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Analysis of the oligomeric state and transactivation potential of TAp73α

LM Luh[1,5], S Kehrloesser[1,5], GB Deutsch[1], J Gebel[1], D Coutandin[1], B Schäfer[1], M Agostini[2], G Melino[2,3,4] and V Dötsch*[,1]

The proteins p73 and p63 are members of the p53 protein family and are involved in important developmental processes. Their high sequence identity with the tumor suppressor p53 has suggested that they act as tumor suppressors as well. While p63 has a crucial role in the maintenance of epithelial stem cells and in the quality control of oocytes without a clear role as a tumor suppressor, p73′s tumor suppressor activity is well documented. In a recent study we have shown that the transcriptional activity of TAp63α, the isoform responsible for the quality control in oocytes, is regulated by its oligomeric state. The protein forms an inactive, dimeric and compact conformation in resting oocytes, while the detection of DNA damage leads to the formation of an active, tetrameric and open conformation. p73 shows a high sequence identity to p63, including those domains that are crucial in stabilizing its inactive state, thus suggesting that p73's activity might be regulated by its oligomeric state as well. Here, we have investigated the oligomeric state of TAp73α by size exclusion chromatography and detailed domain interaction mapping, and show that in contrast to p63, TAp73α is a constitutive open tetramer. However, its transactivation potential depends on the cellular background and the promoter context. These results imply that the regulation of p73′s transcriptional activity might be more closely related to p53 than to p63.
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Detailed functional and structural investigations of p63 and p73, two homologs of the tumor suppressor protein p53, have suggested that p63 is the most ancient member of this protein family, from which the other members have evolved.[1–3] This idea was particularly fueled by the discovery that invertebrates like *Caenorhabditis elegans* have p53-like proteins[4–6] that are more closely related to p63 than to p53.[7–9] In *C. elegans* the Cep-1 protein does not act as a tumor suppressor but is expressed in germ cells where it serves as a quality control factor.[10] This function is also preserved in mammals where p63 is highly expressed in female oocytes.[11] Detection of DNA damage leads to the activation of p63, which results in the elimination of these compromised oocytes.[12] The high expression level of p63 in resting, non compromised oocytes suggested that its transcriptional activity must be inhibited, and only becomes activated upon the detection of DNA damage. In a recent study we had started to investigate the mechanism that keeps p63 inactive.[13] In a series of experiments we could show that TAp63α, the p63 isoform that is expressed in oocytes,[14] is kept in a closed conformation by a network of domain–domain interactions. Furthermore, while it has been shown that the active form of p53 is a tetramer that is created by a highly conserved oligomerization domain (OD), the inactive TAp63α conformation in oocytes is a dimer. This dimeric inactive conformation is maintained by an interaction network including the N-terminal transactivation (TA) domain, the C-terminal transactivation inhibitory (TI) domain and the central OD. Phosphorylation triggers the opening of this closed conformation, enabling the formation of active tetramers that initiate apoptosis.

While p63 mainly seems to be involved in the development of stratified epithelial tissues[15] and in the quality control of oocytes[11] and sperm cells,[16] the role of p73 as a tumor suppressor is better supported.[17,18] Like p63, p73 exists in multiple isoforms[19,20] that are created by the combination of at least two different promoters with different C-terminal splicing variants. Of these isoforms those containing the full-length N-terminal TA domain (TA-isoforms) act pro-apoptotically and those that lack this domain (ΔN-isoforms) have anti-apoptotic effects.[21,22] The complete knockout of all pro- and anti-apoptotic isoforms of p73 in mice displayed severe developmental impairments,[23] including hippocampal dysgenesis, hydrocephalus, chronic infections and inflammation, as well as abnormalities in pheromone-sensory pathways. Surprisingly, however, an increased susceptibility for tumorigenesis was not observed in these mice. In contrast, the selective inactivation of the TA-isoforms increased the susceptibility to induced and spontaneous tumor formation,[18] demonstrating that the TA-isoforms act as tumor suppressors. These studies further revealed that TAp73 knockout mice are infertile due to low quality of oocytes, which show spindle abnormalities leading to multinucleated blastomeres.[17]

[1]Institute of Biophysical Chemistry and Center for Biomolecular Magnetic Resonance, Goethe University, Frankfurt, Germany; [2]Medical Research Council, Toxicology Unit, Leicester University, Leicester, UK; [3]Department of Molecular Biomedical Research, Flanders Institute for Biotechnology, Ghent, Belgium and [4]Biochemistry Laboratory, Instituto Dermopatico dell'Immacolata, Instituto di Ricovero e Cura a Carattere Scientifico and University of Rome 'Tor Vergata', Rome, Italy
*Corresponding author: V Dötsch, Institute of Biophysical Chemistry and Center for Biomolecular Magnetic Resonance, Goethe University, Max-von-Laue-Str. 9, Frankfurt 60438, Germany. Tel: 06979 829 631; Fax: 06979 829 632; E-mail: vdoetsch@em.uni-frankfurt.de
[5]These authors contributed equally to this work.
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The activity of p73 is regulated by a multitude of different factors that include E3 ligases of the ubiquitination system, transcriptional coactivators, kinases, phosphatases, acetyltransferases, prolyl isomerases and other factors.[24] Regulation of the activity of members of the p53 protein family gets further complicated by the formation of oligomers that can include isoforms lacking a transactivation domain exerting a dominant-negative effect on the respective TA-isoform.[14,25,26] Furthermore, even mixed hetero-oligomers between p63 and p73 are possible, making oligomerization an important regulatory mechanism. p73 shows a high sequence homology to p63 and also contains a C-terminal domain with high sequence identity to the TI domain of p63, suggesting that the transcriptional activity of p73 might also be regulated by forming closed and inactive conformations. To address the question of how the activity of p73 is regulated, we have investigated the conformational state and transcriptional activity of TAp73α, the largest isoform.

Results

p73 forms an open tetramer. The closed conformation of TAp63α in oocytes is stabilized by interactions of the C-terminal inhibitory TI domain and the N-terminal TA domain with the central OD.[13] All three domains are present in TAp73α and show high sequence identities of ~55% (OD), ~22% (TA) and ~45% (TI) (Figures 1a and b). To investigate whether TAp73α forms a closed and compact conformation similar to TAp63α, we expressed TAp73α in rabbit reticulocyte lysate and applied size exclusion chromatography (SEC). Fractions containing TAp73α were identified by western blotting and the resulting elution profile was compared with results obtained for TAp63α and ΔNp63α. As we have shown previously, TAp63α elutes at a retention volume corresponding to a closed dimeric conformation, whereas tetrameric isoforms like ΔNp63α or TAp63γ elute significantly earlier. As can be seen in Figure 2a TAp73α elutes at a volume that significantly differs from the elution volume of TAp63α, suggesting that TAp73α forms open tetramers. ΔNp73α and TAp73β elute as well at volumes corresponding to open tetramers, which is comparable with p63 isoforms lacking one of the terminal domains (Figure 2). To validate whether the results obtained with rabbit reticulocyte lysate-expressed TAp73α represent a native state, we performed SEC analyses with the same set of isoforms expressed in Saos-2 cells. Supplementary Figure 1 shows that expression in a cellular environment results in elution profiles virtually identical to the *in vitro* derived ones for all the tested isoforms. For TAp63α these findings are also in agreement with data of endogenous protein obtained from mice ovaries. These results further support the interpretation that TAp73α is – in contrast to TAp63α – a constitutive open tetramer.

Experiments with p63 have shown that open, tetrameric isoforms show interaction in GST pull-down experiments with either an external TA or an external TI domain. For the interaction with GST–TA, the p63 isoform must contain an accessible TI domain (ΔNp63α) and for interaction with GST–

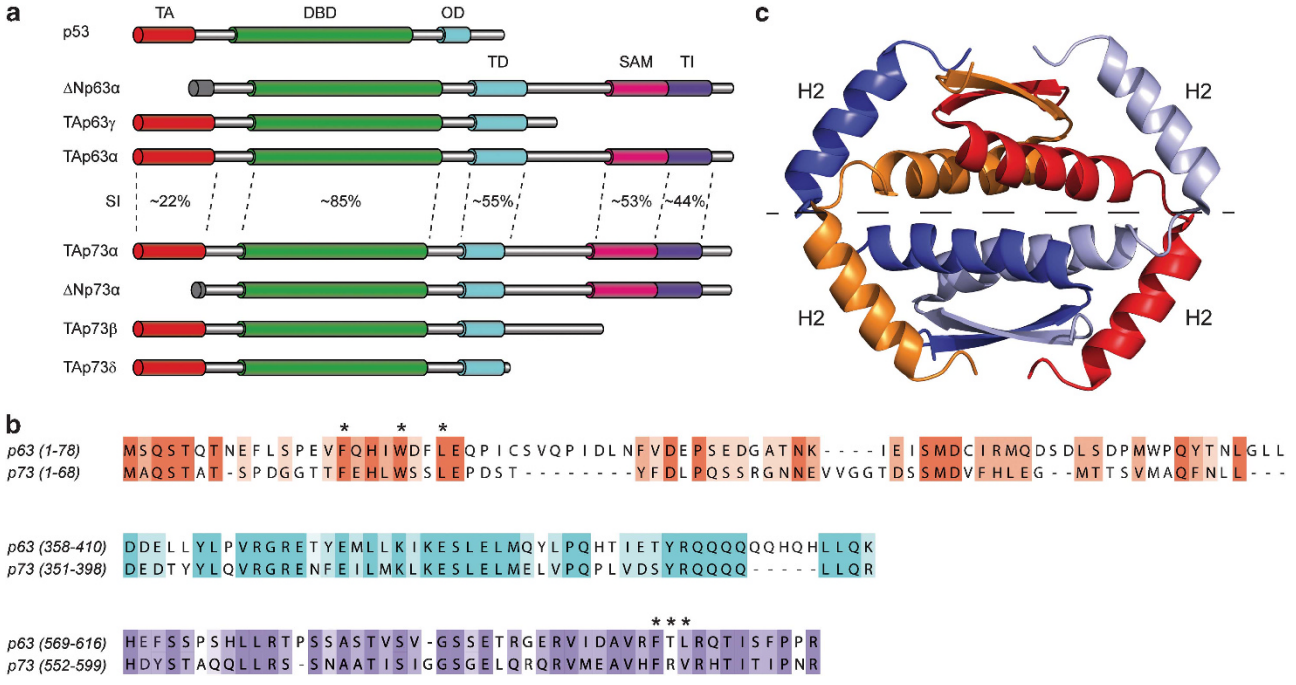

Figure 1 Domain structure and sequence alignment of p73 and p63. (**a**) The domain structure of p53 is compared with the domain structures of several p63- and p73-isoforms used in this study. Corresponding domains have the same color. The degree of sequence identity between the p63 and p73 domains is indicated. (**b**) Sequence alignment of the TA, the tetramerization (TD) and the TI domains of p63 and p73 (coloring according to domain structure in (**a**)). Color intensity indicates the degree of homology. Amino acids mutated in this study are highlighted by asterisks. (**c**) Structure of the TD of p73. The tetramer consists of a dimer of dimers. The dark and light blue monomers form one dimer and the red and yellow monomers the second dimer. In contrast to the OD of p53, the TDs of p63 and of p73 have an additional C-terminal helix (H2) that reaches across the tetramerization interface, depicted by the dashed line

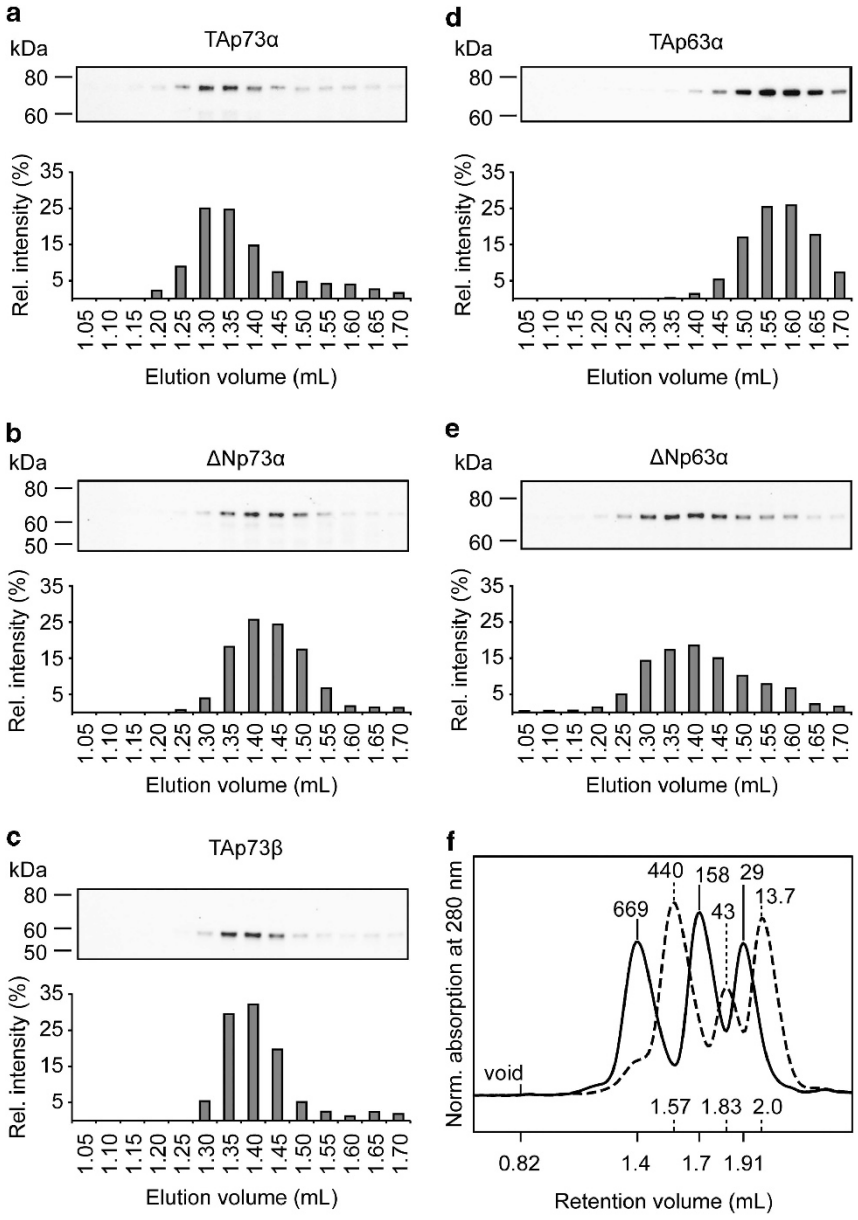

Figure 2 SEC analysis of p73 (**a**–**c**) and p63 (**d** and **e**) isoforms expressed in rabbit reticulocyte lysate. Lysates were applied to a Superose 6 PC 3.2/30 column. For each isoform the western blot analysis of the individual fractions (given in mL of elution volume underneath the bar diagram) and a bar diagram representing the relative intensity of the western blot signals are shown. The sum of the intensity of all individual fractions is set to 100%. (**f**) Calibration of Superose 6 PC 3.2/30 column using thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa) and ribonuclease A (13.7 kDa). Corresponding molecular weights, the respective elution volumes of calibration proteins and the void volume are indicated

TI, an accessible TA domain is a prerequisite (TAp63γ).[25,27] In contrast, the closed, dimeric conformation of TAp63α does neither react with an external TA nor with an external TI domain, suggesting that its TA and TI domains already interact intramolecularly with each other, probably mediated by the OD.[13] In the open tetrameric state of TAp73α, both the TA and the TI domains should be readily accessible for interactions in pull-down experiments. In these experiments, TAp73α indeed binds to an external TI domain, similar to TAp73β that lacks the inhibitory TI domain (Figure 3). ΔNp73α can also bind to an external TI domain through TI–TI interactions as we have seen with ΔNp63α as well, but this pull-down is significantly weaker than the pull-down with the other two isoforms. This additional TI–TI domain interaction explains the higher pull-down efficiency of TAp73α relative to TAp73β. In pull-down experiments with an external TA domain, TAp73α and ΔNp73α also strongly interact while TAp73β displays virtually no pull-down due to the lack of a TI domain.

To further characterize the interaction of the TI domain with the TA and potentially other domains in TAp63α, we had used alanine scanning to identify mutations that would activate the

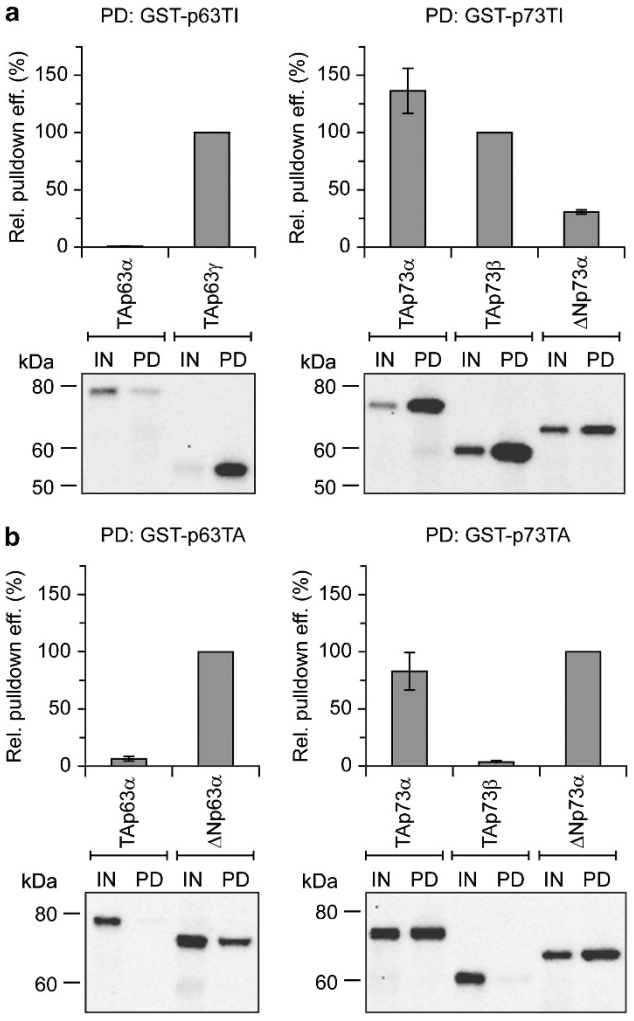

Figure 3 Pull-down experiments with external TA and TI domains confirm the open state of TAp73α. For each pull-down experiment the input signal (IN) and the pull-down signal (PD) of the western blot analysis are shown. Pull-down efficiencies as indicated by the bar diagrams are normalized to either TAp63γ or TAp73β in case of TI-pull-downs or to ΔNα isoforms for the TA-pull-downs. The external TA or TI domains were expressed as GST-fusion proteins and immobilized on glutathione sepharose beads. Panel (**a**) shows experiments with the external TI domains, while (**b**) displays experiments with the external TA domains

protein by releasing the inhibitory interaction and allowing the formation of an open and tetrameric state.[25] In this alanine scan we had identified three amino acids F605, T606 and L607, which when simultaneously mutated to alanine lead to a transcriptionally active and tetrameric form of the protein.[25] The compact dimeric state of TAp63α is further stabilized by interaction of the N-terminal TA domain with the OD. Mutating the three amino acids F16, W20 and L23 to alanine disrupts this interaction, also leading to the formation of an open and tetrameric state. To investigate the importance of the respective residues in TAp73α, we also performed pull-down experiments with corresponding mutants. The control pull-down experiments with p63 showed the expected results, as both the FWL > AAA and FTL > AAA mutants abrogated the TA–TI interaction, although both mutants display an open tetrameric conformation (Supplementary Figure 2).

Although the F15, W19 and L22 motif within the TA domain is conserved, the FTL motif in the TI domain of p63 corresponds to a FRV sequence in p73 (Figure 1b). Mutating either of both sites to triple alanine had identical effects compared with p63. The FWL > AAA mutation reduces the TI-pull-down efficiency to a level observed for ΔNp73α, leaving only the TI–TI interactions intact, while the FRV > AAA mutation abrogated the interaction with an external TA domain (Supplementary Figure 2).

These data support the model that TAp73α forms an open and tetrameric conformation. They, however, also demonstrate that the TA and TI domains of TAp73α can in principle interact with each other as well. Why they do not interact in an intramolecular manner similar to the domains in TAp63α, but only in the intermolecular setting of a pull-down experiment is not quite clear. However, it is a well-known phenomenon that interactions that cannot be observed with isolated molecules in solution can be detected in pull-down experiments when one of the partners is immobilized on a surface. This phenomenon is probably responsible for the observed interaction as well.

The open conformation of TAp73α leads to the formation of heterotetramers with p63. Structural investigations of the ODs of both p73 and p63 have revealed that they differ from the structure of the OD of p53 by the presence of an additional C-terminal helix (H2, see Figure 1c).[28–30] The OD itself consists of a dimer of dimers, with a dimerization interface through which two monomers form dimers and a tetramerization interface through which two dimers interact with each other[31] (Figure 1c). Helix H2 reaches across the tetramerization interface and stabilizes the tetramer. Interaction studies of the isolated ODs of p63, p73 and p53 have revealed that p63 and p73 do not interact with p53. Interestingly, however, p63 and p73 interact strongly with each other, with a tetramer consisting of a p63 dimer and a p73 dimer being thermodynamically the most stable species.[28] If TAp73α adopts an open tetrameric conformation, it should be able to form heterotetramers with open forms of p63 such as ΔNp63α, but not with the closed conformation of TAp63α. We tested this hypothesis by co-transfection of different p63 and p73 isoforms and mutants in Saos-2 cells and subsequent coimmunoprecipitation experiments. The results shown in Figure 4 demonstrate that precipitation of TAp63α is very inefficient with any of the p73 isoforms, consistent with its closed and dimeric conformation. In contrast, TAp73α is able to form heterotetramers with all the open and tetrameric p63 isoforms (ΔNp63α and TAp63γ), and with the FTL > AAA mutant. We obtained virtually identical results with ΔNp73α. To validate that this hetero-oligomerization is mediated by specific interaction via the tetramerization interface, we used the TAp63αMI mutant. It has been shown that the M374Q and I378R mutations destroy the tetramerization interface resulting in the formation of an open dimer with accessible TA and TI domains[13], and that the equivalent mutations in p53 also result in the formation of a dimer.[32] Coimmunoprecipitations (co-IPs) with this mutant show very low co-IP efficiency, suggesting that both the TA and TI domains are dispensable for hetero-oligomerization and support previous findings that p73 and p63 can form stable and specific heterotetramers.[33]

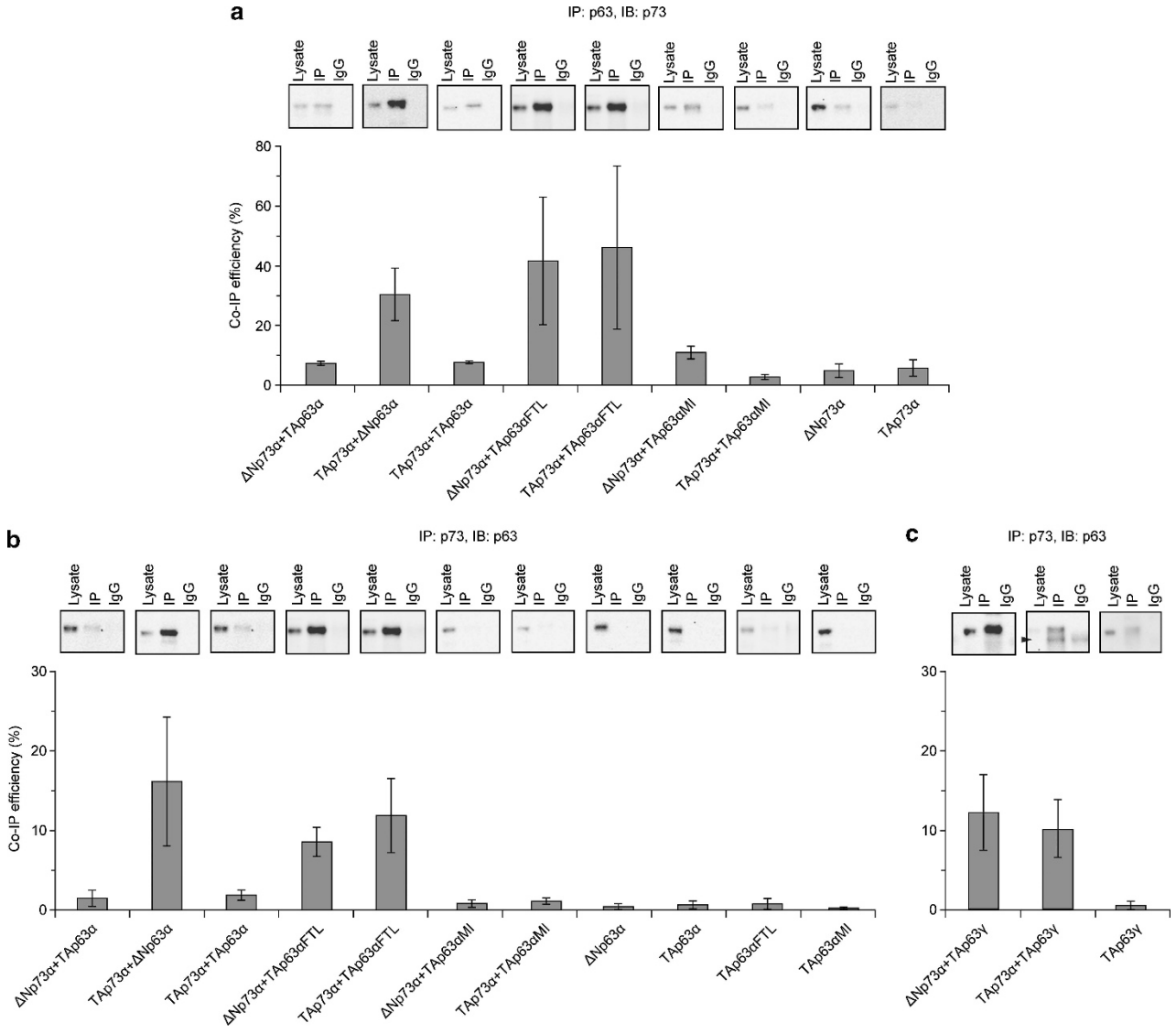

Figure 4 TAp73α forms heterotetramers with tetrameric p63 isoforms and mutants. Different p63 and p73 isoforms or mutants were coexpressed in Saos-2 cells and subsequently coimmunoprecipitated using either (**a**) anti-p63 antibodies (H-129) for precipitation and anti-p73 antibodies (ER-15) for detection or (**b**) vice versa. (**c**) TAp63γ was detected with a different anti-p63 antibody (4A4) due to the lack of the H-129 antibody epitope (resulting IgG heavy chain background is indicated by the arrowhead). Normal IgG was used for the negative controls. The bar diagrams show the efficiency of each coimmunoprecipitation relative to the respective input signal (lysate). TAp63αMI harbors two point mutations in the TD (M374Q and I378R), which leads to an open, yet dimeric conformation, which is not capable to tetramerize. Coimmunoprecipitation experiments with TAp63αMI were therefore used to prove specific heterooligomerization via the TD

Domains within TAp73α do not form an interaction network. The closed and inactive conformation of TAp63α is stabilized by a network of domain–domain interactions involving the TA, TI and OD. Using NMR spectroscopy we had detected specific TA–OD interactions. These results had suggested that in the dimeric state the stabilizing interaction of helix H2 with the core OD is blocked by the presence of the TA. Similar NMR experiments to identify the binding site of the p63 TI domain could unfortunately not be performed due to the high aggregation propensity of this domain. On the basis of indirect, mutation-derived results, however, we were able to suggest a model, in which the TI domain binds to and thereby blocks the tetramerization interface. To test whether similar domain interactions with the OD (the p53-like core domain without the additional helix H2) can occur in TAp73α, we performed analogous NMR titration experiments with peptides representing either the primary transactivation domain (Asp10 to Asp25), the secondary transactivation domain (Asp46 to Met57) or the TI peptide (Glu584 to Arg599). In contrast to p63 no chemical shift perturbations could be detected, indicating that neither the C-terminal nor the N-terminal domain is interacting with the central OD (Supplementary Figure 3). NMR titrations with the full tetramerization domain containing the additional helix H2 showed, as expected, the same result. All NMR-based interaction studies, therefore, further support the model that TAp73α forms an open, tetrameric state.

TAp73α is transcriptionally active. Originally, the inhibitory effect of the TI domain has been identified in transactivation assays performed on the p21 promoter in transiently transfected Saos-2 cells.[27] In these experiments TAp63γ reached a transcriptional activity of more than 60% compared with p53, while TAp63α reached less than 10%.[14] Our detailed analysis of the conformation of TAp63α has revealed that its activity is downregulated both by the formation of a dimeric state, which reduces the DNA binding affinity ~20 fold[13] and by inhibiting the interaction of the transcriptional machinery with the N-terminal TA domain, which is masked by the above mentioned TA–TI and TA–OD interactions.[13] To test the transcriptional activity of TAp73α, we performed transactivation assays with the Bax promoter construct in SK-N-AS cells, a p73-deficient neuroblastoma cell line. In Figure 5 the transcriptional activities of p53 and several p63 isoforms are compared with TAp73α, ΔNp73α, which lacks the N-terminal TA domain, and with the two shorter TA-isoforms TAp73β and TAp73δ, both lacking the TI domain. In these experiments TAp73α showed a similar transcriptional activity as TAp63γ, which was significantly more active than TAp63α. Mutating the three important amino acids F15, W19 and L22 to alanine in the N-TA domain reduced the transcriptional level to that of ΔNp73α, while mutating the amino acids F588, R589 and V590 in the TI domain had only a slight affect, increasing the transcriptional activity to the level of TAp73β. These results show that TAp73α not only adopts an open, tetrameric conformation but is also transcriptionally significant and more active than TAp63α.

Discussion

The current model of the evolution of the p53 protein family suggests that p63 is the most ancient protein[8,34] and that the original function of p63 was the quality control of germ cells.[11]

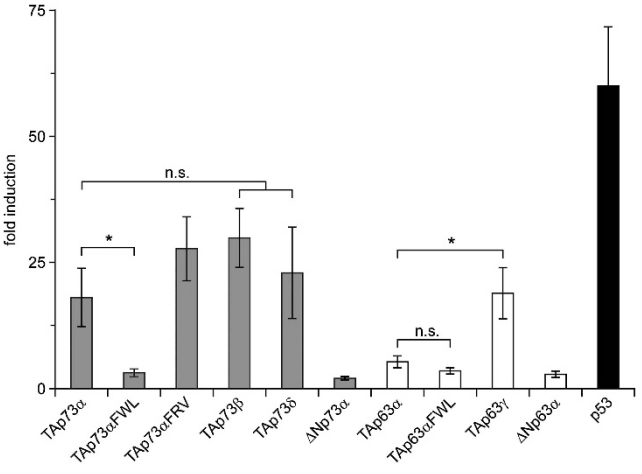

Figure 5 TAp73α is transcriptionally active. The transcriptional activity of different p73 isoforms and mutants were measured on the Bax promoter in transiently transfected SK-N-AS cells and compared with the transcriptional activities of different p63 isoforms and mutants as well as to p53. Bar diagrams indicate fold change of promoter induction compared with empty vector control. Significant differences are indicated with an asterisk and non significant differences are labeled as n.s.

In higher organisms that developed renewable tissues, p63 evolved into a factor additionally involved in the control of epithelial stem cells.[35–37] Furthermore, these organisms developed two more family members, p73 and p53. Both of them are involved in developmental functions,[3,23,38] the main function of p53, however, is that of a tumor suppressor. This tumor suppressor function of p53 has been studied intensively and based on the high sequence identity to p63 and p73, the latter have been suggested to have similar properties.[39] Although the tumor suppressor function of p63 is still debated[40] and its role in tumorigenesis seems to be more that of an oncogene (overexpression of ΔNp63α in squamous cell carcinomas[41]), the tumor suppressor function of p73 is well established.[17,42] This suggests that p73 is, despite its high sequence identity with p63, functionally closer related to p53. This functional resemblance is potentially also reflected in similar mechanisms of regulation (Figure 6). The activity of p53 is mainly regulated via its intracellular concentration. In non stressed cells, p53 is kept at a very low level through the action of E3 ligases, mainly MDM2 and MDMX.[43] Detection of cellular stress results in the stabilization of p53. This switch in stabilization as well as the interaction with other proteins such as the transcriptional machinery of the cell are themselves regulated by post-translational modifications.[44] In contrast, TAp63α accumulates to high concentrations in resting oocytes where it is kept in a closed and dimeric conformation. Important for the formation of this inhibited conformation are domain–domain contacts, in particular between the N-terminal TA domain, the C-terminal inhibitory domain and the central OD. All three domains exist in TAp73α and show high sequence identity to the corresponding p63 domains. Despite this striking sequence homology, we could not detect interactions between the TI or TA domains with the OD using NMR titration experiments. Other experiments have shown that p73 adopts an open and tetrameric conformation. Surprisingly, our pull-down assays have revealed that the N-terminal TA domain and the C-terminal TI domain can interact with the corresponding domain in trans. Why this interaction does not occur within a p73 oligomer leading to a closed and inactive conformation is not obvious. We can only speculate that the formation of such a closed conformation requires additional interaction with the OD, which is possible in p63 but not in p73. Such an interpretation is supported by experiments with the M374Q and I378R double mutant in the OD of p63. This mutant cannot form tetramers due to the destruction of the tetramerization interface. SEC experiments have revealed that it exists as an open dimer, which in pull-down experiments can interact with the external TA and TI domains.[13] An effective intramolecular interaction, however, does not seem possible within this p63 dimer mutant. As we could not detect any interaction of the TA and the TI domain of p73 with its OD, no stable interaction that would also inhibit tetramerization seems possible for p73. The slight reduction in the transcriptional activity of TAp73α relative to TAp73β and in particular to the TAp73α_FRV mutant, however, suggests that transient interactions between the TA and the TI domains might occur in TAp73α resulting in a slight inhibitory effect.

An important factor in regulating the activity of p53 is its concentration. Upon detection of cellular stress, p53′s stability

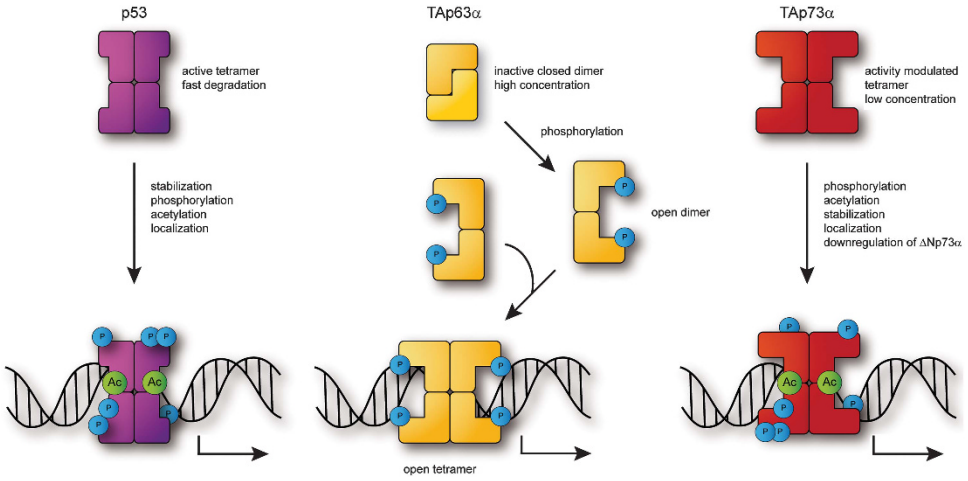

Figure 6 Schematic representation of the proposed activation mechanisms of p53 and the TAα-isoforms of p63 and p73. As the protein level of tetrameric p53 is kept low due to fast degradation, upregulation of p53 target genes requires the stabilization of p53 by phosphorylation and acetylation (simplified, exemplary phosphorylation and acetylation are indicated in blue and green, respectively). TAp63α on the other hand accumulates to high concentrations in an inactive and closed dimeric conformation and becomes activated upon phosphorylation, resulting in the formation of active tetramers. Despite its greater sequence homology to p63, TAp73α can readily form tetramers but like p53 needs further post-translational modifications and coactivators for full transactivation potential

increases, resulting in a higher intracellular concentration. Similarly, the concentration of TAp73α is also kept low. SEC experiments with endogenous TAp73α from cortical neurons or ciliated cells from the upper airway system failed due to the low concentration of TAp73α in these cells. Whether p73 gets induced by genotoxic stress is still debated and might depend on the specific tissue.[38–41] However, even if the intracellular concentration is an important factor for controlling p73′s activity, other mechanisms of regulation exist as well. One such mechanism is a shift in the relative concentrations of pro-apoptotic TAp73α and the anti-apoptotic ΔNp73α isoforms. It has been observed that a shift towards TAp73α can, for example, be achieved by selective degradation of ΔNp73α.[45] Finally, the activity gets further modulated by the interaction with other proteins and can also be dependent on the promoter type. In our cell culture transactivation experiments, TAp73α and the shorter TA-isoforms were active in SK-N-AS cells on the Bax promoter (Supplementary Figure 4). The same isoforms, however, showed a significantly reduced transcriptional activity relative to p63 or p53 on the p21 promoter in the same cells, as well as on the Bax promoter in Saos-2 cells. The fact that all TA-isoforms were similarly affected points to the lack of interaction partners that are important for the activation of p73 (but not p63) in Saos-2 cells or the inhibition of activating interactions on different promoters. Factors that influence the activity of p73 such as Pin1,[46] c-Abl[47,48] or YAP[49] have been described. The exact interaction partner will most likely also depend on the cell type and cellular context and elucidating the activation network of p73 is a future challenge. Our results, however, have established that intramolecular domain–domain interactions that regulate the oligomeric state in case of p63, do not have a role for the regulation of p73.

Materials and Methods

Plasmids. Mammalian cell expression constructs for the p63 isoforms and mutants used in this study have been described earlier.[13,25] TAp73α (NM_005427.3), TAp73β (NM_001204184.1), TAp73δ (NM_001204186.1) and ΔNp73α (NM001126240.2) were cloned accordingly. All mutants were generated employing the Quickchange site-directed mutagenesis protocol. The GST-fusion proteins used for *in vitro* pull-down experiments were produced using pGEX-6p-2 vector encoding p73 amino acids 1–125 (GST–p73TA) and p73 amino acids 552–599 (GST–p73TI), as well as p63 amino acids 1–136 (GST–p63TA) and p63 amino acids 569–616 (GST–p63TI) with an additional C-terminal hexa histidine-tag. Constructs used for expression and purification of the p73 oligomerization or tetramerization domain have been described earlier.[28] For the transactivation assays single copies of the p21 and BAX promoter have been cloned into pGL3 promoter vector.

Size exclusion chromatography. SEC experiments were performed at 4 °C using a Superose 6 PC 3.2/30 column (GE Healthcare, München, Germany) as previously described[13] with an injection volume of 50 μL. Proteins were expressed in an *in vitro* rabbit reticulocyte lysate transcription/translation System (Promega, Mannheim, Germany) or in Saos-2 cells by transient transfection (Effectene, Qiagen, Hilden, Germany) following the manufacturer's instructions. Saos-2 cells were grown in a 10-cm petridish, detached using Accutase, pelleted and resuspended in lysis buffer (50 mM sodium phosphate, pH = 7.2, 150 mM NaCl, EDTA-free protease inhibitor cocktail (Roche, Mannheim, Germany) and phosphatase inhibitor cocktail (Roche). Cells were lysed by freeze/thaw cycles and mechanical force. Cell debris was pelleted by centrifugation and the supernatant analyzed by SEC. All collected SEC fractions were analyzed by western blotting.

Western blotting. Western blotting was performed as previously described.[25] The following antibodies were used: anti-p63 (H-129, Santa Cruz, Heidelberg, Germany), anti-p63 4A4, anti-p73 (ER-15, Merck, Darmstadt, Germany), anti-myc (clone 4A6, Merck-Millipore, Darmstadt, Germany) and anti-GAPDH (Merck-Millipore). Quantification of western blot signals was performed using ImageJ (http://rsb.info.nih.gov/ij/).

Pull-down experiments. Pull-down experiments have been performed as described previously.[25] Pull-down efficiencies were determined by normalizing the pull-down signal to the corresponding input. For TA-pull-downs all isoforms and mutants were further normalized to the respective ΔN-isoform, while TI-pull-downs were normalized to TAp63γ or TAp73β, respectively. Normalization has been performed for each independent experiment. Experiments have been performed in triplicates at least, if not indicated otherwise.

Cell culture and coimmunoprecipitation. The osteosarcoma cell line Saos-2 was maintained in DMEM containing 10% FBS (PAA, Cölbe, Germany)

and 2 mM L-glutamine (PAA) at 37 °C and 5% CO_2. SK-N-AS cells, a neuroblastoma cell line, which is p73 deficient, was grown in DMEM, 10% FBS (PAA), 2 mM L-glutamine (PAA), and 1x MEM nonessential amino acids (Gibco, Darmstadt, Germany). For coimmunoprecipitation assays, Saos-2 cells were co-transfected with different isoforms of p63 (TAp63α, TAp63αFTL, TAp63αMI and ΔNp63α) and p73 (TAp73α, ΔNp73α) using Effectene (Qiagen) according to the manual. Twenty-four hours after transfection cells were harvested and lysed on ice in PBS containing 0.75% NP-40, 1 mM DTT and protease inhibitors. Insoluble parts were removed by centrifugation and the supernatant was incubated with 2 μg of anti-p63 (H-129, Santa Cruz), anti-p73 (ER-15, Merck) or normal IgG (rabbit or mouse, Santa Cruz) overnight at 4 °C. Immunocomplexes were removed from the lysate using Protein G Dynabeads (Invitrogen, Darmstadt, Germany), washed four times with PBS containing 0.75% NP-40 and eluted with LDS-sample buffer (Invitrogen) for 10 min at 70 °C. Samples were analyzed by western blotting. Immunoprecipitation efficiency was calculated by normalization of the IP western blot signal to the respective input signal. Each co-IP was performed in triplicates at least.

NMR titration assay. All NMR experiments were conducted at 25 °C on a Bruker (Karlsruhe, Germany) Avance 700 MHz spectrometer equipped with cryogenic tripleresonance probes. Proteins were expressed and purified as described previously.[28] Proteins were concentrated to 100 μM in a HEP-RES buffer (25 mM HEPES, 50 mM arginine, 50 mM glutamate, 2 mM TCEP, pH 7.5) and peptide was added to a final concentration of 2 mM of TA1 or TA2 peptide or 500 μM of TID peptide. Peptides were synthesized by Genscript (Piscataway, NJ, USA) (hp73_TA1: DGGTTFEHLWSSLEPD, hp_73TA2: DSSMDVFHLEGM) and Coring (Gernsheim a. Rhein, Germany) (hp73_TID: EAVHFRVRHTITIPNR).

Transactivation assay. Transactivation experiments were performed in SK-N-AS and Saos-2 cells using the Promega Dual-Glo Luciferase reporter assay. Cells were transfected with 100 ng DNA per plasmid (Effectene, Qiagen) in 12-well plates, grown for 24 h, harvested and subsequently assayed for Renilla and Firefly luciferase activities in 96-well plates four times. Remaining sample volume has been used for western blot analysis. After determining the Renilla to Firefly ratio, outliers have been identified using Grubb's test and corrected data have been averaged. A total of three independent experiments have been performed. Means were compared using Student's *t*-test.

Conflict of Interest

The authors declare no conflict of interest.

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