Cell Death and Differentiation (2004) 11, 685–687

www.nature.com/cdd

Letter to the Editor

DNA damage induces the rapid and selective degradation of the ΔNp73 isoform, allowing apoptosis to occur

Cell Death and Differentiation (2004) **11,** 685–687. doi:10.1038/sj.cdd.4401376
Published online 19 December 2003

Dear Editor,

p73 belongs to the family of transcription factors that also includes p53 and p63. The three proteins share a significant degree of sequence homology, and these structural similarities are paralleled by a certain degree of functional overlap and all members of the family are capable of inducing cell cycle arrest and apoptosis[1] (reviewed in reference Melino et al.[2]). Unlike p53, the p73 gene gives rise to a number of different mRNAs, which are translated into several different proteins. The different mRNAs arise either by alternative splicing or by the use of alternative promoters. Most of the splicing occurs at the 3′ end (isoforms α to η), in a part of the sequence that is not present in p53, and creates proteins that have different C-termini.[1,3–6] In addition, the second promoter (P2), located in intron 3, controls the expression of ΔNp73s. These N-terminally truncated isoforms lack the transactivation (TA) domain, but have novel sequence coded in an additional exon, exon 3′.[7] Moreover, additional isoforms of p73 that lack the N-terminal TA domain can also arise from alternative splicing of transcripts originating from the first exon.[8–10] While TAp73 isoforms work as transcription factors, the ΔNp73 isoforms that lack the transactivation domain are incapable of directly inducing gene expression and do not induce growth arrest or cell death. However, the ΔNp73 forms have a very important regulatory role, since they exert a dominant negative effect on p53 and TAp73 by blocking their transactivation activity, and hence their ability to induce apoptosis.[7] The relative levels of expression and more importantly the steady-state levels of the ΔNp73 isoforms can therefore determine the function of both TAp73 and p53. It is therefore most interesting that the ΔNp73 promoter (P2) contains a very efficient p53/p73 responsive element and consequently, p53 and TAp73 efficiently induce ΔNp73 expression.[7] This creates a dominant negative feedback loop that regulates the function of both p53 and TAp73 and can fine tune the function of p53 (reviewed in Melino et al.[2]).

Here we show that upon DNA damage, ΔNp73 is rapidly degraded, thus releasing the block exerted on p53 and TA p73 and allowing cell cycle arrest and apoptosis to proceed.

To analyse the stability of the ΔNp73, we generated a TeT-ON-inducible SAOS-2 cell line, SAOS-2(ΔNp73α), overexpressing this protein. Upon introduction of doxycycline in the media, SAOS-2(ΔNp73α) cells express ΔNp73α with a peak at 48 h (data not shown), but high levels of expression are reached even after 18 h of treatment (Figure 1a). In order to study the effect of ΔNp73α on UV-induced apoptosis, we treated SAOS-2(ΔNp73α) cells with doxycycline for 18 h and then with different doses of UV (35, 70 and 140 J/m^2) and analysed the cells 1, 3 and 6 h after UV treatment. Unexpectedly, a Western blot for ΔNp73α showed that ΔNp73α is rapidly degraded upon UV treatment (Figure 1a and b). Degradation is dose dependent, and is specific for the ΔNp73α isoform since treating SAOS-2 cells inducible for TAp73 or p53[11] with the same doses of UV does not result in a reduction of the protein levels (Figure 1c). To confirm that this is not an effect of UV on the mRNA levels or on the inducible system, we performed an RT-PCR specific for the over-expressed ΔNp73α mRNA using a primer in the HA tag sequence and one in the ΔNp73α sequence. As shown in Figure 1d, no changes in the mRNA levels of ΔNp73α or TAp73α (used as a control) are observed in UV-treated cells, confirming that the decreased protein levels are due to rapid degradation in response to UV treatment. Treatment with 35 J/m^2 UV of SAOS2 cells transiently transfected with either ΔNp73α or ΔNp73β and collected after 6 h, shown in Figure 1e, demonstrates that the degradation is independent of the C-terminal region and common to different ΔN isoforms (with or without the SAM domain). In addition, since the transfected proteins are not tagged, this experiments show that the UV-dependent degradation of ΔNp73 (Figure 1a and b) is independent of the tag.

ΔNp73α degradation is not only restricted to treatment with UV but is also observed with a number of different DNA-damaging agents, as shown in Figure 1f. Treatment of induced SAOS-2(ΔNp73α) with doxorubicin and etoposide results in the rapid disappearance of the ΔNp73α protein.

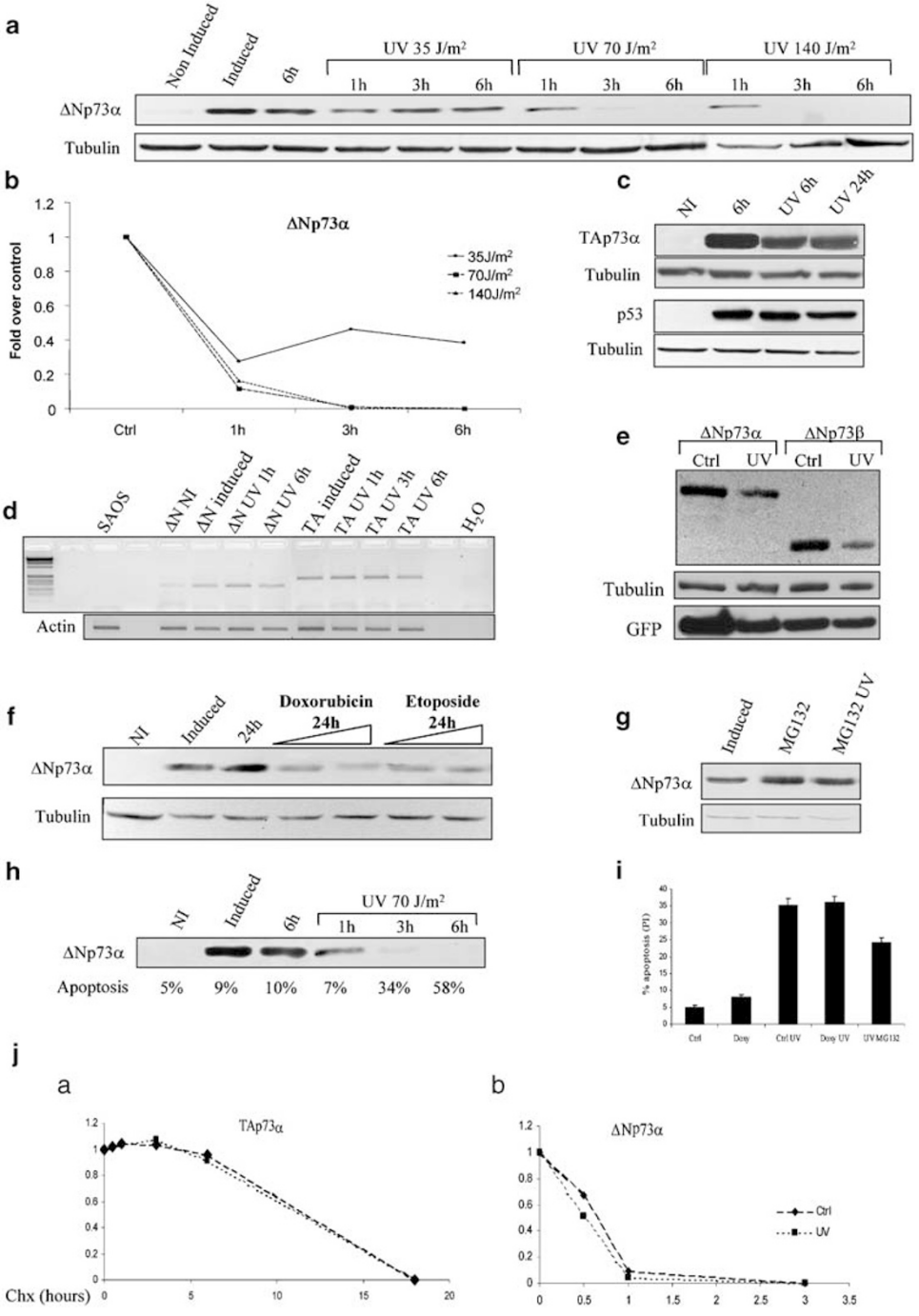
Figure 1g shows that treatment of cells with the proteasome inhibitor MG132 completely blocks UV-dependent degradation, showing that the degradation occurs in a proteasome-dependent manner.

Since it has been shown that ΔNp73α expression is capable of blocking p53- and TAp73-dependent apoptosis and cell cycle arrest, we investigated the effect of its degradation on these two phenomena. As shown in Figure 1h, UV treatment of SAOS2(ΔNp73α) induced to express ΔNp73α results in an increase in apoptosis paralleled by the reduction of ΔNp73α. Treatment with proteasome inhibitor MG132 prevents ΔNp73α degradation (Figure 1g) and rescues cells from

apoptosis measured by cytofluorimetric analysis of cells stained with PI (Figure 1i).

In order to better understand the ΔNp73 degradation process, we investigated ΔNp73 half-life. Figure 1j (panel b) shows a cycloheximide blocking experiment performed on inducible SAOS2 cells demonstrating that, unlike TAp73 (Figure 1j panel a), ΔNp73 has a half-life of about 35′ and after about 1 h of cycloheximide treatment almost all the protein is degraded. In contrast, 18 h are necessary to completely degrade the TAp73 protein. Interestingly, in the presence of cycloheximide UV treatment does not result in an accelerated degradation of ΔNp73 (Figure 1j, panel b), suggesting that UV-dependent ΔNp73 requires protein synthesis.

Since ΔNp73 levels can be controlled by TAp73 and p53,[7] the existence of ΔNp73 represents a safety system preventing inappropriate cell death. However, this direct control implies that ΔNp73 levels increase in response to DNA damage[12] (in a p53/TAp73-dependent manner), preventing p53 and TAp73 from exerting their action on damaged cells. It is therefore



necessary that this safety block is removed when cells are irreversibly damaged. We show for the first time that ΔNp73 is rapidly degraded upon DNA damage, while p53 and TAp73 proteins are not. As a consequence, upon DNA damage, ΔNp73 levels initially rise and then decrease in a dose-dependent manner, while TAp73 and p53 levels are increased and remain high. This results in cell cycle arrest and apoptosis of damaged cells. Our results therefore demonstrate a very important role of ΔNp73 in controlling cell fate.

Our data also show that the ΔNp73 protein has a very short half-life if compared to the TAp73 isoform suggesting that ΔNp73 protein levels are normally kept very low in cells. This is very important since ΔNp73 has been shown to have transforming activity,[13,14] and high levels of ΔNp73 have been associated with a number of tumours[15] and/or with poor prognosis.[16]

Acknowledgements

The work was supported by grants from Telethon (E1224) and Ministero Sanita' to VDL; EU Grants (QLG1-1999-00739 and QLK-CT-2002-01956), progetto Genomica Funzionale COMETA, FIRB 2001, MIUR 2002, Istituto Naz Tumori 8114, AIRC and Ministero Sanita' to GM. We are particularly grateful to RA Knight for helpful suggestions.

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Figure 1 ΔNp73α is rapidly degraded by UV irradiation. (**a**) Western blot of SAOS2(ΔNp73α) cells using anti-HA antibody (BABCO, HA-11). Cells were treated with doxycycline for 18 h (induced). Doxycycline was then removed and cells were UV irradiated with 35, 70 and 140 J/m^2 and harvested after 1, 3 or 6 h. We also included a noninduced (NI) control and a non irradiated control collected 6 h after removing doxycycline (6 h). (**b**) Densitometry analysis of the Western blot shown in (**a**) demonstrating the time and dose dependency of the ΔNp73α degradation. (**c**) Western blot of SAOS2(TAp73α) and SAOS2(p53) cells using anti-HA (BABCO, HA-11) and anti-p53 antibodies (Santa Cruz, DO-1 and 1801). Cells were induced as described above and treated with 140 J/m^2 UV and harvested after 6 or 24 h. We also included a non induced control (NI) and a non irradiated control collected 6 h after removing doxycycline (6 h). (**d**) RT-PCR for TAp73 and ΔNp73 transcripts using mRNA extracted from SAOS2(ΔNp73α and SAOS2(TAp73α) induced (induced) or not (NI) to express ΔNp73 or TAp73, respectively, and then treated with UV (140 J/m^2) as described above and collected after 3 and 6 h. No changes in transcript levels of either p73 isoform are observed after UV treatment. (**e**) Western blot analysis of SAOS-2 cells transfected with plasmids expressing: green fluorescent protein (GFP) and not tagged ΔNp73α or ΔNp73β using Lipofectamine 2000 (Invitrogen Life Technologies, Basel, Switzerland) according to the manufacturer's protocol. Protein extracts of untreated (ctrl) or UV-treated cells (UV) (35 J/m^2) were blotted and stained using the anti-p73 antibody (Neomarker, Ab-4). Both ΔNp73 isoforms are degraded upon UV treatment. (**f**) Western blot analysis using anti-HA antibody (BABCO, HA-11) of SAOS2(ΔNp73α) cells. Cells were treated with doxycycline for 24 h, doxycycline was then removed and cells were harvested immediately (induced) or after 24 h of treatment with Doxorubicin (1 and 3 μM) or Etoposide (25 and 50 μM). We also included a non induced control (NI) and a nonirradiated control collected 24 h after removing doxycycline (24 h). (**g**) ΔNp73α expression was induced by a 24 h doxycycline treatment, doxycycline was then removed and cells were preincubated 3 h with the proteasome inhibitors MG132 (10 μM), and then treated with UV (140 J/m^2) and harvested after 6 h and analysed by immunoblotting using an anti-HA antibody (BABCO, HA-11). A Non irradiated control treated with MG132 was also collected after 6 h (MG132). (**h**) Western blot analysis of SAOS-2(ΔNp73α) cells induced with doxycycline for 24 h, treated with UV (70 J/m^2) and collected after 1, 3 or 6 h. Part of the cells were collected for apoptosis analysis fixed in 70% ethanol. Hypodiploid events were evaluated by flow cytometry using a propidium iodide (PI) staining on a FACS-Calibur flow cytometer (Becton-Dickinson, CA, USA). We also included a non induced control (NI) and a nonirradiated control collected 6 h after removing doxycycline (6 h). (**i**) SAOS-2(ΔNp73α) cells induced with doxycycline for 24 h were pretreated with MG132 for 3 h (I) or directly irradiated with UV (70 J/m^2). Cells were collected after 6 h and fixed in 70% ethanol. Apoptosis was evaluated as above. (**j**) Densitometry analysis of a Western blot for HA-ΔNp73 and HA-TAp73 (performed as described above). ΔNp73αexpression was induced by a 24 h doxycycline treatment, doxycycline was then removed and cells were irradiated (■) with UV (140J/m^2) or not treated (◆). Cells were treated with 20 μg/ml cycloheximide and harvested at different time points. Western blot analysis of HA-ΔNp73 and HA-TAp73 was performed as described above, and densitometry analysis was normalized by tubulin levels