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ORIGINAL PAPER

Apoptin induces apoptosis by changing the equilibrium between the stability of TAp73 and ΔNp73 isoforms through ubiquitin ligase PIR2

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Abstract Apoptin, a protein derived from the chicken anaemia virus, induces cell death in various cancer cells but shows little or no cytotoxicity in normal cells. The mechanism of apoptin-induced cell death is currently unknown but it appears to induce apoptosis independent of p53 status. Here we show that p73, a p53 family member, is important in apoptin-induced apoptosis. In p53 deficient and/or mutated cells, apoptin induced the expression of TAp73 leading to the induction of apoptosis. Knockdown of p73 using siRNA resulted in a significant reduction in apoptin-induced cytotoxicity. The p53 and p73 pro-apoptotic target PUMA plays an important role in apoptin-induced cell death as knockdown of PUMA significantly reduced cell sensitivity to apoptin. Importantly, apoptin expression resulted in a marked increase in TAp73 protein stability. Investigation into the mechanisms of TAp73 stability showed that apoptin induced the expression of the ring finger domain ubiquitin ligase PIR2 which is involved in the degradation of the anti-apoptotic ΔNp73 isoform. Collectively, our results suggest a novel mechanism of apoptin-induced apoptosis through increased TAp73 stability and induction of PIR2 resulting in the degradation of ΔNp73 and activation of pro-apoptotic targets such as PUMA causing cancer cell death.

Keywords Apoptin · p73 · Ubiquitin ligase E3 · PIR2 · RNF144B · PUMA

Abbreviations

AP	Apoptin
DAPI	4′,6-Diamidino-2-phenylindole
Ad-GFP	Adenovirus expressing green fluorescent protein
BH3	Bcl-2 homology3
CIS	Cisplatin
Lenti-GFP	Lentivirus expressing green fluorescent protein
Lenti-GFP-apoptin	Lentivirus expressing green fluorescent tagged apoptin
PKC	Protein kinase C enzyme
PUMA	p53 upregulated modulator of apoptosis

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Introduction

The chicken anaemia virus (CAV) protein apoptin is 121 amino acids, rich in prolines, serines, threonines and basic amino acids [1]. It has the ability to induce cell death in cancer cells derived from different tissues but has negligible toxicity in normal cells including fibroblasts, keratinocytes, smooth muscle cells, T cells, and endothelial cells [2–6]. Although apoptin is not toxic to normal cells, those cells become sensitive to apoptin following oncogenic transformation by SV40 LT antigen suggesting that even a transient exposure to transformation events sensitises cells to apoptin [3].

Apoptin seems to induce apoptosis through the intrinsic pathway inducing cytochrome *c* release from the mitochondria and caspase-3 activation but not caspase-8 [7, 8]. However, the exact mechanisms by which apoptin induces apoptosis and its mode of tumour selectivity remain unclear. So far two clear characteristics of apoptin have been observed; (1) in cancer cells apoptin is localised to the nucleus where it seems to interact with DNA, whereas in normal cells it remains in the cytoplasm and becomes destabilised, (2) apoptin is highly phosphorylated in cancer cells but significantly less in normal cells of various types [9]. The upstream signalling pathways that lead to the activation of apoptin as well as the kinases responsible for apoptin phosphorylation are currently unknown. Several kinases such as PI3-K, AKT and CDK2 have been shown to be able to phosphorylate and functionalise apoptin [10]. Recently, we have shown that in certain tumour cell types apoptin is phosphorylated by protein kinase C enzyme (PKC) [11]. Additionally, apoptin expression results in the activation and cellular redistribution of PKCβ and consequently activation of caspase-9/3, cleavage of PKCδ catalytic domain and downregulation of the MERTK and AKT kinases [11].

Apoptin-induced apoptosis appears to be p53 independent in many cancer cell lines [5, 12, 13]. The p53-independent apoptotic function of apoptin is an important feature for its use as a potential anticancer therapeutic. However, the roles of the other p53 family members including p63 and p73 in apoptin-induced apoptosis have not been fully explored.

p73, first identified in 1997, shares a high level of homology in the DNA-binding domain with the p53 protein family [14]. The *p73* gene is located on chromosome 1p36.3 and expresses seven differentially spliced C-terminal isoforms, p73α-η as well as at least four alternatively spliced N-terminal isoforms that contain different parts of the transactivation domain (TA). The ΔTAp73 is the collective name for four different p73 isoforms lacking TA including ΔNp73, ΔN′p73, Δex2 p73 and Δex2/3 p73 [14–16].

The *p73* gene can be transcribed from two distinct promoters, P1 and P2. The P1 promoter is upstream of exon 1 and drives the expression of transactivating TAp73 isoforms while P2 is the alternative promoter located in intron 3, generating the amino-terminal truncated ΔNp73 isoforms [15]. Similar to p53, TAp73 transactivates genes involved in cell cycle arrest and apoptosis in response to cellular stress signals [17, 18]. In contrast, ΔNp73 isoforms have an anti-apoptotic potential and establish a negative feedback loop that controls the levels of TAp73 and p53 [19, 20]. Mutation of the *p73* gene in cancer cells is very rare [21]. However, frequent loss of heterozygosity of the *p73* gene has been shown in a number of different cancers, suggesting an important role in tumourigenesis [22–24]. Additionally, ΔNp73 has been shown to be up-regulated in human cancers [25–28] conferring an independent prognostic value for such tumours [26, 29].

Under normal physiological conditions, TAp73 protein levels are kept low, but p73 expression and activity increases in response to a subset of DNA damaging agents [30]. The DNA damage-mediated induction and activation of p73 is mainly regulated by post-translational modification such as phosphorylation, acetylation and ubiquitination [16, 31, 32]. In response to DNA damage TAp73 levels increase whilst the ΔNp73 levels diminish, releasing the inhibitory effect of ΔNp73 on TAp73 and p53 [28]. Several proteins including E3 ubiquitin ligases Itch, FBXO45 and PIR2 have been shown to regulate p73 stability and function [33–35]. The change in the equilibrium between pro-apoptotic and anti-apoptotic isoforms of p73 is believed to be important in regulating the induction of cell death under stress conditions. Additionally, phosphorylation of p73 by several kinases such as c-Abl tyrosine kinase, PKCδ and PKCβ serine/threonine kinases has been shown to result in increased p73 activity and apoptosis [36–38].

p53 upregulated modulator of apoptosis (PUMA), one of the Bcl-2 homology3 (BH3)-only subgroup of the Bcl-2 family members, plays an important role in p53-dependent apoptosis induced by genotoxic stress [39, 40]. Similar to p53, p73 can transactivate PUMA by binding to the same p53-responsive element in the PUMA promoter in response to DNA damage [41, 42]. We have recently shown that apoptin expression induces the endogenous protein levels of TAp73 in p53-mutated head and neck squamous cell carcinoma H357 suggesting an important role for p73 in the regulation of apoptin-induced apoptosis [43]. However, the mechanism by which apoptin activates TAp73 remained unknown.

Here we provide direct evidence that p73 is required for apoptin-induced apoptosis as knockdown of p73 using siRNA in HCT116 colon cancer cell lines increased resistance to apoptin-induced killing. Importantly, apoptin expression stabilised TAp73 and its downstream target PUMA causing tumour cell death. The stabilisation of TAp73 by apoptin resulted in increased expression of the ring finger domain ubiquitin ligase, PIR2, consequently

degrading the anti-apoptotic ΔNp73 isoform and activating the p73 mediated apoptotic pathway. Collectively, our data proposes a novel mechanism for apoptin-induced apoptosis, which involves the stabilisation of TAp73 isoform and concurrent reduction in the level of ΔNp73 through PIR2. The resultant change in the equilibrium between the expression of anti- and pro-apoptotic p73 isoforms seems to determine sensitivity of cancer cells to apoptin.

Results

Apoptin induces G2/M arrest and activates TAp73α

Several studies have used apoptin fused to GFP for the convenience of imaging and functional studies. However, there has been some concern that GFP-apoptin may have a somewhat altered function as compared to apoptin alone [44, 45]. Although, GFP-apoptin behaves similarly to native apoptin in cancer and transformed cells, it appears to partially lose tumour cell selectivity. Additionally, in contrast to apoptin, which is mainly cytoplasmic in normal cells, GFP-apoptin when expressed can translocate to the nucleus of some normal cells [44, 46].

We constructed an adenoviral vector expressing Apoptin (Ad-apoptin) using the Ad-easy system (Q-biogene) and infected p53-deleted Saos-2 cells with an MOI 20. Expression of apoptin was detected by indirect immunofluorescence using a phospho-specific antibody against Threonine-108-phosphorylated apoptin (Apoptin-P). Nuclei were counterstained with DAPI. The percentage of apoptin positive cells was determined by scoring of the FITC stained cells (Ad-apoptin) and GFP marker (Ad-GFP) using fluorescent microscopy. The percentage of apoptotic cells was determined by scoring the cells with clear condensed or fragmented nuclei. The results showed that already at 24 h post-infection, 84 ± 5 % of cells were expressing apoptin and 76.5 ± 8 % of cells were expressing GFP. Apoptin mainly localised to the nucleus and was phosphorylated whilst Ad-GFP infected cells showed the presence of GFP both in the nucleus and cytoplasm. Concomitantly, we observed a significant amount of apoptotic nuclei in Ad-apoptin infected cells when compared to Ad-GFP infected cells ($P < 0.05$) (indicated with white arrows, Fig. 1a). Cell cycle analysis by flow cytometry indicated that in contrast to Ad-GFP, Ad-apoptin significantly induced G2/M accumulation at 24 h post infection with a further increase in G2/M arrest after 48 h ($P < 0.05$) (Fig. 1b).

To examine the effect of apoptin on p73 expression, Saos-2 cells were mock infected or infected with either Ad-apoptin or Ad-GFP or treated with cisplatin. Western blot analysis was performed using p73α (p73SAM) antibody. At 48 h post infection, expression of apoptin in Saos-2 cells resulted in the upregulation of endogenous TAp73α protein. At this time point apoptosis occurred in apoptin expressing cells as detected by cleaved PARP p85 fragment when compared to the control and Ad-GFP infected cells (Fig. 1c). These results further confirmed the ability of Ad-apoptin to induce apoptosis and provided clear evidence that apoptin regulates p73 to induce apoptosis in the p53 deficient Saos-2 cells.

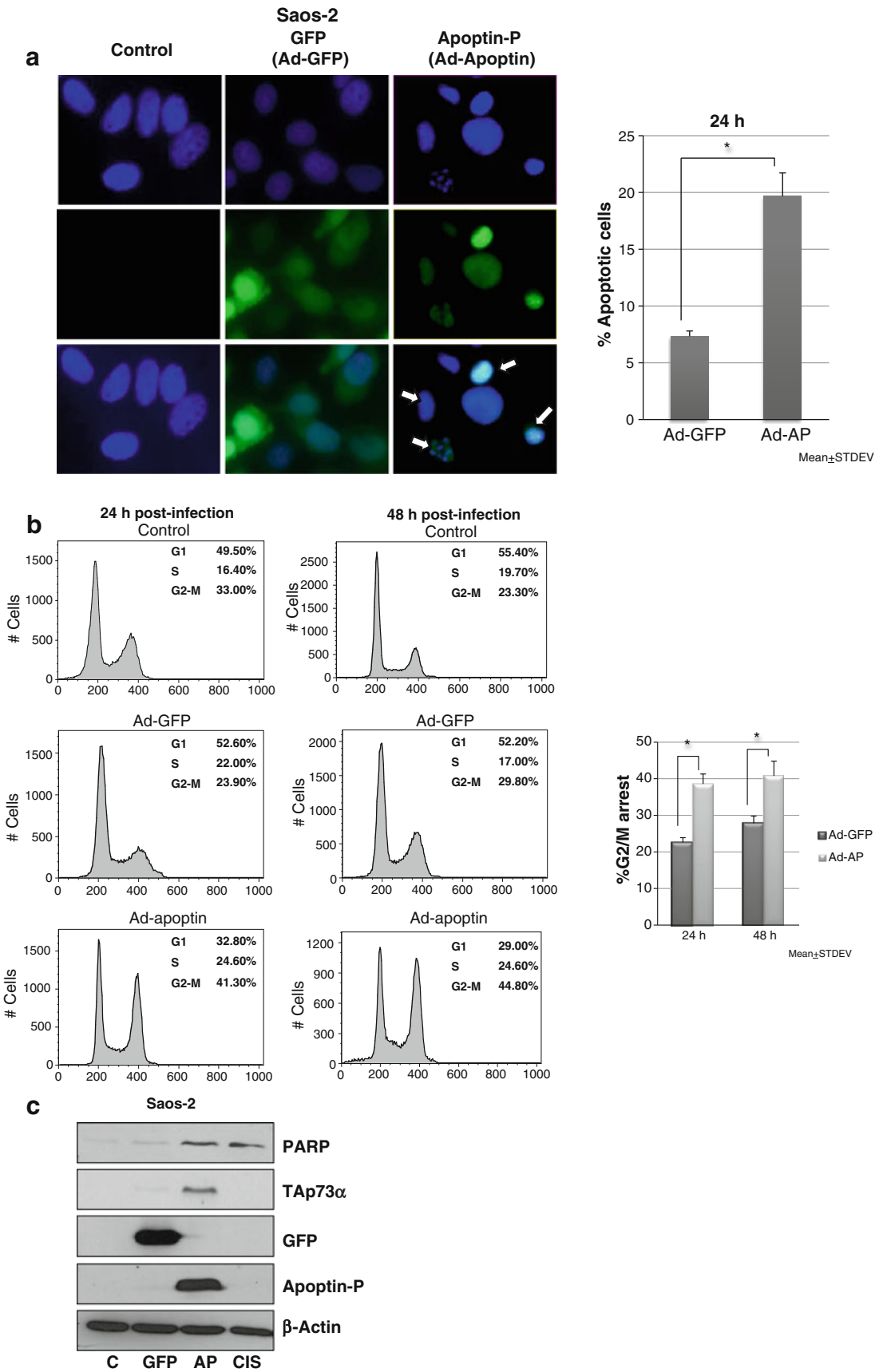
p73 regulates p53 independent apoptin-induced cell death

The importance of p73 in apoptin-induced cell death was further investigated using human colon cancer HCT116 cell lines containing either wild-type p53 or hetero and homozygous p53 ($p53^{+/+}$, $p53^{+/-}$ and $p53^{-/-}$) knockout. The cells were infected with Ad-apoptin and cell viability was measured at 24 and 48 h post-infection by the MTT assay. As shown in Fig. 2a, no significant difference in the sensitivity to apoptin was observed between p53 wild-type and deficient HCT116 cells ($P > 0.05$). This was further confirmed by phase contrast microscopy showing morphological changes associated with cell death in all cultures infected with Ad-apoptin (Fig. 2b). Apoptin expression induced TAp73 protein which was detected at both 24 and 48 h post-infection, in all three HCT116 cell lines, irrespective of their p53 status. p53-independent cell death was detected by cleaved PARP at 48 h post-infection (Fig. 2c).

To examine whether p73 has a direct role in apoptin-induced cell death, $p53^{-/-}$ HCT116 cells were transduced with siRNA molecules directed against TAp73α and then infected with Ad-apoptin at 48 h post-transduction. At 24 h post-infection apoptin induced expression of endogenous TAp73α but no apoptosis was detected in either control siRNA transduced or p73 knockdown cells (data not shown). However, at 48 h post-infection, Western blot analysis showed a clear reduction in PARP cleavage in p73 knockdown cells indicating increased resistance of these cells to apoptin-induced killing (Fig. 2d).

PUMA is important for apoptin-induced cell death

The effect of apoptin on a number of pro-apoptotic targets including Bax, Bak and Noxa has been previously reported [47]. Additionally, we have recently shown that apoptin induces the expression of the p53/p73 pro-apoptotic target PUMA [43]. To further investigate the importance of PUMA in apoptin-induced apoptosis, PUMA knockout HCT116 cells were investigated. MTT assay and phase contrast microscopy showed that PUMA inhibition significantly reduced sensitivity to apoptin-induced apoptosis ($P < 0.05$) (Fig. 3a, b) as compared to PUMA expressing


a
Saos-2
Control
GFP (Ad-GFP)
Apoptin-P (Ad-Apoptin)
24 h
% Apoptotic cells
Ad-GFP
Ad-AP
Mean±STDEV
b
24 h post-infection
48 h post-infection
Control
G1 49.50%
S 16.40%
G2-M 33.00%
G1 55.40%
S 19.70%
G2-M 23.30%
Ad-GFP
G1 52.60%
S 22.00%
G2-M 23.90%
G1 52.20%
S 17.00%
G2-M 29.80%
Ad-apoptin
G1 32.80%
S 24.60%
G2-M 41.30%
G1 29.00%
S 24.60%
G2-M 44.80%
Cells
%G2/M arrest
24 h
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Ad-GFP
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c
Saos-2
PARP
TAp73α
GFP
Apoptin-P
β-Actin
C GFP AP CIS

◀ **Fig. 1** Apoptin induces G2/M arrest and activates TAp73α. **a** Saos-2 cells were seeded in 8-well chamber slides, infected with either Ad-apoptin or Ad-GFP. Cells were fixed after 24 h. Apoptin was detected by a phospho-specific antibody against Threonine-108–phosphorylated apoptin and Goat-anti-rabbit IgG FITC conjugate antibody. Cells were mounted in DAPI-containing mounting medium (Vector Laboratories). Apoptin and GFP stained green and nuclei stained blue. The percentage of apoptosis was measured by scoring apoptin and GFP positive cells which contained condensed or fragmented nuclei. At least one hundred infected cells were counted in triplicate experiments. *Error bars* indicate standard deviation (STDEV). *Asterisk* represents significant difference of $P < 0.05$. **b** Cell cycle analysis of Saos-2 cells by PI FACS staining. The analysis was performed using cell cycle analysis (Dean–Jett–Fox model), FlowJo version 9.4.11 (Tree Star, Inc., USA). The graphs represent the percentage of Saos-2 cells arrested in G2/M after infection with 20 MOI of Ad-apoptin or Ad-GFP after 24 and 48 h. *Error bars* indicate STDEV. *Asterisk* represents significant difference of $P < 0.05$. **c** Apoptin induces endogenous TAp73α protein level. Saos-2 cells were infected with either Ad-apoptin or Ad-GFP at a MOI of 40 or left untreated. Saos-2 cells treated with 10 μg/ml cisplatin for 48 h were used as positive control. The cells were collected at 48 h post-infection and were analysed by Western blot analysis using indicated antibodies

HCT116 cells. Interestingly, Western blot analysis showed that apoptin increased the levels of p73 as well as p53, which are upstream of PUMA, in both PUMA wild-type and knockout HCT116 cells. However, diminished apoptosis was only observed in the PUMA knockout cells (Fig. 3c) suggesting that the activation of the p53/p73 pathway is not sufficient for the full induction of apoptosis by apoptin and that PUMA is one of the crucial mediators of apoptin cell death induction. As apoptosis was not completely inhibited in PUMA knockdown in HCT116 cells, there are likely to be other pro-apoptotic targets involved in cell killing by apoptin. Interestingly, the level of apoptin phosphorylation was reduced in PUMA knockdown cells, this effect was not due to an altered infection or expression efficiency of PUMA knockdown cells as confirmed by comparable levels of GFP expression in these cells (Fig. 3c).

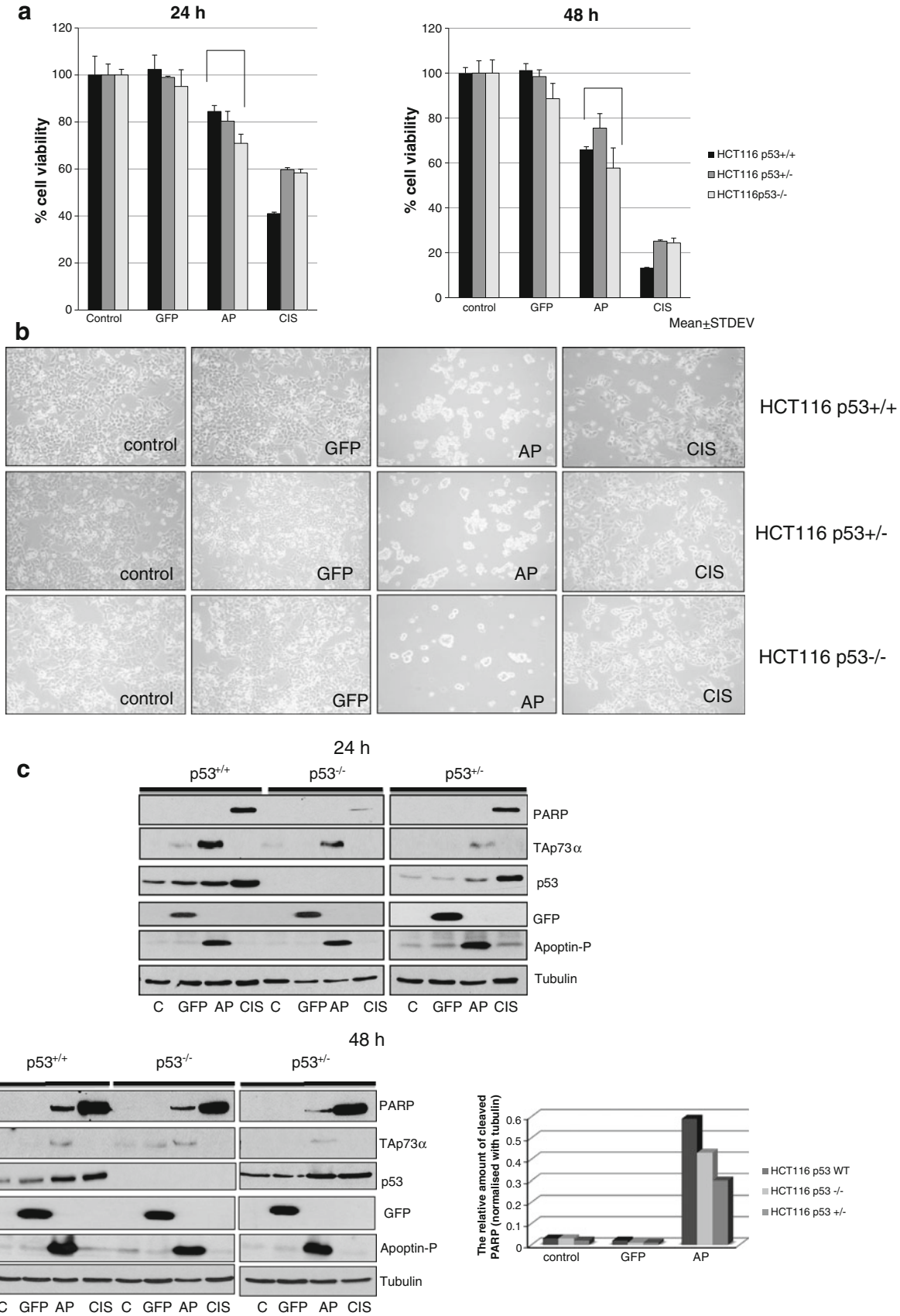
Apoptin stabilises the pro-apoptotic TAp73α isoform

We have previously shown that apoptin has no effect on the transactivation of p73 isoforms [43]. We therefore investigated the effect of apoptin on the protein stability of TAp73α and ΔNp73α isoforms using p73 isoform specific inducible Saos-2 cells. As shown in Fig. 4a, expression of apoptin increased TAp73α stability for up to 12 h, the maximum time point in this experiment, as compared to cells expressing Ad-GFP and non-treated controls. Importantly, apoptin did not increase the stability of the oncogenic ΔNp73α isoform. Immunoprecipitation studies demonstrated that apoptin interacted with both TAp73 and ΔNp73α isoforms with higher affinity to TAp73α than ΔNp73α (data not shown).

The role of specific p73 isoforms in sensitivity to apoptin-induced cell death was also examined using doxycycline inducible Saos-2 cells expressing either TAp73α or ΔNp73α. Cells were infected with either Lenti-GFP, Lenti-GFP-apoptin or left untreated in the presence (+ doxy) or absence (− doxy) of doxycycline induction. Cells were analysed by indirect immunofluorescence and apoptotic cells were scored. Doxycycline induction of TAp73α but not ΔNp73α resulted in some cell death in Saos-2 cells (Fig. 4b, c). However, apoptin expression induced a significantly higher percentage of apoptosis in cells expressing TAp73α as compared to apoptin expression without TAp73α induction (Fig. 4d, $P < 0.05$). Conversely, the percentage of apoptotic cells induced by apoptin in cells expressing ΔNp73α was significantly decreased as compared to apoptin expression without ΔNp73α induction ($P < 0.05$) (Fig. 4d). The above results indicate that apoptin regulates the p73 activity by increasing the stability of TAp73α but not ΔNp73α. Additionally, the expression of TAp73α sensitises cells while ΔNp73α expression inhibits apoptin-induced cell death.

Ubiquitin ligase PIR2 acts as a switch in apoptin-induced apoptosis by increasing the degradation of ΔNp73

As discussed in the introduction, the level of TAp73 is believed to be regulated at post-translational level by various modifications [32]. In order to identify the mechanism of apoptin-induced TAp73 stability the effect of apoptin on the expression of known p73 modulators was investigated. We found that apoptin had no significant effect on the level of ubiquitin ligase proteins, Itch and FBXO45 (data not shown). However, apoptin expression resulted in a clear increase in the level of ubiquitin ligase PIR2 and induction of apoptosis in TAp73α inducible Saos-2 cells, as detected by cleaved PARP. This effect was specific to apoptin as the induction of p73 alone or the expression of control GFP had no effect on PIR2 expression suggesting that cooperation with apoptin is necessary for increased PIR2 expression (Fig. 5a). Interestingly, PIR2 expression alone resulted in increased TAp73 expression and the induction of apoptosis in Saos-2 cells. Co-expression of PIR2 together with apoptin in Saos-2 cells led to increased TAp73 and apoptosis at a significantly higher levels than those reached by expressing either apoptin or PIR2 alone (Fig. 5b). We also investigated the effect of apoptin on the level of c-Abl and c-Jun proteins, both of which have been implicated in the stabilisation of p73 [48, 49]. No significant changes in the levels of either of


a
24 h
48 h
% cell viability
120
100
80
60
40
20
0
Control
GFP
AP
CIS
control
HCT116 p53+/+
HCT116 p53+/-
HCT116p53-/-
Mean±STDEV
b
control
GFP
AP
CIS
HCT116 p53+/+
HCT116 p53+/-
HCT116 p53-/-
c
24 h
p53+/+
p53-/-
p53+/-
PARP
TAp73α
p53
GFP
Apoptin-P
Tubulin
C GFP AP CIS C GFP AP CIS C GFP AP CIS
48 h
The relative amount of cleaved PARP (normalised with tubulin)
0.6
0.5
0.4
0.3
0.2
0.1
0
HCT116 p53 WT
HCT116 p53 -/-
HCT116 p53 +/-

◀ **Fig. 2** p73 regulates p53 independent apoptin-induced cell death. **a** Human colorectal cancer cells HCT116 wild-type and its p53 knockdown derivative were left untreated or infected with either Ad-GFP or Ad-apoptin at a MOI 20 or treated with 10 μg/ml cisplatin. Cell survival was measured by the MTT assay at 24 and 48 h post-infection. Results are shown as percentage of viable cells with respect to viable non-treated cells. All experiments were performed in triplicate, *error bars* indicate STDEV. **b** Microscopic pictures of wild type, $p53^{+/-}$ and $p53^{-/-}$ HCT116 cell lines 24 h post-infection with either Ad-apoptin or Ad-GFP at a MOI 20, mock infected or treated with 10 μg/ml cisplatin. **c** HCT116 wild-type and its p53 knockdown HCT116 cells were left untreated or infected with indicated recombinant adenoviruses and lysed 24 and 48 h post-infection and analysed by Western blot. The percentage of cleaved PARP p85 fragment was quantified using ImageJ (NIH, USA). **d** HCT116 $p53^{-/-}$ cells were transfected with either siRNA against TAp73 or siRNA control 24 h after transfection, cells were induced with doxycycline overnight after which the cells were mock infected or infected with either Ad-GFP or Ad-apoptin at a MOI 20 or treated with 10 μg/ml cisplatin. Cells were lysed at 48 h after infection and analysed by Western blot using the indicated antibodies. The percentage of cleaved PARP p85 fragment was quantified using ImageJ (NIH, USA)

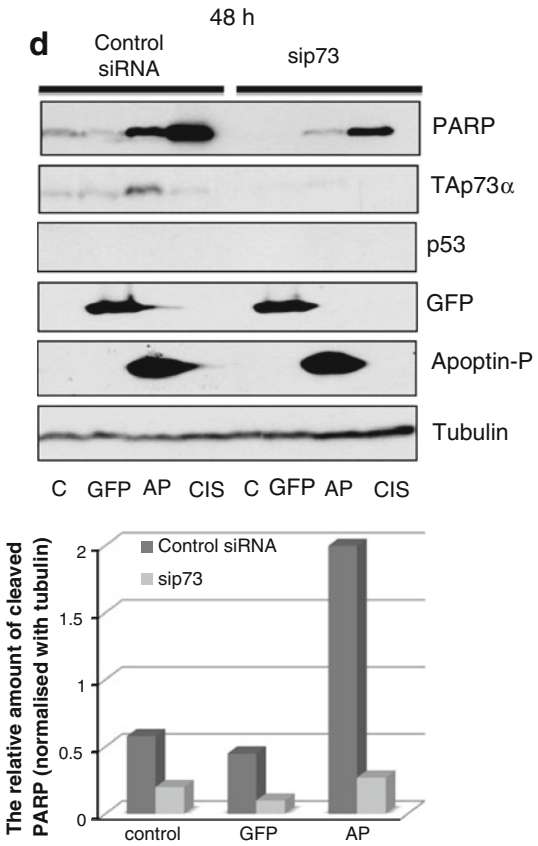


Fig. 2 continued

these proteins were observed with or without apoptin expression (Fig. 5a).

To investigate a direct role of PIR2 in apoptin-induced cell death, PIR2 was silenced by siRNA in TAp73α Saos-2 inducible cells. As shown in Fig. 5a, apoptin expression resulted in increased levels of PIR2 and apoptosis in TAp73α inducible Saos-2 cells both at 24 and 48 h post-infection. PIR2 knockdown showed reduced apoptin-induced apoptosis measured by the level of PARP cleavage as compared with apoptin expression in siRNA control transduced cells (Fig. 5c, 24 and 48 h).

In contrast to TAp73 inducible cells, expression of apoptin in ΔNp73α inducible Saos-2 cells failed to induce PIR2 levels with or without induction of ΔNp73α. Furthermore, apoptin induced apoptosis only in cells lacking ΔNp73α expression as shown by PARP cleavage (Fig. 5d). These results indicated that PIR2 is induced by apoptin downstream of TAp73α but not ΔNp73α hence upregulation of TAp73α by apoptin leads to PIR2 induction and consequently apoptosis. In agreement with a recent study [33], we found that overexpression of PIR2 resulted in the degradation of the anti-apoptotic ΔNp73 isoform consequently inducing apoptosis in ΔNp73α overexpressing Saos-2 cells (Fig. 5e). Taken together, these data suggest that apoptin modulates TAp73α isoform function through protein stabilisation resulting in the induction of its downstream target ubiquitin ligase PIR2. The destabilisation of ΔNp73α by PIR2 removes ΔNp73α inhibitory effect on the proapoptotic TAp73 isoforms triggering the apoptotic machinery.

Discussion

Many viruses are able to reprogram cellular processes to allow viral replication and propagation. Interaction of certain viral proteins with key cellular components such as p53 and pRB causes deregulation of the cell cycle program and consequently cellular transformation. Additionally, there is clear evidence for the role of viruses, through the specific function of viral proteins such as E1A of human adenovirus, in the induction of tumour specific cell death [43, 50, 51]. The potential use of viruses and viral derived proteins in cancer therapy is of major interest at present. Therefore, understanding the cellular mechanisms which determine the pro- and anti-apoptotic cellular response to viral proteins is crucial for exploring their therapeutic potential. In this study we have investigated the cellular mechanisms responsible for the cytotoxic effect of CAV derived protein VP3/apoptin, which shows tumour selective cytotoxicity. Previously, it has been shown that apoptin-induced cell death was not dependent on p53 and involved the intrinsic apoptotic pathway. However, both the upstream signalling mechanism and the downstream apoptotic components which regulate apoptin function are currently unknown.

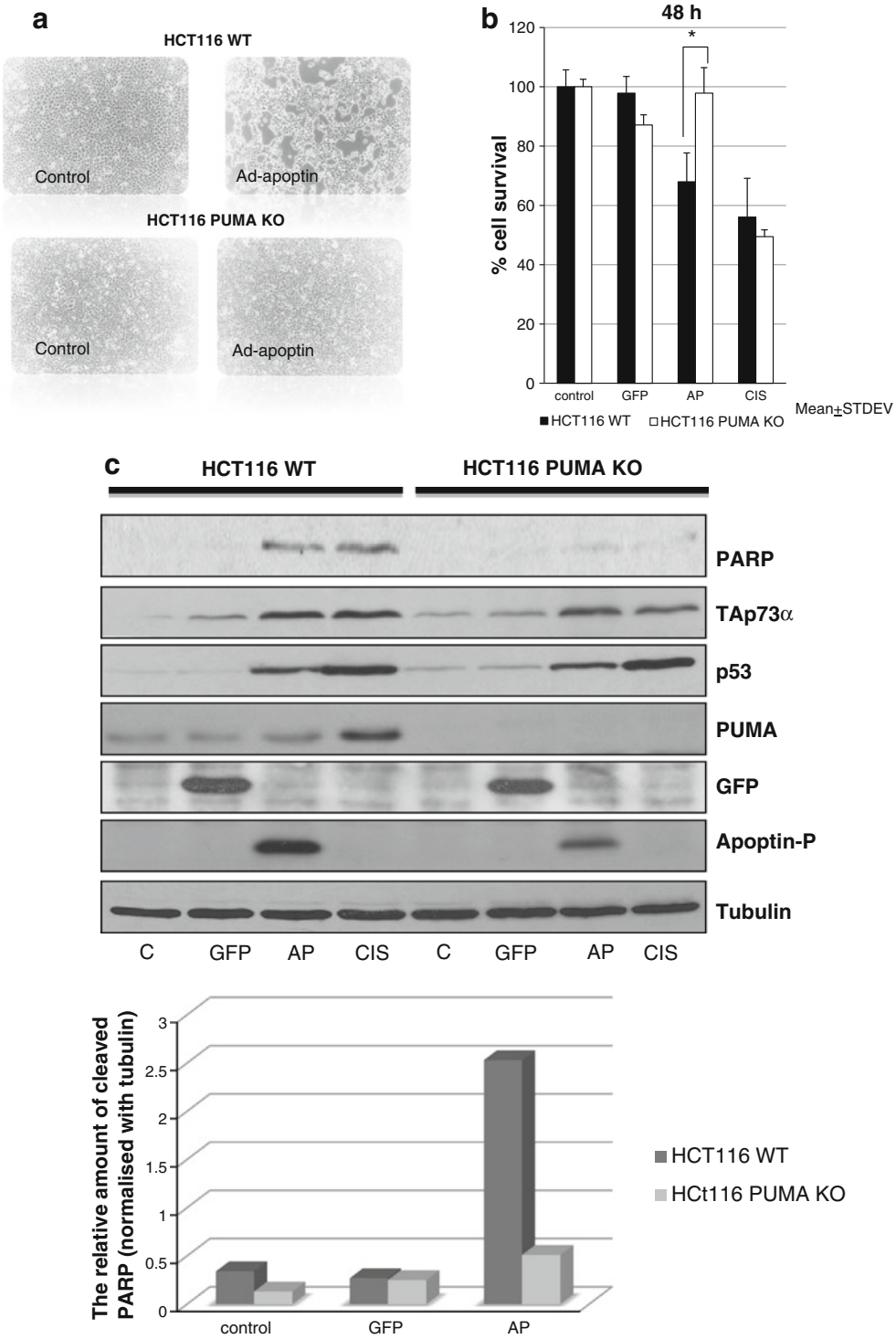


Fig. 3 PUMA is important for apoptin-induced cell death. **a** Phase contrast microscopic pictures of HCT116 cells and its PUMA$^{-/-}$ cell lines at 24 h post-infection with either Ad-apoptin at a MOI 10 or untreated cells. **b** The cells were left untreated or infected with either Ad-GFP or Ad-apoptin at a MOI 10 or treated with 10 μg/ml cisplatin. Cell survival was measured by the MTT assay at 48 h post-infection. Results are shown as percentage of viable cells with respect to viable non-treated cells. All experiments were performed in triplicate, *error bars* indicate STDEV. *Asterisk* indicates a significant difference of $P < 0.05$ **c** HCT116 cells and its PUMA$^{-/-}$ cell lines were infected with Ad-GFP or Ad-apoptin at a MOI 10 or left untreated or treated with 10 μg/ml cisplatin. After 24 h post-infection, cell lysates were prepared and analysed by Western blot using the indicated antibodies. The percentage of cleaved PARP p85 fragment was quantified using imageJ (NIH, USA)

It is known that the function of p73 is dependent on the p53 status of the cell and differs in the presence/absence of wild-type or mutated p53 [52]. Therefore, to investigate the possible role of p73 in apoptin induced cytotoxicity we used either heterozygous or homozygous p53 knockout HCT116 cells. Apoptin expression resulted in the induction of TAp73 and consequently apoptosis regardless of the cellular p53 status (Fig. 2b). Whilst p53 knockout did not reduce the cytotoxicity of apoptin, downregulation of p73 by siRNA significantly reduced apoptin-induced cell death. One of the key features of apoptin is its selectivity for cancer cells; the basis for this specificity might therefore involve the regulation of p73 pathway. Indeed, apoptin resulted in an increase in the endogenous TAp73α in human SV40 large T-antigen

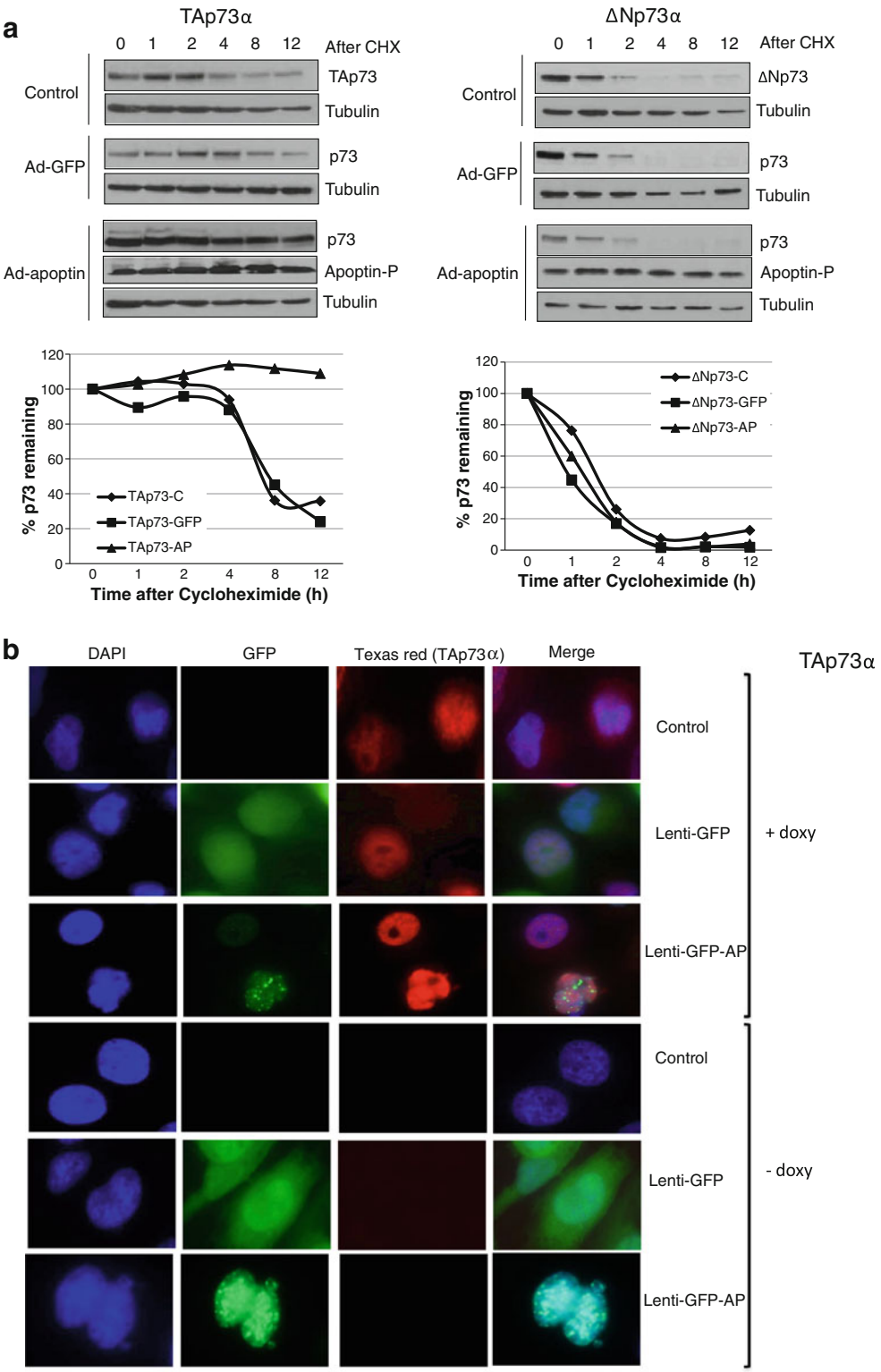


Fig. 4 Apoptin stabilises the pro-apoptotic TAp73α isoform. **a** TAp73α or ΔNp73α inducible-Saos-2 cell lines were used to study the half-life of p73. Cells were treated with 20 μg/ml cycloheximide (CHX) and left untreated or infected with either Ad-GFP or Ad-apoptin at a MOI 10. 24 h post-infection, cells were collected at the indicated time points (0, 1, 2, 4, 8, 12 h). Cell lysates were analysed by Western blot. Saos-2 cells inducible for TAp73α (**b**) and ΔNp73α (**c**) were infected with either Lenti-GFP or Lenti-GFP-apoptin at a MOI of 4 in the presence or absence of doxycycline. Cells were fixed at 24 h after infection and stained for p73 isoforms by immunofluorescent staining using mouse anti-HA primary antibody and Texas red-labeled secondary anti-mouse antibody. Cells were mounted in DAPI-containing mounting medium. p73 stained red and Lenti-GFP-apoptin stained *green*. **d** The percentages of apoptotic cells in Saos-2 inducible TAp73α and ΔNp73α cell lines treated with apoptin in the presence or absence of doxycycline. Apoptosis was examined by scoring the GFP positive cells which contained condensed or fragmented nuclei. At least one hundred infected cells were analysed and experiments were performed in triplicate. *Error bars* indicate STDEV. *Asterisks* represent significant difference of $P < 0.05$

transformed fibroblasts 1BR3N but not in the matched normal human fibroblasts 1BR3 cells (data not shown). Additionally, apoptin phosphorylation levels were lower in normal cells. However, as the regulation of different p73 isoforms in normal, unstressed cells remains unclear, the precise role of p73 in increased sensitivity of tumour cells to apoptin and its involvement in apoptin phosphorylation needs further investigation in different normal cell types.

Our previous studies using luciferase reporter assays showed that apoptin expression has no effect on the

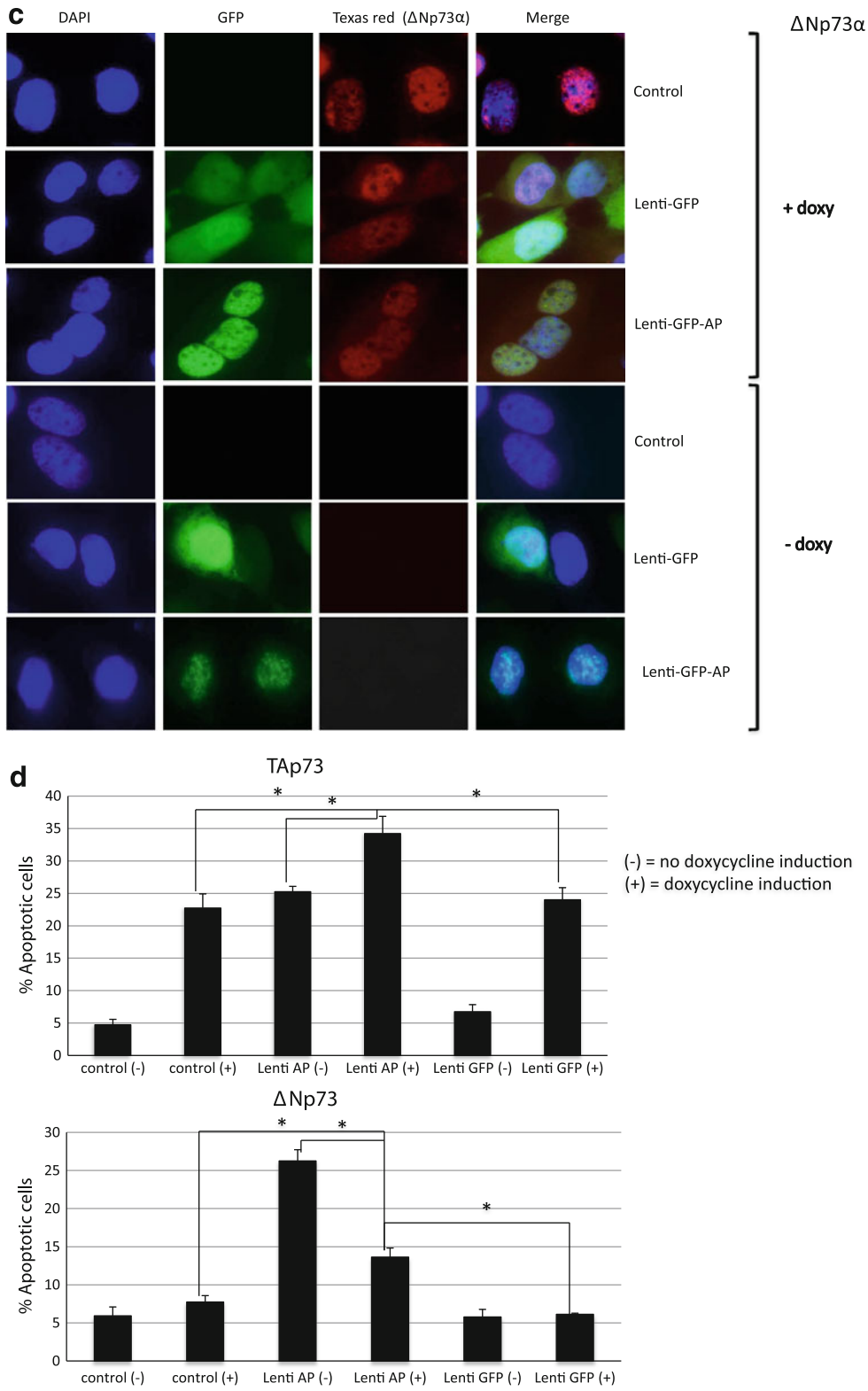


Fig. 4 continued

transactivation of p73 [43]. Here we clearly demonstrate that apoptin expression resulted in substantially increased stability of the pro-apoptotic TAp73 but had no significant effect on the stability of the anti-apoptotic ΔNp73 isoform indicating the true pro-apoptotic function of apoptin.

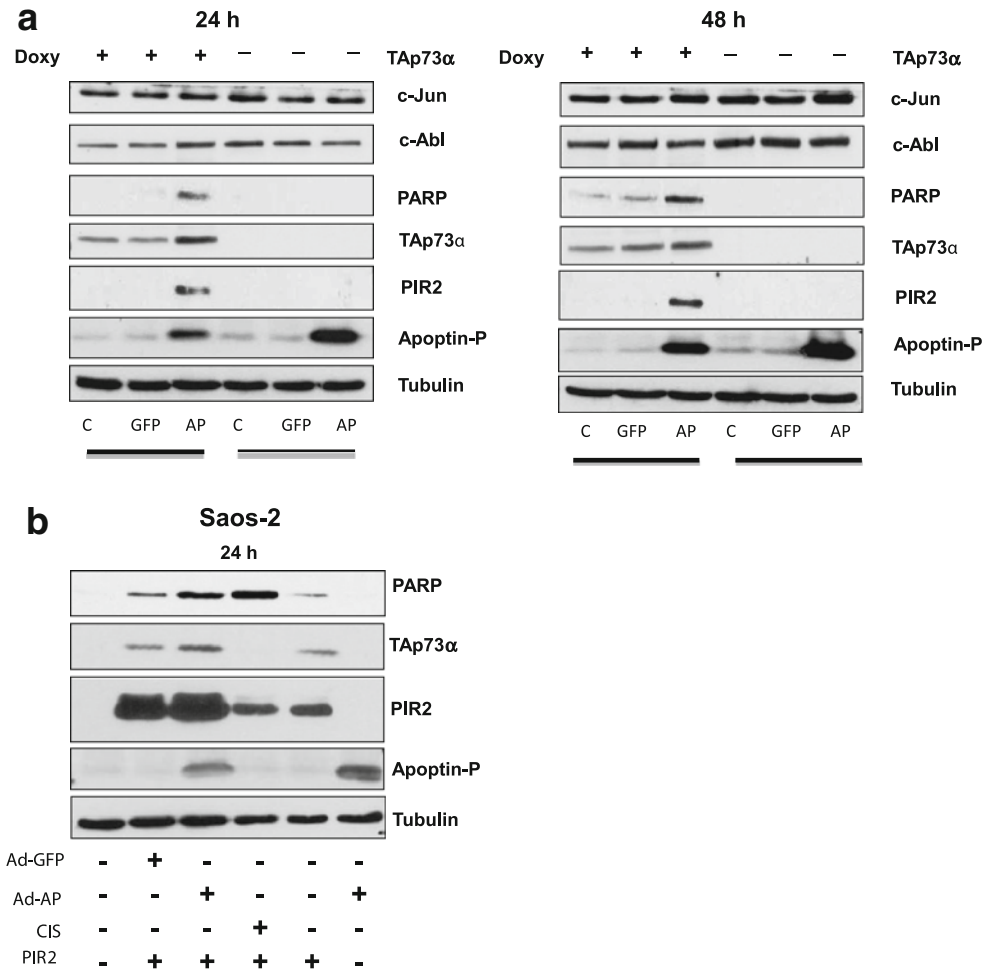


Fig. 5 Ubiquitin ligase PIR2 acts as a switch in apoptin-induced apoptosis by increasing the degradation of ΔNp73 levels. **a** Saos-2 inducible cells expressing TAp73α were left untreated or infected with indicated recombinant adenoviruses at a MOI 10 in the presence and absence of doxycycline and lysed at 24 and 48 h post-infection and analysed by Western blot. **b** Saos-2 cells were transfected with plasmid expressing PIR2 and 24 h post-transfection, the cells were treated with indicated adenoviruses at a MOI 20 or treated with 10 μg/ml cisplatin. Cells were lysed after 24 h treatment and subjected to Western blot analysis with the indicated antibodies. **c** siRNA PIR2 knockdown cells show increased resistance to apoptin-induced apoptosis. TAp73α Saos-2 inducible cells were transfected with either control siRNA or siRNA against PIR2. At 48 h post-transfection, cells were infected with either Ad-apoptin or Ad-GFP or treated with cisplatin. Cells were collected at 24 and 48 h post-infection for Western blot analysis. The percentage of cleaved PARP p85 fragment was quantified using ImageJ (NIH, USA). **d** ΔNp73α inducible-Saos-2 cells were left untreated or infected with either Ad-GFP or Ad-apoptin at a MOI 10. Cells were lysed at 48 h post-infection and subjected to Western blot analysis with indicated antibodies. **e** ΔNp73α inducible-Saos-2 cells were induced with doxycycline and after 24 h the cells were transfected with either empty vector or plasmid expressing PIR2. Cells were lysed at 24 h post-transfection and subjected to Western blot analysis with indicated antibodies. Anti-Myc antibody was used to detect PIR2 expression

Differential protein stability is believed to be the main mechanism that regulates the pro- and anti-apoptotic activity of p73 isoforms thereby determining cellular fate. This led us to investigate the role of E3 ubiquitin ligases that are known to be involved in p73 stability. The ring finger domain ubiquitin ligase, PIR2 was shown to be directly regulated by TAp73 upon cellular stress and an increase in the level of PIR2 leads to degradation of the ΔNp73 isoform [33]. We observed that the induction of TAp73 by apoptin leads to an increase in the PIR2 level and subsequent apoptosis. PIR2 appears to play a role in apoptin-induced apoptosis because PIR2 knockdown showed increased resistance of cells to apoptin cytotoxicity. Importantly, exogenous expression of PIR2 in ΔNp73 overexpressing cells, which are resistant to apoptin-induced cell death, by itself was toxic further proposing an important role for PIR2 in regulating the TAp73 protein stability and ΔNp73 degradation to achieve an apoptotic response. Moreover, our data suggests that TAp73 alone is not sufficient to activate PIR2 expression and its activation/stabilisation by apoptin prompts p73-mediated induction of PIR2. The mechanism by which apoptin modulates

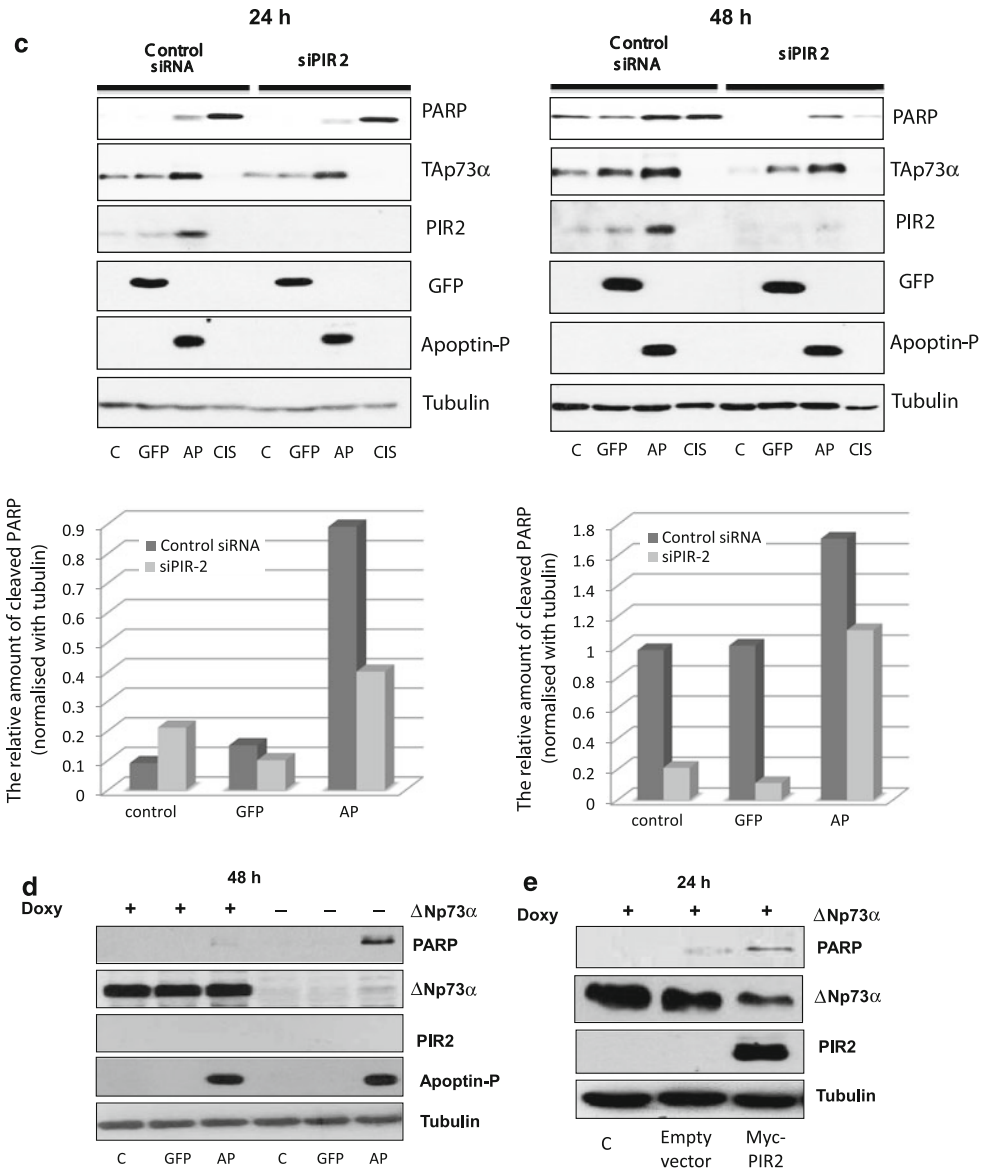


Fig. 5 continued

transcriptional regulation of PIR2 through TAp73 remains currently unclear.

We have shown that the expression of apoptin increases the levels of endogenous TAp73 and its pro-apoptotic target PUMA leading to apoptosis in p53 mutant HNSCC cells [43]. In this study we further investigated the importance of PUMA using PUMA KO HCT116 cells. The results confirmed that despite the activation of p53 and/or p73 by apoptin, inhibition of PUMA significantly increased the resistance of tumour cells to apoptin-induced killing when compared to PUMA wild type cells suggesting the importance of PUMA in mediating apoptin-induced apoptosis. Interestingly, we found that the level of apoptin phosphorylation in PUMA knockout cells was significantly lower than wild-type cells. The GFP control showed that this was not due to differences in infection efficiency or expression levels in the knockout cells (Fig. 3c) indicating a link between PUMA inhibition and apoptin phosphorylation. Recently, PUMA has been shown to be modulated post-translationally through phosphorylation [53]. Whether there is a functional interaction between PUMA and apoptin phosphorylation needs further investigation.

Currently the upstream signalling pathways which stabilise p73 in response to apoptin are not understood. A recent study has shown that apoptin is activated by DNA damage response (DDR) involving ATM and DNA-PK

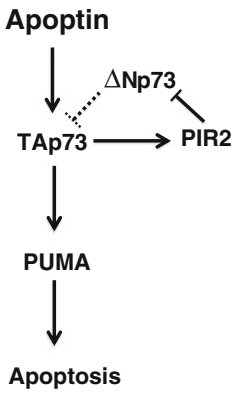


Fig. 6 Proposed model for p53 independent, p73-mediated induction of apoptosis by viral protein apoptin. Apoptin expression leads to activation of pro-apoptotic TAp73 which results in upregulation of the downstream target PUMA and induction of cell death. The model proposes that apoptin induces apoptosis by increased TAp73 stability via the ring finger domain ubiquitin ligase PIR2. Consequently PIR2 expression, degrades anti-apoptotic ΔNp73 isoform to release ΔNp73 on TAp73 isoform

[54]. Also independent studies have shown that p73 stabilisation is involved in DDR through ATM [55, 56]. Another mechanism involved in p73 stabilisation is through phosphorylation and several kinases have been shown to phosphorylate and hence stabilise p73 including Abl, JNK, PKCδ and PKCβ. Recently we have shown the activation of PKCδ and PKCβ by apoptin. However, whether any of these kinases causes p73 phosphorylation and are responsible for apoptin-induced TAp73 increased stabilisation will form part of our future studies.

In summary, our findings suggest a novel pathway for the induction of cell death by apoptin and possibly other apoptotic inducing agents. Figure 6 demonstrates the proposed model summarising the components which may be involved in p53 independent, p73-mediated induction of apoptosis by viral protein apoptin. Further investigation of this mechanism will be very important for the design of effective anticancer therapeutics.

Materials and methods

Cell lines, plasmids and reagents

Saos-2 cells with inducible TAp73α and ΔNp73α, Human colon cancer HCT116, its PUMA$^{-/-}$, p53$^{-/-}$, p53$^{+/-}$ knockout and Osteosarcoma Saos-2 cells were cultured as described previously [43]. Inducible TAp73α and ΔNp73α were under the control of the TET-on system and induced by the addition of 2.5 μg/ml doxycycline. The following constructs were used: pcDNA4/TO/myc-His-PIR2, Lentivirus-GFP-apoptin, Lentivirus-GFP, Adenovirus-apoptin and Adenovirus-GFP. Adenovirus amplification and purification was essentially done as described previously [51].

Flow cytometry

Cells were plated in 6-well plates and infected with adenovirus at a MOI 20. The samples were collected at 24 and 48 h after infection. Cells were fixed in 70 % ethanol and stained with PI FACS staining solution. The samples were incubated at 37 °C for 30 min then analysed on a FACSCalibur within 24 h.

Immunofluorescent microscopy

Saos-2 cells were seeded in 8-well culture slides (Becton–Dickinson, Oxford, UK) and infected with Ad-GFP or Ad-apoptin. After 24 h phospho-specific antibody against Threonine-108-phosphorylated apoptin and Goat-anti-rabbit IgG FITC conjugate secondary antibody (Sigma, Gillingham, UK) were used to detect Ad-apoptin. For the Ad-GFP control GFP expression was directly detected by fluorescence microscopy. Saos-2 cells with inducible TAp73α and ΔNp73α were seeded in Falcon 8-well culture slides and infected with Lenti-GFP-apoptin or Lenti-GFP. After 24 h, TAp73α and ΔNp73α expression was detected by mouse anti-HA antibody (Sigma, Gillingham, UK) and followed by goat anti-mouse IgG (whole antibody) Texas-Red (Vector Laboratories, Peterborough, UK). Fixation and preparation of slides were carried out as described previously [51].

MTT assay

Cell survival was measured by the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously [51].

siRNA knockdown

The following siRNA reagents were used: On-TARGET plus® control siRNA GAPD: Human (Thermo Scientific Dharmacon®), Ambion Silencer® Select Pre-designed siRNA: RNF144B (Applied Biosystems), On-TARGET plus SMARTpool TP73 (Thermo Scientific Dharmacon®). siRNA transfection was performed using Amaxa™ Nucleofector™ as recommended by the manufacturer (Lonza Biologics, Cambridge, UK).

Western blot analysis

Western blot analysis was performed as described previously [51]. The antibodies used were: antibody against Threonine-108-phosphorylated apoptin, raised in rabbit

against peptide H2NSLITTT(PO3H2)PSRPRTA-CONH2 derived from the apoptin amino acid sequence (Eurogentec) [11], mouse anti-Myc (Santa cruz), Rabbit anti-c-jun (Santa Cruz), rabbit anti-GFP (cell signalling), rabbit anti-p73SAM [57], rabbit anti-PIR2 [33], mouse anti-p53 clone DO7 (Novacastra Laboratories), mouse-anti-Abl (BD Biosciences), mouse anti-tubulin (Sigma), mouse anti-β actin (Sigma), rabbit anti-PARP p85 fragment clone G734A (Promega, Southampton, UK), mouse anti-HA (Sigma), secondary anti-mouse (Sigma) and anti-rabbit antibodies linked to horseradish peroxidase (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK).

Determination of p73 half-life

Determination of p73 protein turnover was performed by adding 20 μg/ml cycloheximide to TAp73α and ΔNp73α inducible Saos-2 cell lines 24 h after induction with 2.5 μg/ml doxycycline and infection with either Ad-apoptin or Ad-GFP or mock infected. Protein levels were determined by collecting cells at different time points and performing Western blot analysis as described above.

Statistical analysis

For statistical analysis, the Student's *t* test was carried out. Statistically significant difference was defined as $P < 0.05$.

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Conflict of interest The authors declare no conflict of interest.

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