605A/T606A/L607A/T610A/I611A/S612A)). The first lane on the left of the graph shows a molecular weight marker. (b) Quantitative analysis of the pulldown experiments shown in (a). TAp63 γ and TAp63 α mutants with a mutated TI domain that show high transcriptional activity are bound efficiently to the GST–TID fusion protein. TAp63 α mutants that are not transcriptionally active do not bind to the external TI domain. Pulldown experiments with a mutated TID fused to GST do not result in detectable amounts of protein. Each experiment was carried out in triplicate

Mutation of the sumoylation site (TAp63 α ^{K637L}) did not affect the amount of protein bound. The intramolecular interaction of TAp63 α ^{K637L} therefore remained intact and the amino acids that constitute the sumoylation site do not contribute to the intramolecular interaction. TAp63 α ^{SVG>AAA}, as an additional negative control, also bound to the GST–TID resin with an efficiency similar to TAp63 α ^{wt}. Mutation of amino acids in the core TI domain (TAp63 α ^{FTL>AAA} and TAp63 α ^{TIS>AAA}) led to a 5–6-fold increase of protein bound to the resin, which is a result comparable to TAp63 γ . As an additional control to demonstrate that the interaction between GST–TID and TAp63 γ , TAp63 α ^{FTL>AAA} and TAp63 α ^{TIS>AAA} is sequence specific, the pull-down assays were also performed with a mutated version of the TID, GST–TID^{mut} (F605A/T606A/L607A/T610A/I611A/S612A) (Figure 4). The amount of protein bound to GST–TID^{mut} was undetectable in all samples and thus confirmed that the C-terminal sequence between A602 and S612 of TAP63 α is responsible for the intramolecular interaction.

Mutations in the core of the TI domain affect the dominant-negative behavior of Δ Np63 α . The initial characterization of the transcriptional activity of the different p63 isoforms had revealed that Δ Np63 α shows a strong dominant-negative behavior towards TAp63 γ on the p21 promoter in SAOS2 cells.² As Δ Np63 α is only weakly dominant negative and in low concentrations even enhances the transcriptional activity of TAp63 γ , the inhibitory function of Δ Np63 α could be assigned to the C-terminus of the α -isoform. Although the biological relevance of this dominant-negative behavior is currently not clear, this effect provides another interesting assay to further investigate the behavior of mutations in the TI domain. We, therefore, created a mutant of Δ Np63 α with the TA-binding site mutated (Δ Np63 α ^{FTL>AAA}) and tested this mutant in titration assays against TAp63 γ . The plasmid DNAs for Δ Np63 α constructs were mixed with TAp63 γ at ratios of 1:4, 1:2, 1:1, 2:1 and 4:1, transfected into SAOS-2 cells, and assayed on the p21 promoter. The transcriptional activity was normalized with the help of western blot analysis. As reliable western blot signals could be obtained only for concentrations of the Δ N forms above a ratio of 1:1, only these data are shown in Figure 5. The unnormalized raw data and the corresponding western blots are shown in Supplementary Figure S6.

Wild-type Δ Np63 α potently inhibited TAp63 γ , and at a plasmid ratio of 1:2 (Δ Np63 α :TAp63 γ) over 90% of the activity was removed. Mutation of the TID (Δ Np63 α ^{FTL>AAA}) resulted in a slightly reduced ability to inhibit the transcriptional activity of TAp63 γ , reaching approximately 10% activity at the highest expression level of Δ Np63 α ^{FTL>AAA}. Separate investigation of the transcriptional activities of Δ Np63 α and Δ Np63 α ^{FTL>AAA} on the p21 promoter in SAOS2 cells showed that both are transcriptionally inactive (Supplementary Figure S7; it should, however, also be mentioned that Δ Np63 α has been shown to be transcriptionally active on other promoters). As both Δ N forms accumulate to much higher intracellular levels than TAp63 γ , the inhibitory effect of both proteins could at least in part be due to competition for promoter binding sites. To distinguish between the direct inhibition through the α

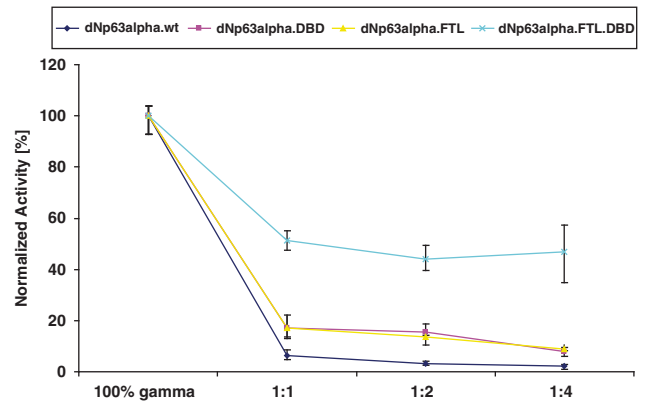


Figure 5 Inhibition of the transcriptional activity of TAp63 γ by increasing amounts of different Δ Np63 α mutants. The relative amount of the Δ Np63 α forms was varied from 4:1 (TAp63 γ : Δ Np63 α) to 1:4. The ratios are shown on the x-axis. The leftmost point corresponds to the transcriptional activity of TAp63 γ alone, which is set to 100%. dNp63alpha.wt: wild type; dNp63alpha.FTL: Δ Np63 α (F605A/T606A/L607A); dNp63alpha.DBD: Δ Np63 α mutant R279H incapable of binding to DNA; dNp63alpha.FTLDBD: R279H mutant of Δ Np63 α (F605A/T606A/L607A). The transcriptional activity was normalized relative to the intracellular concentration of Δ Np63 α with the help of western blot analysis. Each measurement was carried out in triplicate

C-terminus and the indirect inhibition through competition for DNA binding sites, we repeated these experiments with DNA-binding domain mutants of both Δ N forms (Figure 5). Whereas the DNA-binding mutant of Δ Np63 α still resulted in approximately 90% inhibition of the TAp63 γ activity, the inhibitory potential of the DNA-binding mutant of Δ Np63 α ^{FTL>AAA} was significantly reduced, levelling off at 50% transcriptional activity at the highest protein level. These results showed that the inhibitory effect of Δ Np63 α is to a large extent due to the α C-terminus and mutation of the FTL stretch significantly reduces the inhibitory effect of the TID. Still 50% inhibitory potential of the DNA-binding mutant of Δ Np63 α ^{FTL>AAA} remained. However, at high protein levels of Δ Np63 α ^{FTL>AAA} most of the TAp63 γ will form mixed tetramers with Δ Np63 α ^{FTL>AAA}. As dimers are formed co-translationally,³⁰ most likely the dominant form is a hetero-tetramer consisting of TAp63 γ and Δ Np63 α ^{FTL>AAA} homo-dimers. Owing to the DNA-binding mutation of Δ Np63 α ^{FTL>AAA}, the mixed tetramers effectively behave as dimers. Mutation of the tetramerization domain creating TAp63 γ dimers results in 50% transcriptional activity (Supplementary Figure S8). Interestingly, a similar 50% decrease in transcriptional activity was reported for p53 dimers.³¹

Discussion

Two of the six p63 isoforms contain an extended C-terminus with two distinct regulatory elements that combine to auto-inhibit TAp63 α and impart a dominant-negative ability to Δ Np63 α . Although the biological relevance of the dominant-negative function of Δ Np63 α towards TAp63 γ is unclear, the recent discovery that TAp63 α has a major role in protecting the female germ line¹² suggests a clear biological function of the TID for TAp63 α . During oogenesis cells arrest in the prophase of meiosis I (dictyate arrest) until they are recruited for ovulation. This period, which can last up to several

decades in humans, is characterized by strong expression of TAp63 α in the arrested cells. Experiments with mice showed that in these arrested oocytes TAp63 α is transcriptionally inhibited. The binding affinity to DNA increased \sim 20-fold upon exposure to ionizing radiation, which was accompanied by p63 phosphorylation and death of the oocytes. Combining these mouse studies with our biochemical investigation of the TID suggests that TAp63 α is kept in a transcriptionally inhibited conformation by the TID. Essential for keeping the protein in this conformation is a short stretch of highly conserved amino acids that inhibits the transcriptional activity of TAp63 α by directly binding to other p63 domains. The existence of several highly conserved threonines and serines in the TID suggests that phosphorylation might be a possible mechanism of relief of inhibition. A simple mutation of threonine 606 to glutamate, however, did not result in a significant increase in the transcriptional activity of TAp63 α , showing either that this mutation is not a good mimetic of phosphorylation or that the activation mechanism is more complex. Obviously, to answer this question, the identification of kinases that phosphorylate the C-terminus of p63 would be essential.

In our previous investigation, we had shown direct interaction between the bacterially expressed TA and the TI domains, suggesting a masking of the transactivation domain as the basis for transcriptional inhibition.¹⁵ The data reported by McKeon and colleagues¹² show that phosphorylation increases the DNA-binding affinity, suggesting that transcriptional repression might be caused by inhibiting several functions of the molecule. Such a multiple inhibitory mechanism could be realized by forming a closed conformation of the protein in which – in addition to the TA–TI interaction – several other domain–domain interactions occur.

Our investigation has revealed that the TI domain harbors a second inhibitory function, which is based on sumoylation of K637. Sumoylation, however, does not seem to be directly involved in suppressing the intrinsic transcriptional activity of p63, but acts indirectly by controlling the intracellular level. Several investigations have shown that endogenous Δ Np63 α becomes sumoylated in several different cell lines and if transfected in SAOS2 cells.¹⁸ Furthermore, direct interaction of TAp63 α and Δ Np63 α with Ubc9 was demonstrated in transiently transfected human embryonic retina 911 cells.¹⁷ Association between p63 and SUMO-1 could be completely abolished by a K637E mutation and the same mutation led to a dramatic increase in the transactivation of TAp63 α on the RGC promoter. Similar to p63, sumoylation has also been observed for the highly homologous protein p73 and it was shown that sumoylation regulates the intracellular concentration.¹⁹ All together, these different investigations by different research groups using different cell lines and promoters strongly suggest that sumoylation is involved in regulating the activity of p63. Our new study shows that at least in SAOS2 cells the influence of sumoylation on the transcriptional activity is due to regulation of the intracellular concentration while the inhibitory effect of the TID that is based on direct binding is not affected.

Our current and previous studies highlight an interesting correlation between high transcriptional activity and low intracellular concentration, which has also been observed by others.²⁶ This observation suggests that additional

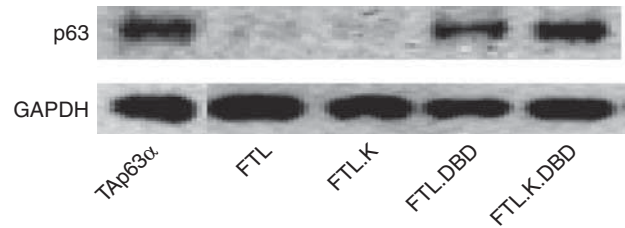


Figure 6 Comparison of the intracellular concentrations of the active triple-alanine mutants TAp63 α ^{FTL>AAA} and TAp63 α ^{FTL/IKEE>AAA/ILEE} with the concentrations of their corresponding DBD mutants. TAp63 α is shown as a control. Each measurement was carried out in triplicate

mechanisms exist to decrease the concentration of transcriptionally active forms without affecting transcriptionally repressed forms. The existence of such different mechanisms is further supported by the observation that the concentration of the transcriptionally active forms TAp63 α ^{FTL/IKEE>AAA/ILEE} and TAp63 α ^{FTL>AAA} that are barely detectable in western blots strongly increases when they are rendered transcriptionally inactive by an additional mutation in the DNA-binding domain (Figure 6). These results also suggest that the formation of a transcriptionally active complex on the DNA is important for the degradation of p63. It is tempting to speculate that the intracellular concentration of transcriptionally inactive forms is mainly regulated by sumoylation whereas that of transcriptionally active forms is dominated by ubiquitination by Itch, MDM2 or other E3 ligases that have been shown to interact with p63.^{32–38} Differences in the mechanism of degradation between transcriptionally inactive and active forms have been observed for other proteins such as the estrogen receptor hER α as well.³⁹

Although no point mutations in the core of the TID have been identified to date in human syndromes, mutations just N-terminal to this stretch (R598L, D601V) in patients with AEC syndrome have been reported.⁴⁰ In addition, patients with split-hand split-foot malformation type 4 who have nonsense mutations that create protein forms truncated by only a few amino acids (eight in Q634X and three in E639X) have been described,⁴¹ which leads to the destruction of the sumoylation site. Several explanations for the surprising absence of missense mutations in the core of the TID exist. First, such mutations could be lethal. Second, a syndrome would arise only if the mutations would create either a dominant-negative or a gain-of-function effect. If the main effect of mutations in the TID is a loss of the dominant-negative behavior toward other transcriptionally active forms, expression from the second, non-mutated allele might be enough to sustain the biological function of Δ Np63 α during development and maintenance of epithelial tissue. Finally, it is also possible that transcriptional inhibition is only important for TAp63 α , which monitors the genetic stability of oocytes, and not for Δ Np63 α 's role in maintaining epithelial stem cells. The effect of mutations on p63's role in oocyte quality control, however, has just begun to be investigated.

Materials and Methods

Plasmids. The mammalian expression vectors encoding N-terminally tagged TAp63 α , Δ Np63 α , and TAp63 γ in pCDNA3.1 and the pGL3 vector were obtained from Frank McKeon (Department of Cell Biology, Harvard University). The fusion of

GST and the TI domain amino acids 569–616 in pGEX-6p-2 (GE Healthcare, München, Germany) has been described earlier.¹⁵ All mutants were generated using the Quickchange site-directed mutagenesis protocol. The second-generation library, clones 18–32, was generated by adding the K637L mutation to clones 1–16 (Table 1).

Transactivation assays. All transactivation experiments were performed in SAOS-2 cells using the Promega (Mannheim, Germany) Dual-Glo Luciferase reporter assay. For the transfection assays, a construct with a single copy of the p21 promoter obtained from human genomic DNA was cloned into the pGL3 vector. Cells were obtained from the ATCC and maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum at 37°C under an atmosphere of 5% CO₂. Cells were transfected with 133 ng DNA per plasmid (Effectene, Qiagen, Hilden, Germany) in 96-well plates, grown for 24 h and assayed for *Renilla* and *Firefly* luciferase activities. For titration assays, the concentration of TAp63 α was kept constant at 66 ng per assay and the amount of Δ Np63 α plasmid was varied from a ratio of 1:4 to 4:1. The total amount of DNA transfected was kept constant with empty pCDNA3.1. The transcriptional activity was normalized with the help of western blots analysis. As reliable western blot signals could be obtained only for concentrations of the Δ N forms above a ratio of 1:1, only these data are shown in the unnormalized raw data and the corresponding western blots are shown in Supplementary Figure S6. A total of three independent experiments were performed, each in triplicate. The linear relationship between intracellular protein concentration and transcriptional activity, which was important for normalization of the transcriptional activity, was tested by comparing the transcriptional activity and concentration obtained with the plasmid amount used in the experiments with the results from experiments with half or twice the amount of plasmid transfected. For both TAp63 α and TAp63 α ^{FTL>AAA}, these tests showed a linear relationship between the amount of plasmid, the intracellular concentration, and the transcriptional activity (Supplementary Figure S9). Error margins are given as the normalized difference between the maximum value and the average value. In the case of the concentration-normalized transcriptional activities, the error resulting from the determination of the concentration was added to the error obtained from the determination of the transcriptional activity, weighted by the ratio of normalized activity and relative activity.

Immunohistochemistry using an anti-myc antibody coupled to a Cy3-fluorophore (Sigma, Hamburg, Germany) showed that all mutants tested, as well as wild-type TAp63 α and TAp63 γ , were exclusively nuclear (Supplementary Figure S10).

Western blotting analysis. As further analysis, the concentrations of TAp63 α and GAPDH were quantified by densitometry to produce a protein concentration-based normalization factor for the transcriptional activity of each clone. Normalizing this concentration-based normalization factor for transfection efficiency using the *Renilla* data led to the identification of the exactly same stretch of amino acids, albeit with different intensities as compared with Figures 1b and 2b. The activity data for the experimental series with both mutant libraries were re-processed with these normalization factors, thus removing a concentration-dependent influence on the measured activity (Figures 1b and 2b, Supplementary Figures S3 and S4). SAOS-2 cells were transfected in the same manner as for the transactivation assays with different p63-containing pCDNA3.1 plasmids (Effectene, Qiagen). Cells were harvested after 24 h, resuspended, and lysed in M-PER reagent (Pierce, Schwerte, Germany) for 5 min. After addition of 15 μ l of 4 \times SDS buffer containing 20% β -mercaptoethanol, samples were heated for 5 min at 95°C. A volume of 7 μ l of the lysate was loaded onto a 17-well NuPage (Invitrogen, Karlsruhe, Germany) 4–12% Bis-Tris (SDS) polyacrylamide gel. Samples were transferred to a PVDF membrane (Immobilon-P 0.45 μ M) (Millipore, Schwalbach, Germany) using an XCell II blot module. The blot was blocked in 5% skim milk and probed with mouse anti-myc antibody clone 4A6 (Millipore), anti-ubc-9 rabbit polyclonal antibody (Cell Signaling, Frankfurt, Germany), or anti-GAPDH (Chemicon International). Detection was performed using an HRP goat anti-mouse IgG peroxidase conjugate (Sigma). The blots were quantitated using the Biometra BioDocAnalyze 2.0 software (Biometra, Göttingen, Germany). Each experiment was repeated three times.

siRNA experiments. SAOS2 cells were transfected either with siRNA targeting Ubc9 or with siRNA with a randomized sequence as a control experiment. The following sequence was used to target Ubc-9 (reference sequence: NM_003345) 5'-AAGGGATTGGTTGGCAAGAA-3'. The unmodified siRNAs were purchased from Qiagen and transfected using RNAifect transfection reagent (Qiagen) at a final concentration of 60 nM following the manufacturer's

instructions. At 24 h after siRNA transfection, cells were transfected with p63-coding plasmids as mentioned before. Cells were harvested after 24 h and the transcriptional activity and protein concentration were determined as described above.

Protein–protein interaction assays. All TI domain mutants described above were expressed in an *in vitro* rabbit reticulocyte lysate transcription/translation system and pulldown experiments were performed using a bacterially expressed and purified *E. coli* GST–TID fusion protein bound to a glutathione-based affinity resin. The GST fusion protein system (GE Healthcare) was used to detect protein–protein interactions between p63 proteins and immobilized domains. The GST–TID protein used as bait was made by cloning p63 amino acids 569–616 into the pGEX-6p-2 vector. An additional His-tag was added at the C-terminus. The GST–TID protein was expressed in BL21 (DE3) cells. The expressed protein was purified over a 10-ml Ni-IDA Fast Flow column using a gradient from 50 to 400 mM imidazole. The eluent was diluted with 30% glycerol, aliquoted into 1 ml fractions and frozen at –80°C for later use at a concentration of approximately 0.5 mg/ml. Pulldown assays were performed in centrifugal filtration units using a Durapore membrane with a pore size of 0.65 μ m (Millipore UFC30DV00) and a table top centrifuge operating at 1000 r.p.m. In all, 250 μ l of the GST–TID protein was incubated for 15 min with 50 μ l of Glutathione resin pre-equilibrated in Pull-Down Buffer A (180 mM NaCl, 0.1% Tween-20, 50 mM Tris pH 8.0). The resin was washed four times with 400 μ l of Pull-Down Buffer A to remove excess GST–TID. The p63 proteins used as inputs for the assay were generated in an *in vitro* transcription/translation rabbit reticulocyte lysate system (Promega) with 1 μ g of template DNA. A volume of 5 μ l of the reaction mixture was removed for use as an input control. The remaining 55 μ l was incubated with the GST–TID resin for 3 h at 4°C and then centrifuged. The resin was washed 4 times with 400 μ l of Pull-Down Buffer A and the bound proteins were eluted with 2 \times 20 μ l of 2 \times hot SDS-PAGE buffer. Analysis after SDS-PAGE was performed by western blotting as described above.

Conflict of interest

The authors declare no conflict of interest.

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